FELINE STEM CELL FACTOR: ISOLATION AND CHARACTERISATION OF BIOLOGICAL ACTIVITY

By

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To Mum and Dad for their constant support and encouragement

SUMMARY

Cytokines are small proteins produced by many tissue types and have wide ranging effects on the haemopoietic and immune systems. The cloning of human cytokines has facilitated the production of recombinant cytokines in quantities sufficient to enable detailed study of their biological properties. An understanding of the biological effects of these cytokines has led to their introduction as novel therapeutic agents with widespread potential uses, including the treatment of cancer, cytopenias and viral infections. The use of heterologous cytokines in domestic species has been of only limited success, in part due to the variable degree of interspecies conservation. In order to fully realise the potential for cytokines as therapeutic agents and to facilitate further studies of the role of cytokines in diseases of domestic species, the isolation of species specific cytokines is desirable. This thesis describes the approach used to isolate and clone feline stem cell factor (fSCF) and subsequently express the recombinant protein and characterise its biological properties.

Stem cell factor is the ligand for the tyrosine kinase receptor encoded by the *c-kit* gene. It has wide ranging actions on cells of the haemopoietic, reproductive and nervous systems and melanocytes, in particular promoting the survival and development of primitive cells. cDNA clones encoding two isoforms of fSCF were isolated using RT-PCR and their sequences determined. The cDNAs encode a predicted full length fSCF protein of 274 amino-acids and a shorter isoform of 246 amino acids. Feline SCF shows a high degree of homology to the SCFs of other species at both the nucleic acid and protein level. Feline SCF was expressed as a soluble protein using the glutathione S-transferase fusion protein system and purified by affinity, anion exchange and gel filtration chromatography. Murine MC/9 and human TF-1 cells were used to assay fSCF biological activity. The recombinant protein supported the growth of feline granulocyte-macrophage colony forming cells *in vitro* and in combination with feline phytohaemagglutinin lymphocyte conditioned medium increased colony numbers and sizes were seen. Administration of the recombinant protein to cats produced increases in circulating colony forming cells,

induced extramedullary haemopoiesis in the spleens of treated cats and led to increased mast cell numbers at the site of injection.

In order to enable assessment of the effects of frSCF upon primitive haemopoietic cells, the production of polyclonal antiserum to CD34 (a transmembrane glycoprotein expressed predominantly on primitive haemopoietic cells) was attempted. Rabbits were used to raise antisera to conserved intracellular epitopes of the CD34 molecule by inoculation with immunogenic peptides. This was of limited success; whilst the antisera recognised the synthetic peptides against which they had been raised, they showed poor affinity for the native protein.

These studies provide the basis for further investigations of the potential of this cytokine in the treatment of feline disease, particularly cytopenias associated with neoplasia, chemotherapy or viral disease (e.g. FeLV, FIV) and in the development of peripheral stem cell transplantation. The ability of fSCF to synergise with other cytokines *in vitro* suggests that it may be combined with other haemopoietic cytokines *in vivo* to provide more potent haemopoietic stimulation. Furthermore, the recombinant cytokine may be usefully employed to support *in vitro* growth of haemopoietic cells in this species and so facilitate their study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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DECLARATION

The studies described in this thesis were carried out in the Department of Veterinary Pathology at the University of Glasgow Veterinary School between October 1993 and March 1997. The author was personally responsible for all the work described herein except for the following. Serum biochemistry analyses described in chapter eight were performed by the staff of the Department of Veterinary Biochemistry, University of Glasgow Veterinary School. Haematology analyses described in chapter eight were performed by Ronnie Barron and Kenny Williamson in the Department of Veterinary Haematology, University of Glasgow. Examination of bone marrow, liver and spleen sections described in chapter eight was performed by Dr Sarah Toth in the Department of Veterinary Haematology, University of Glasgow. Processing of tissues for histopathology was performed by Mr I. Macmillan and the staff of the Histopathology Laboratory, University of Glasgow. Statistical analyses of results presented in chapters six and eight was performed by Professor Stuart Reid, Department of Veterinary Informatics and Epidemiology, University of Glasgow Veterinary School and University of Strathclyde. Endotoxin assays described in chapter five were performed by Q1 Biotech Ltd., West of Scotland Science Park, Glasgow.

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CHAPTER ONE - GENERAL INTRODUCTION

1.1 INTRODUCTION

The cytokines are a large family of proteins with pleiotropic regulatory effects on many cell types involved in host defence and repair processes. Cytokines affect cells in a complex network of both positive and negative interactions. An understanding of the actions of a single cytokine is not possible without consideration of these interactions. This concept is encompassed in the following insight by Rene Dubos: "in the most common and probably the most important phenomena in life, the constituent parts are so interdependent that they lose their character, their meaning and indeed their very existence when dissected from their functional whole" (quoted by De Maeyer and De Maeyer-Guignard, 1988). Consequently, this chapter aims to give an overview of the cytokine family and their role in haemopoiesis prior to considering the biological functions and therapeutic applications of the cytokine, stem cell factor, in greater detail.

1.2 THE CYTOKINES - AN OVERVIEW

1.2.1 THE CYTOKINES

The rapidly expanding field of cytokine research has arisen from the former independent disciplines involving the study of lymphokines, interferons, haemopoietic growth factors and the more classical growth factors. The study of soluble protein mediators, produced by lymphocytes in response to antigenic stimulation, was originally the domain of the immunologist; these substances were termed 'lymphokines' (Dumonde *et al.*, 1969). The recognition that monocytes produced similar proteins soon led to the complementary term 'monokines'. Interferons were originally described, in 1957, as proteins able to confer cellular resistance to viral infection (Isaacs and Lindenmann, 1957). Eight years later a functionally related, virus-inhibitory protein produced by mitogen activated T lymphocytes was described (Wheelock, 1965). This later became known as interferon-γ and subsequently its actions, and those of the other interferons, as regulators of a variety of immunological functions were recognised (reviewed by De Maeyer and De Maeyer-Guignard, 1994). The haemopoietic growth factors, were initially termed colony stimulating factors due to their effects on the promotion of colonies of granulocytes or macrophages from

bone marrow precursor cells (Robinson *et al.*, 1967); subsequently they have been shown to exert additional regulatory effects upon mature cells of the immune system. Classical growth factors, such as platelet derived growth factor, whilst having a largely different spectrum of activity also have effects which make them comparable to proteins within the other groups. In recognition of the role of these proteins as mediators between a variety of cell types within the immune system the descriptive term 'cytokine' was introduced (Cohen *et al.*, 1974). The term interleukin and a numbering system, introduced in 1979, helped to remove some of the confusing nomenclature (Aarden *et al.*, 1979).

It is clear that the cytokines form a large group of regulatory molecules with diverse functions, such that it is difficult to formulate a precise definition as to what constitutes a cytokine. Vilcek and Le (1994) proposed a working definition of cytokines as "regulatory proteins secreted by white blood cells and a variety of other cells in the body; the pleiotropic actions of the cytokines include numerous effects on cells of the immune system and modulation of inflammatory responses". This short definition is necessarily vague, however, consideration of key features exhibited by the cytokines enhances an understanding of their basic properties (Table 1.1).

Cytokines are polypeptides, which may be glycosylated. They are generally less than 30 kDa in size although may form oligomers.
The production of cytokines is not generally constitutive, but is induced by altering the level of transcription or translation.
Production of cytokines is generally transient and actions are mediated at close range in either an autocrine or paracrine fashion.
Actions are mediated via binding to high affinity cell-surface receptors.
Actions due largely to modulations in gene expression in target cells, leading to alterations in cell proliferation, differentiation or function.
Whilst their actions are often diverse, they generally include effects upon haemopoietic cells.

Table 1.1: The characteristic features of cytokines.

The production of each cytokine is not generally limited to a specific cell type. The range of target cells and spectrum of actions of most of the cytokines is usually broad, leading to the description of cytokines as pleiotropic. In addition the spectrum of actions of structurally dissimilar cytokines often overlap, leading to a large degree of redundancy. These features serve to differentiate the cytokines from the more classical endocrine hormones which are produced by specialised cells, act on target cells at some distance and typically show distinct and specific effects. The generation of specific responses by cytokines may, in part, rely upon their local production and sphere of action. Cells producing cytokines are often located close to their target cells (Metcalf, 1991a). Cytokines are produced in small quantities and production may be directed toward the responder cells (Poo *et al.*, 1988). Following interaction with its receptor, the cytokine is generally destroyed by receptor mediated endocytosis (Nicola *et al.*, 1988). Additionally, the effects of cytokines may be localised due to sequestering by extracellular matrix components or by their retention by the producing cell as membrane associated cytokines (Gordon, 1991). Circulating, soluble receptors may serve as a 'safety net' to inhibit the actions of cytokines that inadvertently reach the circulation or may serve as a mechanism to downregulate receptor levels (reviewed by Heaney and Golde, 1996).

1.2.2 CLASSIFICATION OF CYTOKINES

In order to rationalise the classification of cytokines, a system based upon their three-dimensional protein structure has been proposed (Bazan, 1990b; Bazan, 1991b; Parry *et al.*, 1991; Young, 1992; Boulay and Paul, 1993; Sprang and Bazan, 1993; reviewed by Nicola, 1994). This classification leads to four main groups of cytokines, as summarised in Table 1.2. Using this classification system common biological actions may be seen within groups of cytokines. This is suggested to arise, in part, due to shared receptor subunits (Nicola and Metcalf, 1991; Bazan, 1993; Gearing and Ziegler, 1993) or common intracellular signalling mechanisms (Murakami *et al.*, 1991).

Short chain 4-α-helical bundles cytokines (group 1) show effects involving immunohaemopoiesis. Cytokines, within this group acting via the common γ receptor

(e.g. IL-2, IL-4 etc.) influence acquired immunity via regulation of T/B lymphocyte or macrophage function. Those using the common β chain affect innate immunity via modulation of macrophage, neutrophil, eosinophil and mast cell activity. M-CSF and SCF affect the development of immature haemopoietic cells within the bone marrow. Those cytokines with a long chain 4-α-helical bundle structure are somewhat contradictory in nature. In the case of cytokines acting via the shared gp130 receptor subunit (IL-6, leukaemia inhibitory factor, etc.) they are highly pleiotropic. Other cytokines within the group are, however, highly specific (e.g. erythropoietin). This group also includes growth hormone and prolactin which are more typical of endocrine hormones.

Group 2 cytokines (long chain β-sheet structures) such as the nerve growth factor (NGF) and fibroblast growth factor (FGF) families are involved in growth and differentiation of a range of neural, epithelial and endothelial tissues. The tumour necrosis factor (TNF) family and IL-1 family of cytokines, however, are more immunomodulatory in action, with particular effects in the acute phase response to disease or injury.

The group 3 cytokines (short chain α/β) include the chemokines, the largest sub-family of cytokines, which act primarily as modulators of the innate arm of the immune system, involved in the attraction and activation of macrophages, neutrophils and eosinophils. This group also includes the epidermal growth factor class of cytokines, involved in epithelial cell proliferation and wound healing.

The group 4 cytokines (mosaic structures) exhibit effects that are reflected by at least one component of their mosaic structure. IL-12 has immunomodulatory properties typical of the 4-α-helical cytokines whilst the neuregulins (which have an immunoglobulin and an epidermal growth factor [EGF] like domain) have EGF like functions.

LEGEND:

BDNF	brain-derived neurotrophic factor
CNTF	ciliary neurotrophic factor
CSF	colony stimulating factor
CSF-1	colony stimulating factor-1 (M-CSF)
EGF	epidermal growth factor
EPO	erythropoietin
FGF	fibroblast growth factor
Flt	fms-like tyrosine kinase
G-CSF	granulocyte colony stimulating factor
GGF	glial growth factor
GH	growth hormone
GM-CSF	granulocyte macrophage colony stimulating factor
GRO	growth-related oncogene
HGF	hepatocyte growth factor
HRG	heregulin
IFN	interferon
IGF	insulin like growth factor
IL	interleukin
KGF	keratinocyte growth factor
LIF	leukaemia inhibitory factor
M-CSF	macrophage colony stimulating factor
MCP	macrophage chemoattractant protein
MIP	macrophage inhibitory protein
NDF	Neu differentiation factor
NGF	nerve growth factor
NT	neurotopin
OSM	oncostatin-M
PDGF-A/B	platelet derived growth factor
PRL	prolactin
RANTES	regulated upon activation, normal T cell expressed and secreted
SCF	stem cell factor
TGF	transforming growth factor
TNF	tumour necrosis factor
VEGF	vascular endothelial growth factor

CYTOKINE GROUP	*SUBSET*	*EXAMPLES*	*COMMENTS*
Group 1: 4-α helical bundle	Short chain 4-α helical bundle	IL-2, IL-4, IL-7, IL-9, IL-13.	share γ-chain receptor subunit.
		IL-3, GM-CSF, IL-5.	share β-chain receptor subunit
		SCF, M-CSF, flt-3.	Tyrosine kinase (III) receptor
		IFN-γ.	
	Long chain 4-α helical bundle	IL-6, LIF, OSM, CNTF, IL-11.	share gp130 receptor subunit
		EPO, G-CSF.	
		GH, PRL.	
		IL-10, IFN-α/β.	
Group 2: long chain β-sheet	Cysteine knot (dimers)	TGFβ, activin, inhibin.	
		PDGF-A/B, VEGF.	
		NGF, BDNF, NT-3.	
	β-jellyroll	TNF-α, TNF-β, FASL.	
	β-trefoil	IL-1α/β. FGF, KGF.	
Group 3: short chain α/β	S-S rich β-meander	EGF, TGF-α.	
	S-S rich α/β	IGF-I, IGF-II, insulin.	
	chemokines α/β/γ	CXC (α): IL-8, GRO, PF-4.	small protein mediators, sub-classified according to position and number of conserved cysteine residues
		CC (β): MCP-1/2/3, MIP-1α, RANTES.	
		Two cysteine (γ): lymphotactin	
Group 4: mosaic	Ig-EGF-TM-CYT (cell surface)	NDF, GGF, HRGs.	Effects generally compatible with at least one component of structure HGF and IL-12 are heterodimers
	4-kringle + serine protease like	HGF.	
	4-α helical + haemopoietin domain	IL-12.	

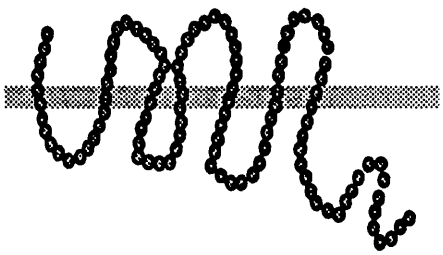
Table 1.2: Classification of the cytokine family based upon structural relationships. Adapted from Nicola (1994).

1.2.3 CYTOKINE RECEPTORS

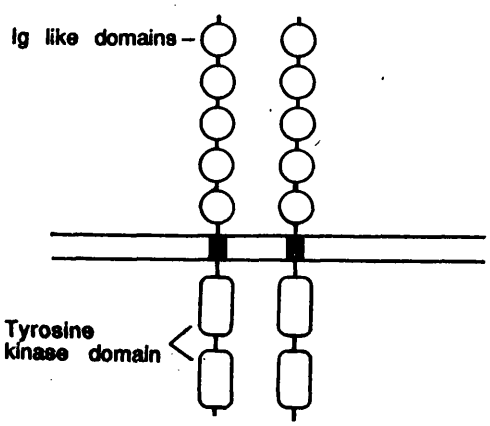
The ability of cytokines to influence cell growth and differentiation relies upon their interaction with specific receptors. Ligand-receptor interaction initiates a cytoplasmic signalling pathway which leads ultimately to changes in cellular function (Bazan, 1990b; Bazan, 1990a). Four major families of receptors have been described based upon similarities in protein sequence, predicted structure and biochemical function(s) (reviewed by Hilton, 1994): the haemopoietin/interferon receptor family, the receptor kinase family, the TNF/NGF receptor family and the family of G-protein coupled receptors. The key features of these receptors are shown in Figure 1.1.

1.2.3.1 G protein coupled receptors

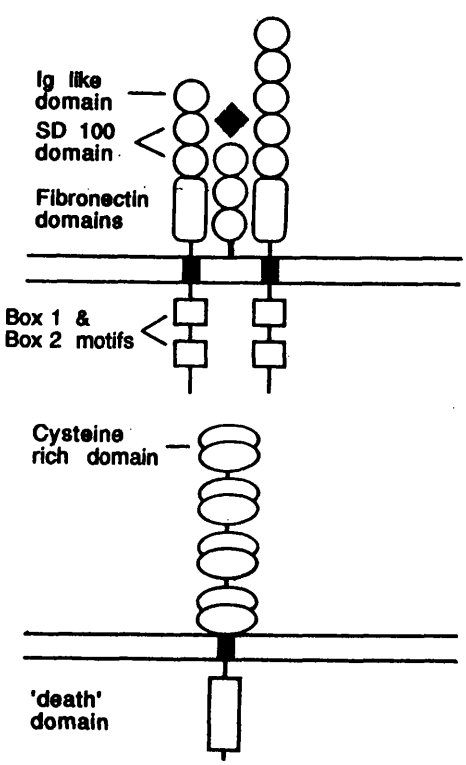
A variety of receptors mediate their intracellular actions via interaction with guanine nucleotide-binding proteins (G proteins), including those for a number of hormones (e.g. LH, PTH), neurotransmitters (e.g. adrenaline) and cytokines (e.g. IL-8). Despite this wide divergence, the G-protein coupled receptors show considerable structural homology based on the presence of seven hydrophobic areas of 20 - 25 amino acids, which form transmembrane α helices (Lefkowitz and Caron, 1988; Ross, 1989). The ligand binding domain may be extracellular or transmembrane in location. (Strader *et al.*, 1994). The binding of a ligand to its receptor produces a conformational change in the intracellular part of the receptor, permitting it to interact with a G protein. Binding of the G protein leads to activation of its α subunit, associated with the exchange of GTP for GDP (Bourne *et al.*, 1990; Bourne *et al.*, 1991). Upon activation the α subunit transduces an intracellular signal, the nature of which depends upon the subtype of the α subunit. Examples include the increase or decrease of cAMP levels (the biological effects of which are numerous, including the regulation of kinase activity) or the regulation of ion channels (Spiegel, 1992).



G-protein coupled receptors
Receptors have a transmembrane region which traverses the membrane seven times e.g. IL-8; endothelin receptors.



Receptor kinases
Receptors have cytoplasmic catalytic kinase domain which phosphorylates either tyrosine or threonine/serine residues.
e.g. SCF, M-CSF receptors.
(class III tyrosine kinase receptor shown)



Haemopoietin/interferon receptors
Receptors have common extracellular domains composed of 200 a.a. termed D200. High affinity receptor generally formed as multimeric complex. e.g. IFN, IL-2, EPO receptors.
(CNTF receptor shown)

NGF/TNF receptors
Extracellular region contains multiple copies of cysteine rich domains
e.g. TNF-R, CD40, CD30, FAS.

Figure 1.1: Classification of the cytokine receptor families and important characteristic features.

1.2.3.2 The receptor kinases

The receptor kinase family of receptors share a number of conserved structural features: a large glycosylated extracellular ligand binding domain, a single hydrophobic transmembrane region and a conserved cytoplasmic domain with intrinsic catalytic function, which catalyses the phosphorylation of either tyrosine or serine and threonine residues (Hanks *et al.*, 1988; Yarden and Ullrich, 1988; Williams, 1989; Ullrich and Schlessinger, 1990). The tyrosine kinase receptors may be further sub-classified based upon sequence and structural similarities (Figure 1.2).

These receptors share a common mechanism of activation and signal transduction (Schlessinger, 1988; Williams, 1989). Receptor - ligand interaction induces receptor oligomerisation which leads to apposition of the cytoplasmic domains of the oligomers and activation of their intrinsic kinase activity (Ullrich and Schlessinger, 1990). This activation leads to phosphorylation of specific amino acid residues in the cytoplasmic domain of the receptor. The phosphorylated residues are then able to interact with various cytoplasmic proteins and so initiate an intracellular signalling pathway which ultimately results in alteration of cellular function.

1.2.3.3 The haemopoietin/interferon receptor family

The haemopoietin/interferon receptor family comprises the majority of cytokine receptors and has thus been named the cytokine receptor superfamily. The family may be further subclassified into class I (haemopoietic or cytokine/growth hormone/prolactin receptor family) or class II (interferon family) (Bazan, 1990a). The receptors are thought to have arisen from a common ancestral gene which has been extensively modified and duplicated and so share certain common features (Nakagawa *et al.*, 1994). The receptors have a single hydrophobic transmembrane domain, a highly variable cytoplasmic domain and an extracellular domain with certain conserved features. Many of the receptors contain extracellular immunoglobulin-like or fibronectin type III domains, however, the major region of homology lies within one or two domains of 200 amino acids (D200). The D200 domain may be further divided into homologous sub-domains of 100 amino acids (SD100) (Bazan, 1990a;

Thoreau *et al.*, 1991). The intracellular domains have regions of limited homology in the membrane-proximal region, known as box 1 and box 2 motifs (Bazan, 1990a).

The extracellular domain of the class I receptors is further defined by the presence of four positionally conserved cysteine residues and a Try-Ser-Xaa-Try-Ser (WSXWS) motif (Bazan, 1990a). The conserved cysteine residues are thought to form disulphide bonds which contribute significantly to receptor tertiary structure. The function of the WSXWS motif, however, remains elusive, but it may serve to direct proper intracellular transport (Yoshimura *et al.*, 1992) or efficient folding of the precursor protein (Hilton *et al.*, 1996). The class II receptors show similar characteristic cysteine residues, in pairs, at the amino and carboxyl termini of their SD-200 domains (Bazan, 1990a)

The members of this receptor family are predicted to adopt a similar tertiary conformation, whereby the SD-100 domains form a 'barrel' like structure. The ligand interaction site is predicted to be formed by the hinge region connecting two adjacent 'barrels' (Bazan, 1990a). This prediction was confirmed in the case of growth hormone receptor and its ligands, growth hormone and prolactin, upon the discovery of the crystal structure of the receptor-ligand complex (De Vos *et al.*, 1992; Somers *et al.*, 1994).

These structural studies also served to introduce the concept of receptor oligomerisation as a necessary prerequisite to signal transduction; one molecule of growth hormone was found to form a complex with two receptor molecules (De Vos *et al.*, 1992). Subsequently, receptor oligomerisation has been demonstrated in the majority of cytokines within this family. In most cases, receptors consist of a multichain complex, formed by different subunits, utilising a 'private' ligand-specific receptor chain and a 'public' class-specific signal transducing chain (Kishimoto *et al.*, 1994). For example, GM-CSF, IL-3 and IL-5 each bind to a specific α-subunit with low affinity. A high affinity receptor complex, capable of signal transduction, is formed upon subsequent interaction with a common β-subunit (reviewed by Miyajima *et al.*, 1992a, Miyajima *et al.*, 1992b).

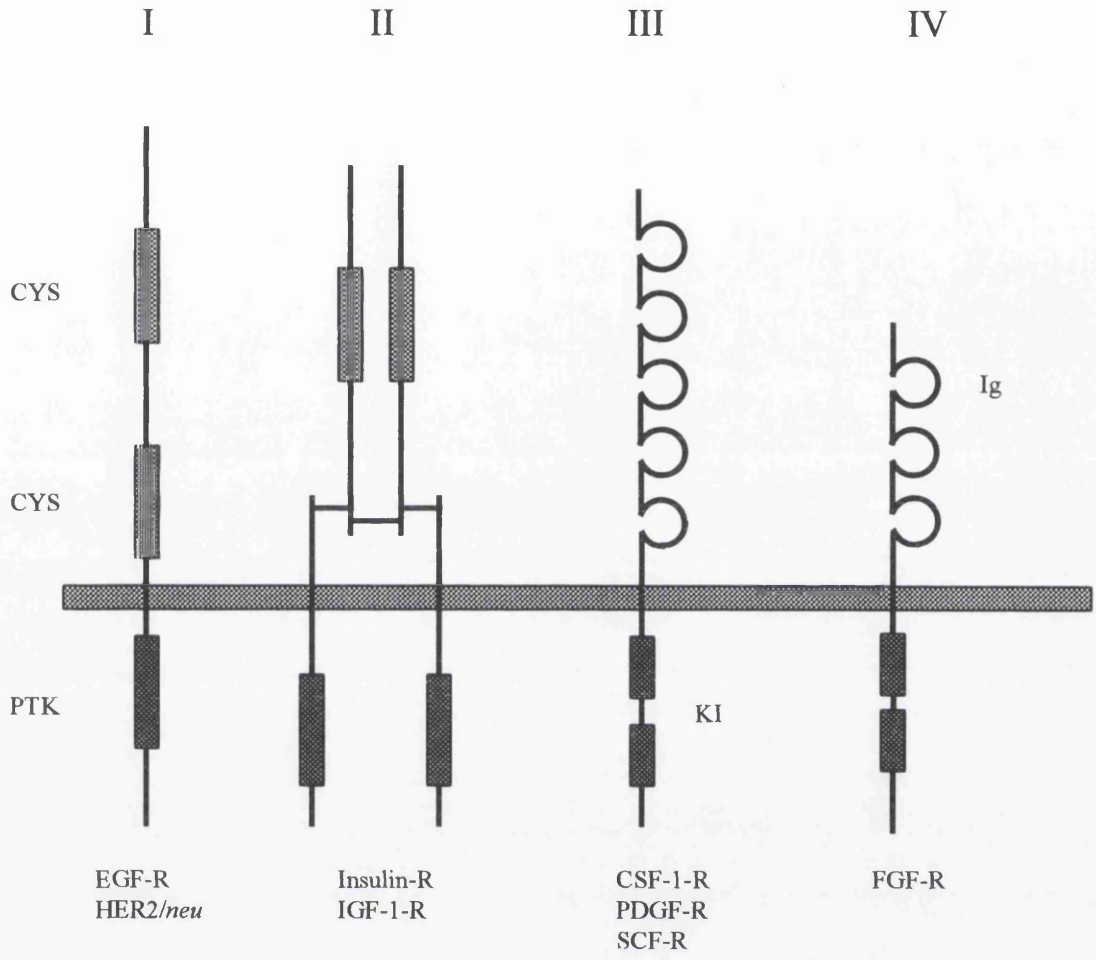


Figure 1.2: Classification of the tyrosine kinase receptor superfamily: diagrammatic representation of the receptor sub-classes. Examples of the members of each receptor subclass are shown. Class I receptors have two cysteine rich extracellular domains. Class II receptors have a heterotetrameric structure ($\alpha_2\beta_2$) with similar cysteine rich domains. Class III and IV receptors are characterised by the presence of five or three extracellular immunoglobulin-like domains, respectively and the presence of a split tyrosine kinase domain. Adapted from Ullrich and Schlessinger, (1990). Up to six further classes of tyrosine kinase receptor have been recognised, although all the cognate ligands have yet to be cloned (Hilton, 1994).

LEGEND:

CYS: cysteine rich domain
Ig: immunoglobulin like domain
PTK: tyrosine kinase domain
KI: kinase insert.

CSF1-R: colony stimulating factor - 1 receptor
SCF-R stem cell factor receptor
PDGF-R: platelet derived growth factor receptor
FGF-R fibroblast growth factor receptor
EGF-R: epidermal growth factor receptor
IGF-1-R: insulin like growth factor receptor

1.2.3.4 TNF/NGF receptors

The TNF/NGF receptor superfamily includes the type I and II TNF receptors, the p75 subunit of the NGF receptors, and the CD-30, CD-40 and FAS receptors. The members of this family exhibit low homology (25 - 30%) but are grouped together due to the presence of conserved extracellular cysteine rich domains (CRD) (reviewed by Bazan, 1993, Smith *et al.*, 1994, Gruss and Dower, 1995). The receptors have variable numbers of CRDs (typically four), each of which has approximately six cysteine residues within a stretch of 40 amino acids. In addition, limited sequence similarity exists within the cytoplasmic domain of the p55 TNF receptor and FAS over a region of 65 - 80 amino acids. This region has been called the 'death domain' due to its role in the generation of apoptotic cell death (Tartaglia *et al.*, 1993). Functional membrane associated receptors form a trimeric or multimeric structure, stabilised by intermolecular disulphide bonds (Banner *et al.*, 1993). In addition many members of this family of receptors (e.g. TNFR-I, TNFR-II, CD30, CD40, FAS) also exist as soluble moieties. The generation of the soluble isoform may occur via proteolytic cleavage of the membrane associated receptor or by the use of alternatively spliced mRNAs (reviewed by Heaney and Golde, 1996).

1.3 HAEMOPOIESIS

Circulating blood cells are generally short lived. The circulating lifespan of neutrophils in the cat is approximately 5 - 10 hours (Prasse *et al.*, 1973). Haemopoiesis (or haematopoiesis) is the process whereby mature blood cells are continuously produced throughout life. During embryonic life haemopoietic cells develop within the yolk sac from mesodermal cells. Haemopoiesis occurs in 'blood islands' which consist of an outer endothelial layer, a central core of haemopoietic cells and an inner layer of endoderm. As fetal development progresses the main site of haemopoiesis becomes the fetal liver and later the bone marrow. In the adult mammal, the primary organ of haemopoiesis is the bone marrow, although the spleen and liver retain a capacity for extra-medullary haemopoiesis, which may be required during periods of increased demand. These changes in the predominant site of haemopoiesis are associated with the migration of immature stem cells and subsequent "seeding" of a new site of production (reviewed by Zon, 1995). Mature blood cells of all lineages ultimately originate from a select population of pluripotent stem cells. These stem cells develop into a series of increasingly specialised progenitor cells by a process of clonal proliferation and differentiation. The progenitor cells subsequently differentiate to produce mature blood cells which are released into the circulation. Thus, within the bone marrow a structured hierarchy of developing cells may be envisaged. This is represented diagrammatically in Figure 1.3. These developing cells do not exist in isolation within the bone marrow, but are found in association with stromal cells which form the haemopoietic microenvironment.

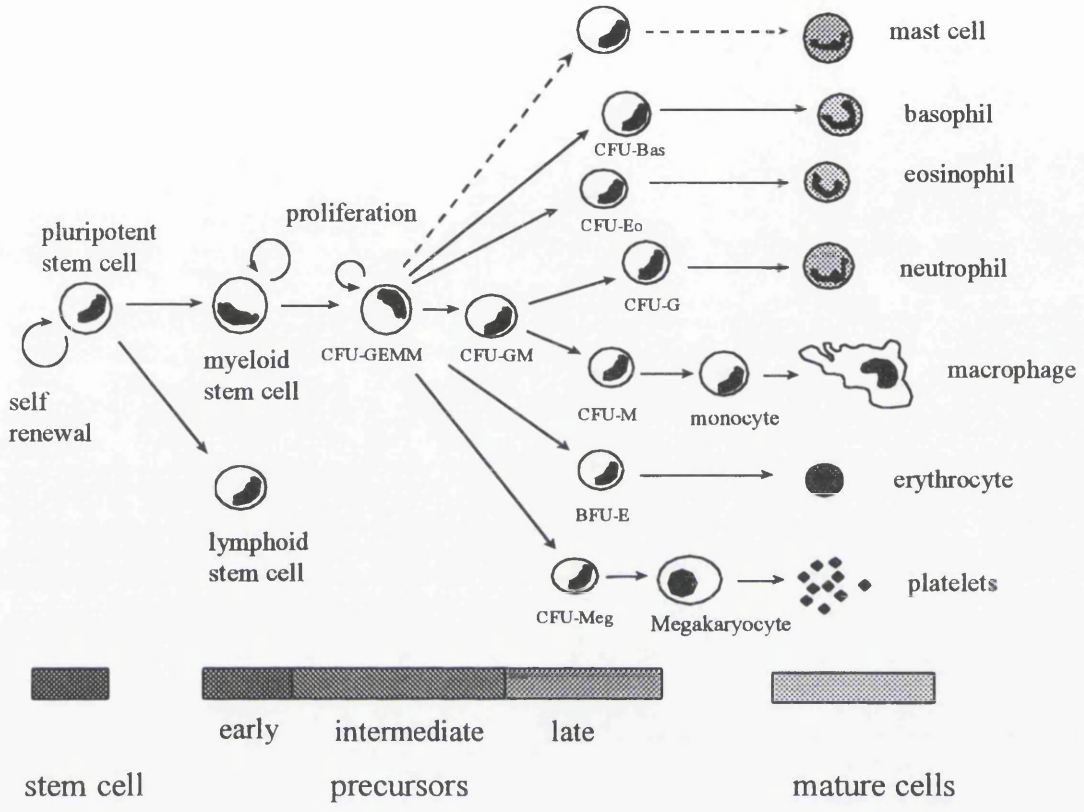


Figure 1.3: Diagrammatic overview of haemopoiesis. Bone marrow haemopoietic cells consist of three main populations of cells: stem cells, progenitor cells and mature cells. The stem cells are the least differentiated and have the highest capacity for self renewal. The more primitive progenitor cells are capable of differentiation into cells of a number of lineages whilst the more committed progenitors are lineage restricted.

LEGEND:

BFU-E	burst forming unit erythroid
CFU	colony forming unit
CFU-Bas	colony forming unit - basophil
CFU-Eo	colony forming unit - eosinophil
CFU-E	colony forming unit - erythroid
CFU-GEMM	colony forming unit - granulocyte erythroid macrophage monocyte
CFU-GM	colony forming unit - granulocyte macrophage
CFU-M	colony forming unit - monocyte
CFU-Meg	colony forming unit - megakaryocyte

1.3.1 PLURIPOTENT HAEMOPOIETIC STEM CELLS

Pluripotent haemopoietic stem cells (PHSCs) have been defined as "cells with an extensive proliferative potential and a capacity to develop into cells of all lineages" (Quesenberry, 1992; Orlic and Bodine, 1994, and references therein). PHSCs are able to proliferate and produce either more stem cells (a process known as self renewal) or may differentiate to produce progenitor cells. This potential for self renewal provides the capacity for the generation of mature blood cells throughout life. The initial evidence for the existence of PHSCs originated from studies on patients with myeloid leukaemia. Cells of all lymphohaemopoietic lineages showed an identical chromosomal abnormality (the Philadelphia chromosome) suggesting that the cells were derived from a single leukaemic cell. Subsequently, the existence of normal PHSCs was provided by demonstration of an identical, radiation induced chromosomal abnormality in all lymphohaemopoietic lineages in mice following bone marrow transplantation (Wu *et al.*, 1968).

The study of the biological properties of the PHSC is fundamentally difficult due to the low numbers of stem cells present in the bone marrow (about 1 in 10^4 nucleated bone marrow cells) and absence of a distinct morphology. Early studies into stem cell biology were prompted by the use of radiation for military and peaceful purposes. It was recognised that mice given a lethal dose of irradiation suffered bone marrow failure which could be reversed by the injection of non-irradiated bone marrow cells. Recovery from lethal irradiation was associated with the development of macroscopic nodules within the spleen, consisting of multiple cell lineages. The cells responsible for generating these nodules were termed colony forming unit-spleen (CFU-S) (Till and McCulloch, 1961). Furthermore, using radiation induced chromosomal markers it was demonstrated that all cells within a single colony were derived from a single CFU-S (Becker *et al.*, 1963; Wu *et al.*, 1967). Thus the concept of cells with the potential to produce large numbers of daughter cells of multiple lineages was firmly established. However, it soon became apparent that CFU-S constituted a heterogeneous group of cells, some more primitive than others and with different degrees of repopulating ability. The CFU-S which produce later appearing colonies (day 12 CFU-S) share many of the characteristics of PHSCs. However, they differ

from PHSCs in several important respects. No study has convincingly demonstrated that CFU-S are the progenitors for cells of the lymphoid system and the frequency of murine PHSC appears lower than that of day 12 CFU-S (Jones, 1992; Keller, 1992). Moreover, studies using the cytotoxic agent, 5-fluorouracil (5-FU) have demonstrated that 99.5% of CFU-S are killed by a single injection of 5-FU but that cells with long term repopulating ability survive (Orlic and Bodine, 1994).

In vitro assays have provided a great deal of information regarding haemopoiesis in general. In long term bone marrow cultures, the long-term culture initiating cell (LTC-IC) has been shown to have many of the properties of the PHSC. It can give rise to multipotential clonogenic cells for several months in culture and exists in the bone marrow at a similar frequency to PHSCs. However, the ability of cultured cells to repopulate mice declines in these long term cultures, likely associated with a decrease in PHSC number (Orlic and Bodine, 1994).

The study of PHSCs has been advanced further by the development of techniques to purify these cells. Methods used include separation based on physical characteristics such as cell density, sensitivity to drugs (e.g. 5-FU) and display of surface glycoproteins. However the use of monoclonal antibodies to cell surface antigens has provided a more potent tool with which to attempt identification and isolation of PHSCs. Initial separation techniques used included immune adherence and immunomagnetic bead separation, but the development of fluorescent-activated cell sorting (FACS) has provided a more powerful method to attempt purification of the PHSC. The PHSC population is enriched in cells lacking specific lineage makers [e.g. Mac (myelomonocytic cells), CD4, CD8 (T lymphocyte), B220 (B lymphocyte)]. Other important markers used to identify PHSCs include Sca-1, CD34, Thy-1, CD33, HLA, and *c-kit.* However the isolation of a definitive PHSC, in terms of phenotype, may be impossible due to the heterogeneity of the PHSC population, when assessed in this way (Leftwich *et al.,* 1992; Orlic and Bodine, 1994).

An important subset of PHSCs are those present in the peripheral blood at approximately 2 - 10% of the levels present in bone marrow (Goodman and Hodgson, 1962; McCredie *et al.,* 1971). These cells are termed peripheral blood stem cells

(PBSCs) and are able to successfully repopulate the bone marrow of transplanted hosts (Goodman and Hodgson, 1962). The numbers of PBSC are increased following high dose chemotherapy and by a number of haemopoietic growth factors. PHSC are also present in the adult liver (Taniguchi *et al.*, 1996) and placental and umbilical cord blood (Nakahata and Ogawa, 1982).

1.3.2 PROGENITOR CELLS

The developing cell population consists of more primitive multipotential progenitor cells [e.g. colony forming unit - granulocyte erythroid macrophage monocyte (CFU-GEMM)] which differentiate into a series of unipotential progenitor cells [e.g. colony forming unit - monocyte (CFU-M)]. These more mature progenitors have a high capacity for cell division, with each progenitor cell capable of producing up to 10^5 immature cells of a specific lineage (Metcalf, 1989). These immature cells develop further to give functionally mature cells. The numbers of precursor cells and their high proliferative capacity likely provides the haemopoietic system with a large reserve for the generation of mature blood cells, which may be utilised in the event of increased demand (Necas *et al.*, 1995).

The study of progenitor cells has been possible due largely to the development of techniques to culture the cells *in vitro*. Progenitor cells can be grown in agar or methylcellulose assays in the presence of stimulating factors. These factors were initially provided by feeder cells or from culture medium conditioned by the growth of certain cells (e.g. lectin stimulated lymphocytes). Subsequently, the production of purified haemopoietic growth factors, by purification to homogeneity or more recently by recombinant DNA techniques has provided valuable reagents with which to culture progenitor cells. Progenitor cells cultured *in vitro* proliferate and differentiate to produce phenotypically and functionally mature cells. Clonal assays may therefore be used to define the nature of and quantify numbers of progenitor cells.

As mentioned earlier a development hierarchy of haemopoietic cells exists within the marrow. *In vitro* colony assays have demonstrated the presence of distinct cell types with different degrees of differentiation and potential for proliferation. Amongst the

most primitive of cells to be cultured in this assay system is the high proliferative potential colony forming cell (HPP-CFC). These cells are relatively resistant to 5-FU, require multiple growth factors for growth support and give rise to large macroscopic colonies of macrophages (over 5 x 10^4 cells). They are able to generate myeloid, erythroid and megakaryocytic precursors in culture (Bertoncello, 1992). The generation of mixed colonies in culture is also a feature of the primitive progenitor CFC-GEMM (CFC-Mix). More differentiated progenitors may give rise to two cell types (e.g. CFC-GM) or a single cell type. Erythroid progenitors with high (BFU-E) or lower (CFU-E) proliferative potential have also been recognised (Stephenson *et al.*, 1971; Heath *et al.*, 1976). The relationship of progenitor and stem cells, as identified by different bioassays is shown in Figure 1.4.

The development of cell lines with characteristics of stem and progenitor cells has provided a powerful model enabling study of mechanisms of differentiation and self-renewal (Dexter *et al.*, 1980; Spooncer *et al.*, 1986). Cell lines, designated factor dependent continuous cell lines, Paterson Laboratories (FDCP), require growth factors for survival (typically IL-3), are non-malignant and can differentiate into cells of multiple lineages. In the absence of IL-3, FDCP-Mix cells die by apoptosis (Williams *et al.*, 1990). However, when cultured over a layer of feeder cells (3T3 cells) they can survive and differentiate in the absence of IL-3, the feeder cells supplying the necessary survival signals (Yamazaki *et al.*, 1989). Differentiation of FDCP-Mix cells into mature haemopoietic cells can be effected by the addition of growth factors and the lineage into which these cells develop can be influenced by the growth factors used. For example, in the presence of IL-3, EPO and haemin, cells develop along the erythroid lineage, whilst IL-3, GM-CSF and G-CSF stimulate development of these cells into neutrophils (Kan *et al.*, 1993).

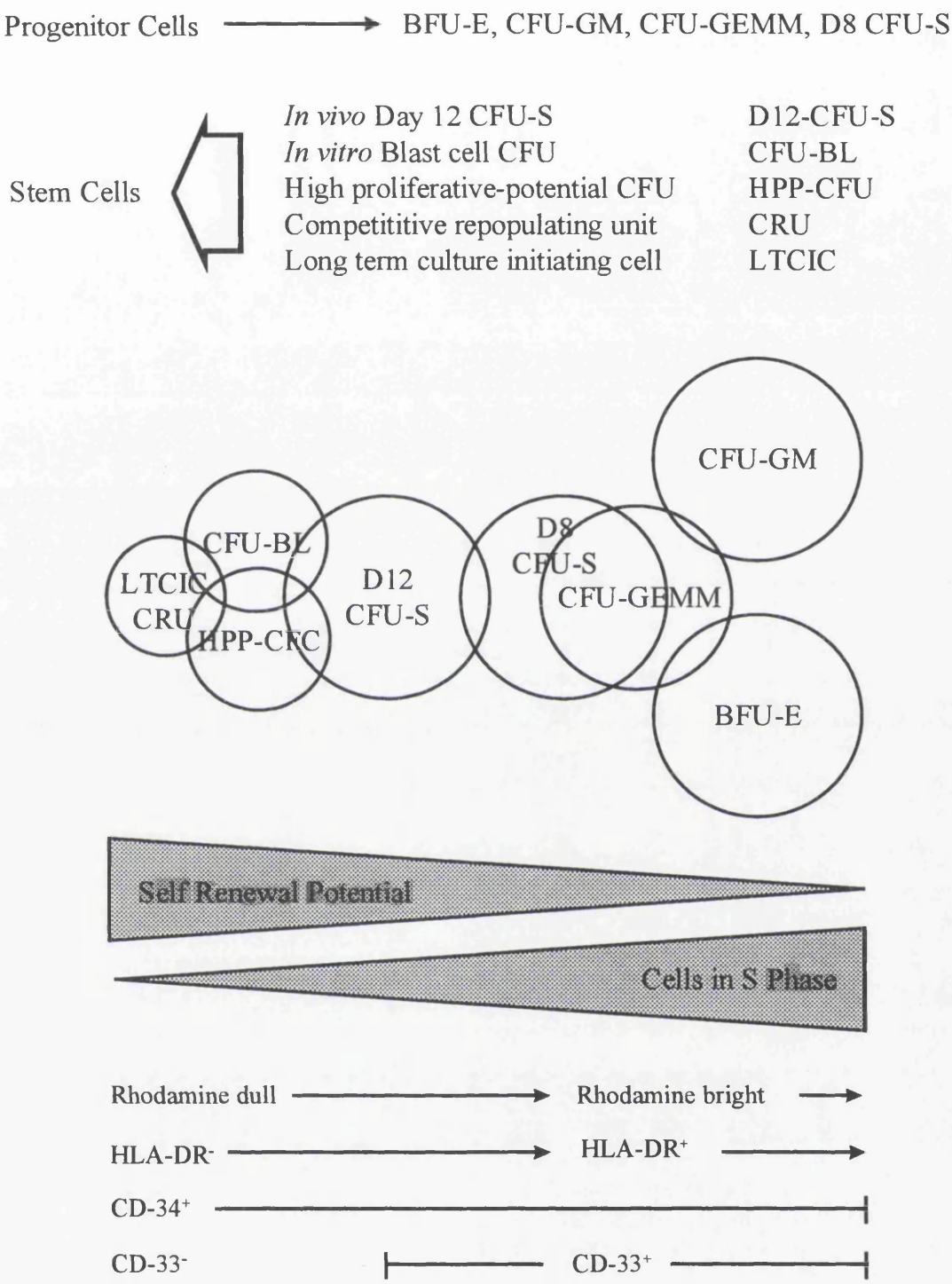


Figure 1.4: Diagram to show inter-relationships between stem and progenitor cell populations as determined by a number of assays. Adapted from Moore (1991).

1.3.3 THE HAEMOPOIETIC MICROENVIRONMENT

Haemopoiesis within the bone marrow of adult mammals occurs within extravascular spaces between marrow sinuses. The sinus wall is composed of an inner luminal layer of endothelial cells and an outer layer of reticular adventitial cells. The endothelial cells form a complete layer, with adjacent cells overlapping and having distinct cell junctions. The adventitial reticular cells have extensively branching cytoplasmic processes which envelop the outer wall of the sinus to form an adventitial sheath. This sheath provides incomplete coverage of the abluminal surface of the sinus; regulation of this coverage may be important in controlling cell egress from the bone marrow. The reticular cells provide physical support for haemopoietic cells and may have inductive and regulatory functions. Together the endothelium and reticular cells form the "blood-bone barrier". Endothelial and reticular cells belong to a subset of non-haemopoietic cells within the bone marrow called stromal cells. Other stromal elements include marrow fibroblasts, adipocytes and macrophages (Lichtman, 1984).

Studies of stromal cell cultures have provided evidence establishing their importance in haemopoiesis. Long term bone marrow cultures (LTBMC) established in a nutrient rich medium with hydrocortisone and horse serum (Dexter cultures) consist of an adherent stromal cell layer and adherent and non-adherent haemopoietic cells. The stromal cells form a heterogeneous population consisting of cells which morphologically resemble endothelial cells, fibroblasts, adipocytes and macrophages. The stromal layer supports the growth of mature myeloid cells, multipotent myeloerythroid progenitors, progenitors of B and T lymphocytes and even pluripotent stem cells (Dexter *et al.*, 1984). Murine lymphoid (B-cell) cultures can be established using the system established by Whitlock and Witte (1982), where cultures are established in a nutrient poor medium using fetal calf serum with no hydrocortisone. It has further been established that the stromal layers established in either culture system are able to support the growth of haemopoietic cells supported in the other system following appropriate changes in the growth medium. Such cultures are termed 'switch cultures'. The development of stromal cell lines have also provided a valuable tool to study stromal cells *in vitro*.

Stromal cells appear to support haemopoiesis in three main ways: direct cell-cell interactions, production of extracellular matrix and production of soluble mediators. Direct examination of bone marrow has shown that stromal cells and haemopoietic cells are intimately related. Reticular cell processes interact with developing granulocytes, whilst developing red blood cells are found in association with clusters of macrophages in "erythroblastic islets". Similar associations of stromal and haemopoietic cells are seen in LTBMC, where progenitor cells develop in close association with the adherent stromal layer. Stromal cells express surface molecules such as vascular cell adhesion molecule 1 (VCAM-1), fibronectin and glycoproteins; these molecules have been implicated in mediating attachment of progenitor cells to the stroma. The attachment of CD34+ cells expressing very late antigen-4 (VLA-4) to stromal cell VCAM-1 can be blocked by antibody to VLA-4 resulting in delayed myelopoiesis in Dexter cultures (Quesenberry, 1992; Deryugina and MullerSieburg, 1993). Stromal cells also express membrane associated cytokines including M-CSF and SCF. These may mediate attachment of cells expressing the appropriate receptor and may also act as growth factors for developing haemopoietic cells. The extracellular matrix produced by stromal cell lines contains collagen, laminin, fibronectin and proteoglycans such as heparan sulphate and chondroitin sulphate. Such components may facilitate adhesion of haemopoietic cells, aid transfer of nutrients and result in the local sequestering of cytokines. It has recently been shown that the survival of human LTC-ICs in long term stromaless culture (in the presence of picogram quantities of cytokines) is greatly enhanced by the addition of purified, stromal cell derived, heparan sulphate (Gupta *et al.*, 1996).

Stromal cell lines produce a variety of different cytokines, both constitutively and following induction by external factors. Cytokines produced include GM-CSF, G-CSF, M-CSF, SCF, IL-6, IL-7, IL-8, IL-11 and TGF-β (Deryugina and MullerSieburg, 1993 and references therein). The use of LTBMCs, where the stromal layer is physically separated from haemopoietic progenitors by a microporous membrane have shown that direct stromal cell contact is not essential for the maintenance of haemopoiesis. In fact, primitive progenitors are conserved to a greater extent in such conditions, than when cultured in direct contact with the

stroma. However, absence of contact with a stromal layer results in the accumulation of granulocyte-macrophage progenitors at the expense of a decrease in production of mature blood cells (Verfaillie, 1992). Additionally, enriched populations of primitive human haemopoietic cells ($CD34^+$, $HLA\text{-}DR^-$) may be maintained in long term suspension cultures, in the absence of an adherent stromal layer by the regular addition of cytokines such as IL-1α, IL-3 and IL-6 (Brandt *et al.*, 1990). Similarly, cytokines including IL-3 and G-CSF enhance the survival of CFU-S in suspension cultures; an effect which can be specifically blocked by addition of the appropriate antibody (Bodine *et al.*, 1991).

1.3.4 DEVELOPMENT OF FUNCTIONALLY MATURE CELLS

Committed progenitor cells generate mature blood cells by a process of further cell division and cellular differentiation. The developing cells have characteristic morphological features that may be identified by light microscopy. The generation of mature neutrophils, for example, progresses through a series of morphologically distinct cell types: myeloblast, promyelocyte, myelocyte, metamyelocyte, band neutrophil and neutrophil. This entire process from myeloblast to mature neutrophil, takes approximately 125 hours in the cat (Testa *et al.*, 1983). During maturation the cells become functionally mature and show changes in various cellular attributes such as cell surface receptor expression, glycoprotein expression, histocompatability antigens and elements of the cytoskeleton. Band and segmented neutrophils within the marrow constitute the marrow granulocyte reserve. This reserve comprises the majority of nucleated cells within feline bone marrow and provides the capacity for a rapid response to increased demand for circulating neutrophils. Mature cells exit the bone marrow through the endothelial cells of the marrow sinuses. Release of neutrophils from the bone marrow is influenced by a number of factors, including anatomical location of the cell, reticular cell activity, cell characteristics (e.g. surface charge, deformability) and the influence of neurohumoral factors. Mature neutrophils show decreased cell surface charge and increased deformability and motility which induces their release from the marrow (Jain, 1993a).

The development of mature erythrocytes is associated with the extrusion of their cell nuclei and phagocytosis by adjacent macrophages (nurse cells). Platelets are produced in mammals by cytoplasmic budding from megakaryocytes. Mature, productive megakaryocytes extend cytoplasmic processes through the endothelial lining of the sinusoids into the lumen and platelets are released by a "pinching-off" process. The elaboration of mature circulating blood cells in the domestic species is discussed further by Jain, (1993b).

1.3.5 CONTROL OF HAEMOPOIESIS

A number of lines of evidence, discussed above, implicate the importance of known cytokines and potentially other, as yet uncharacterised soluble mediators in the control of haemopoiesis. *In vivo* the situation is likely to be extremely complex, with all cells in the marrow potentially able to produce cytokines that may modulate haemopoiesis either directly or by modulating the cytokine profile produced by other cells. The haemopoietic system is able to respond to increased demand for mature blood cells by increasing cell production and release appropriately. This modulation of function is mediated largely by cytokines. Perhaps the most understood example of cytokine control of haemopoiesis is the control of red blood cell (RBC) production by erythropoietin (EPO). Tissue hypoxia (for example caused by anaemia or altitude) leads to increased EPO production (by peritubular interstitial cells of the kidney) resulting in an increased in RBC production and thus an increase in oxygen carrying capacity of the blood. A similar modulatory mechanism occurs in response to bacterial infections. Infection with gram negative bacteria provides a potent stimulus for the production and release of neutrophils from the bone marrow mediated by the effect of bacterial cell wall lipopolysaccharide (endotoxin). Endotoxin causes release of a number of cytokines including IL-1β, IL-6, TNF-α and G-CSF, the latter of which is potent inducer of neutrophil production and release from the bone marrow (Hack *et al.*, 1989; Cannon *et al.*, 1990; Dale *et al.*, 1992). The role of cytokines in the regulation of haemopoiesis is discussed further in chapter six.

A potential role of the central nervous system in the regulation of haemopoiesis has been postulated, based on the observation of afferent and efferent nerve fibres within

the marrow and their apparent connection to stromal cells. Substance P (SP) is an eleven amino acid neural peptide belonging to the tachykinin family. Stromal cells display high-affinity SP receptors and SP has been shown to increase stromal production of SCF and IL-1. In addition SP promotes the release of IL-3 and GM-CSF from bone marrow mononuclear cells (reviewed in R & D Systems Bulletin, 1995).

1.4 BIOLOGICAL FUNCTIONS OF STEM CELL FACTOR AND ITS RECEPTOR *C-KIT*

Stem cell factor is the ligand for the tyrosine kinase receptor encoded by the *c-kit* gene. Stem cell factor (SCF) has also been named kit ligand (KL), mast cell growth factor (MGF) and steel factor (SLF or SF); the term SCF is used throughout this thesis. An understanding of the biological importance of SCF and its receptor (SCF-R) has been derived from three main areas. The occurrence of spontaneous mutations in inbred mice causing multiple defects was recognised long before the cloning of the genes for SCF and SCF-R. Subsequent to the cloning of SCF cDNAs it has been possible to use recombinant DNA technology to produce large amounts of recombinant protein with which to study the effects of SCF both *in vitro* and *in vivo*.

1.4.1 HEREDITARY ANAEMIAS OF MICE

The study of mice harbouring mutations at the steel (*Sl*) locus on chromosome 10 and the dominant white spotting (*W*) locus on chromosome 5 has provided great insights into the physiological role of stem cell factor and its receptor *c-kit*. Mutations at each locus lead to similar phenotypes, with an array of abnormalities encompassing haemopoietic, pigment and germ cells (reviewed by Russell, 1979).

1.4.1.1 Mutations at the *Sl* locus

A mutation at the *Sl* locus causing abnormalities in haemopoiesis, fertility and coat colour was first described by Sarvella and Russell in 1956. Homozygous mice have a severe macrocytic anaemia that develops prenatally and leads to death *in utero*. The mice lack primordial germ cells (Bennett, 1956) and transplantation experiments have shown that their skin is unable to support the growth of melanocytes (Mayer and

Green, 1968). Heterozygous *Sl*/+ carriers have a mild macrocytic anaemia and diluted hair pigment but remain fertile although their gonads are reduced in size. Other mutations at the *Sl* locus include Sl^d (Steel-Dickie); affected homozygotes are severely anaemic but survive to adulthood, have black eyes and white hair and are sterile (Bernstein, 1960).

1.4.1.2 Mutations at the *W* locus

Since the first mutation at the *W* locus was reported by de Alberle in 1927, a large number of mutations have been independently identified at this locus. Mice homozygous for the original *W* mutation die perinatally with a severe macrocytic anaemia, lack coat pigment and show failure of germ cell development. Heterozygous *W*/+ carriers have normal haematological parameters and are fertile but may be identified by the presence of a white spot, frequently occurring on ventral abdomen. Other mutations at the *W* locus give rise to viable homozygotes with a variable phenotype. Mice may show abnormalities in all of skin, haemopoietic and reproductive tissues, with varying severity, or may have abnormalities largely confined to two of these systems (Little and Cloudman, 1937; Guenet and Mercier-Balaz, 1975; Geissler *et al.*, 1981).

1.4.1.3 Haemopoiesis in *Sl* and *W* mutant mice

Analysis of bone marrow cellularity in W/W^v and Sl/Sl^d adult mice compared to their normal counterparts reveals a reduction in cell numbers affecting all of myeloid, erythroid and megakaryocyte precursors. The bone marrow of W/W^v or Sl/Sl^d mice shows a reduction in cellularity to between 50% and 75% of normal levels (Ebbe *et al.*, 1972; Ruscetti *et al.*, 1976). Despite the pancellular nature of the marrow hypocellularity, circulating levels of granulocytes remain normal in affected W/W^v mice (Lewis *et al.*, 1967), whilst Sl/Sl^d mice show only a modest reduction (Ruscetti *et al.*, 1976). Similarly, the number and size of circulating platelets in both W/W^v and Sl/Sl^d mice is comparable to +/+ mice (Lewis *et al.*, 1967; Ebbe *et al.*, 1972; Ebbe *et al.*, 1973). Mast cell numbers are profoundly reduced in both W/W^v and Sl/Sl^d mice at all body sites, with levels in the skin reduced to less than 1% when compared to +/+ mice and mast cells rarely found at other sites (e.g. stomach, bone marrow, liver) (Kitamura *et al.*, 1978; Kitamura and Go, 1979).

A key property of the haemopoietic system is the ability to respond to alterations in the demand for mature circulating cells of all lineages by the appropriate modification of bone marrow or splenic activity. This homeostatic function has been investigated in *W/W^v* and *Sl/Sl^d* mice with respect to erythroid and megakaryocyte lineages. The induction of erythrocyte production by hypoxia, blood loss or phenylhydrazine treatment has been demonstrated in mice of both genotypes, although mutant mice generally showed a delayed erythropoietic response (Harrison and Russell, 1972). *W/W^v* mice and *Sl/Sl^d* mice also show effective regulation of platelet numbers in response to the induction of thrombocytopenia by injection of anti-platelet serum (Ebbe and Phalen, 1978; Ebbe *et al.*, 1978). These studies show that whilst SCF is important in the regulation of haemopoiesis in normal mice, homeostasis is maintained to a large degree in mutant mice, presumably due to the presence of other compensatory mechanisms.

1.4.1.4 Correction of defects in *Sl* and *W* mice by tissue transplantation

The injection of histocompatible haemopoietic cells derived from bone marrow, spleen or fetal liver into *W/W^v* mice leads to a permanent resolution of their anaemia (Russell *et al.*, 1959). Analysis of these mice, two to four months after marrow transplantation, reveals a shift in cell population of haemopoietic tissues to that of the donor genotype (Harrison and Cherry, 1975; Harrison and Astle, 1976) and also cure of their mast cell deficiency (Kitamura *et al.*, 1978). Conversely, whilst the injection of sufficient numbers of *W/W^v* marrow or spleen cells into lethally irradiated +/+ mice was able to prevent death in recipients, surviving mice showed haematological values typical of *W/W^v* mice (Harrison, 1972). Such experiments provided strong evidence localising the defect in *W/W^v* mice to the haemopoietic cells themselves, at or near the level of the pluripotent stem cell.

Implantation of histocompatible +/+ marrow cells into *Sl/Sl^d* mice does not reverse their characteristic anaemia (Bernstein, 1970). However, transplantation of *Sl/Sl^d* marrow into lethally irradiated +/+ recipient mice resulted in their survival and transplantation into *W/W^v* mice led to cure of their anaemia, suggesting that the haemopoietic cells of *Sl/Sl^d* mice were normal, but their environment was either

suppressing or failing to stimulate their development (McCulloch *et al.*, 1964). Further studies showed that the anaemia of *Sl/Sl*^{d} mice could be reversed by the intraperitoneal implantation of a +/+ or *W/W*^{v} spleen (Bernstein, 1970); by assessing the number of heme-containing nucleated cells, the donor spleen was identified as the primary site of the reconstitutive erythropoiesis rather than the native *Sl/Sl*^{d} marrow or spleen (Harrison and Russell, 1972).

Such transplantation experiments have also been applied to non-haemopoietic tissues. When *W/W*^{v} skin is grafted onto a *Sl/Sl*^{d} or +/+ recipient it becomes populated with a normal level of mast cells whereas *Sl/Sl*^{d} skin grafted onto *W/W*^{v} or +/+ mice does not support mast cell development (Kitamura and Go, 1979). More recently, Fujita *et al.* (1989) suggested that the microenvironmental defect responsible for the abnormal development of mast cells in *Sl/Sl*^{d} mice could be localised to fibroblasts. The cloned fibroblast cell line 3T3, derived from +/+ mouse embryos, was able to support the growth of mast cells *in vitro* and *in vivo*, following implantation into *Sl/Sl*^{d} mice. A similar cell line derived from *Sl/Sl*^{d} mice was unable to support such growth.

The reciprocal nature of *Sl* and *W* mutations, suggested by such transplantation experiments, led Russell (1979) to propose that *Sl* and *W* encoded interacting structures such as a receptor and its ligand.

1.4.2 Molecular Biology of the SCF Receptor

1.4.2.1 *V-kit* and *c-kit* genes

The gene encoding the receptor for stem cell factor, *kit*, was first described as a viral oncogene (*v-kit*) of a feline retrovirus (Hardy-Zuckerman 4 feline sarcoma virus) isolated from a feline fibrosarcoma. Partial homology to tyrosine-specific protein kinase oncogenes was recognised and the presence of a cellular DNA sequence, homologous with *v-kit*, was demonstrated in feline, human and murine tissues by Southern blot hybridisation (Besmer *et al.*, 1986). The viral oncogene was proposed to have been generated by transduction of its cellular counterpart, designated as *c-kit*. This was confirmed with the isolation of both murine and human cDNAs encoding the protooncogene *c-kit* (Yarden *et al.*, 1987; Qiu *et al.*, 1988). Analysis of the deduced

protein sequence revealed features typical of a transmembrane tyrosine kinase receptor with significant homology to the receptors for platelet derived growth factor (PDGF) and colony stimulating factor-1 (CSF-1).

The receptor has subsequently been assigned as a member of the type III tyrosine kinase growth factor receptor superfamily which also includes the CSF-1, PDGF, flk-1, flk-2, flt-1 and flt-4 receptors. The members of this family are characterised by the presence of five immunoglobulin (Ig) like regions within the extracellular domain of the receptor and an intracellular kinase domain which is divided by the insertion of a hydrophilic stretch of 70 - 100 amino acids (see Figure 1.2). The extracellular domain for the SCF receptor has a signal peptide and a ligand binding domain which likely forms five Ig-like domains, with tertiary structure influenced by the presence of intramolecular disulphide bonds and *N*-linked glycosylation. A hydrophobic domain, characteristic of a membrane spanning region, separates extracellular from intracellular regions. The intracellular region has a kinase domain, an ATP binding site, an autophosphorylation site and a hydrophilic kinase insert (KI) domain (Yarden *et al.*, 1987; Qiu *et al.*, 1988). The KI domain is thought to function as a binding site for the SH2 domain of cytoplasmic signal transduction proteins (discussed in section 1.4.3). Figure 1.5 shows the structure of the SCF gene and the predicted SCF receptor.

A number of alternative SCF-R proteins have been described, generated by alternate splicing of *c-kit*. Analysis of murine mid-gestation placenta and mast cells *in vitro* has shown cells to express two *c-kit* transcripts, one of which has an in-frame deletion of codons 510 - 513 which encodes Gly-Asn-Asn-Lys, in the extracellular domain of the SCF-R (Yarden *et al.*, 1987; Reith *et al.*, 1991). The homologous isoforms have also been detected in human cells (Yarden *et al.*, 1987; Giebel *et al.*, 1992). Preferential expression of one isoform has been demonstrated in a number of tissues; the shorter mRNA transcript predominates in murine mast cells, bone marrow cells and fetal liver cells (Reith *et al.*, 1991) and in human erythroleukaemia cells and melanocytes (Giebel *et al.*, 1992). Analysis of receptor affinity for soluble SCF has shown that both receptor isoforms have similar binding affinities and subsequent levels of autophosphorylation and association with both phosphatidylinositol 3'-kinase (P13'K)

and phospholipase C-γ1 (PLC-γ1). However, the presence of low-level constitutive autophosphorylation and association with P13'K and PLC-γ1 has been shown with respect to the shorter, but not the longer isoform. It has been suggested that in some cell types, low-level, constitutive, activation of the SCF receptor may provide the cell with a necessary survival signal when SCF - SCF-R interaction is lacking and that in such conditions, the shorter SCF-R isoform may be preferentially expressed (Reith *et al.*, 1991; Williams *et al.*, 1992).

Alternatively spliced *c-kit* transcripts are also expressed in the mouse associated with gametogenesis. Post-meiotic, haploid spermatids express two shorter *c-kit* transcripts of 3.5 and 2.3 kb rather than the full length 5.5 kb transcript (Sorrentino *et al.*, 1991). Cloning of the 3.5 kb isoform showed that the alternative transcript lacked coding sequences for the extracellular and transmembrane domains, the ATP binding site and part of the kinase domain. Any functional roles must therefore be ligand and kinase independent; it has been proposed that the truncated receptor may facilitate the interaction of intracellular signalling proteins (Rossi *et al.*, 1992).

The SCF receptor also exists as a soluble isoform in human serum at relatively high concentrations (mean +/- SD = 340 +/- 114 ng/ml). The soluble receptor comprises the majority of the extracellular domain of the membrane bound receptor. It is glycosylated with both *N*- and *O*- linked carbohydrates. The soluble receptor can be purified by immunoaffinity chromatography and is able to inhibit the binding of SCF to membrane bound receptor *in vitro*. This suggests a possible role for the soluble receptor in the modulation of SCF activity *in vivo*. The generation of the soluble isoform *in vivo* probably occurs via proteolyic cleavage of the membrane bound receptor. The soluble isoform may downregulate the activity of SCF by competitive binding of the ligand, may interfere with dimerisation (and therefore signal transduction) by the membrane bound receptor, may be generated in order to downmodulate cellular SCF-R levels or may act as a chaperone for circulating soluble SCF (Wypych *et al.*, 1995).

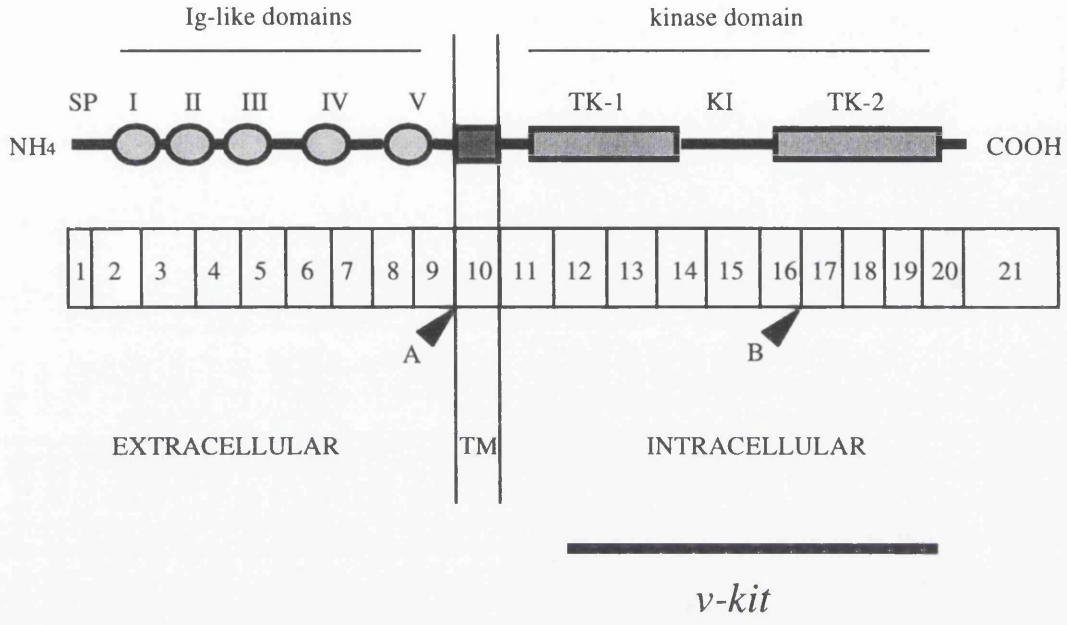


Figure 1.5: Diagrammatic representation of the c-kit gene and structure of the predicted SCF receptor. The exons, 1 - 21, of the *c-kit* gene are shown boxed, with the location of the ATG start and TGA stop codons indicated below. Regions of particular interest within the predicted SCF receptor are shown above the exons which encode them, these include: the signal peptide (SP), immunoglobulin like domains (I - V) within the extracellular ligand binding domain, the transmembrane (TM) region, and the tyrosine kinase domain (TK1 and TK2) split by the intervening kinase insertion region (KI). Alternative isoforms are generated by **A.** deletion of codons 510 - 513 in murine and human tissues and **B.** use of alternate promoter in intron 16 to produce 3.2 kb transcript in mouse spermatids. The location of the virally transduced, truncated *v-kit* oncogene is indicated below the exons. Adapted from Galli *et al.* (1994).

1.4.2.2 Chromosomal localisation of *c-kit* - allelism between the murine *W* locus and *c-kit*

The human *c-kit* gene maps to the centromeric region of chromosome 4, between 4q11 and 4q21. This corresponds to a conserved area on mouse chromosome 5 which includes the *W* gene locus (Yarden *et al.*, 1987). This finding prompted Chabot *et al.* (1988) to investigate a possible relationship between *c-kit* and *W*. They showed that *c-kit* mapped closely to the *W* locus. In addition a radiation-induced *W* mutant mouse, W^{19H}, was shown to have a deletion which included the *c-kit* protooncogene (Chabot *et al.*, 1988; Geissler *et al.*, 1988a). Further evidence for the *W* locus encoding *c-kit*, was provided by the demonstration that two further *W* mutants, W^{44} *and* W^{x}, had distinct genetic rearrangements of the *W* locus (Geissler *et al.*, 1988b). The W^{x} mutant showed a likely gene rearrangement within the region encoding amino acids 342 - 791. The W^{44} mutant, however, showed a mutation which resulted in the production of a full length (5.5 kb) mRNA, albeit at a greatly reduced level, as demonstrated by Northern analysis. Subsequently, further *W* mutant mice have been shown to have mutations in the *c-kit* gene, including a number comprising single base pair mutations in the tyrosine kinase domain of the SCF receptor (Nocka *et al.*, 1990b; Tan *et al.*, 1990; Tsujimura *et al.*, 1993). Additionally, it has been demonstrated that *c-kit* expression is seen predominantly in tissues that are known to be affected by *W* mutations, namely mast cells, fetal and adult erythropoietic tissues and neural-crest derived melanocytes (Nocka *et al.*, 1989).

1.4.2.3 Distribution and regulation of SCF receptor expression

Studies of mutations at the murine *W* locus have provided evidence for the extensive nature of SCF-R expression. However, using immunohistochemical or Northern hybridisation methods, SCF-R expression has been demonstrated in a number of tissues which are not phenotypically affected in *W* mutant mice, suggesting a more widespread importance for SCF and its receptor. SCF-R expression has been implicated as important in the development of cells of the nervous system, placenta, heart septum, lung, and kidney during embryonal and fetal development in addition to cells of haemopoietic, melanocyte and germ cell lineages (Matsui *et al.*, 1990; Orr-Urtreger *et al.*, 1990; Keshet *et al.*, 1991). Such studies have suggested that the

SCF-receptor - ligand complex provides a homing mechanism for stem cells during their migration in early development and affects stem cell proliferation, differentiation, or survival in later development.

In adult tissues SCF receptor distribution is again widespread. Within the haemopoietic system the majority of cell lineages show SCF receptor expression at some point in their development. Studies using ^{125}I-labelled SCF showed the SCF-R to be present in high numbers on blast cells and in lower numbers on immature granulocytic, monocytic, and eosinophilic cells and some lymphocytes (Metcalf and Nicola, 1991). In general, expression of the SCF-R proceeds from low levels on primitive, dormant multipotent progenitors to high levels on more mature, actively cycling progenitors, and decreases to very low or undetectable levels on most mature blood cells (Katayama *et al.*, 1993). However, both mature mast cells (Katayama *et al.*, 1993) and platelets (Grabarek *et al.*, 1994) express the SCF-R and are responsive to SCF stimulation. In murine gonadal tissues expression is highest in growing oocytes in the ovary and in proliferating spermatogonia and Leydig cells in the testis (Manova *et al.*, 1990). Other tissues expressing the SCF-R, in the human adult, include brain astrocytes and glial cells, renal tubules, parotid cells, thyrocytes, and breast epithelium (Natali *et al.*, 1992).

At the cellular level, a number of stimuli, including various cytokines, are able to alter the level of SCF-R expression. TNF-α and TGF-β1, for example, both decrease *c-kit* mRNA stability so shortening its half life and thus decreasing cellular receptor levels (Khoury *et al.*, 1994; Heinrich *et al.*, 1995). This effect, however, is only transient, lasting for up to 72 hours. The control of basal and cell specific SCF-R expression is poorly understood, but appears complex, involving the interaction of a number of activators and repressors (Vandenbark *et al.*, 1996). The murine *c-kit* promoter has been cloned and partially characterised (Yasuda *et al.*, 1993). The principal transcription initiation site (TIS) is located 58 bp upstream from the translation initiation codon (ATG). The main promoter elements are contained within the proximal 200 bp of 5' flanking DNA. This region contains no CCAAT or TATA elements but includes consensus binding sites for Sp1 and AP-2 transcription factors. Several short GA-rich elements were also identified as putative transcription factor

binding sites. The human *c-kit* promoter region contains similar putative transcription factor binding sites, including sites for AP-2, Sp1, basic helix-loop-helix proteins, Myb and GATA-1. The major transcription start sites in the human gene are 62 bp and 58 bp upstream of the translation start site (Yamamoto *et al.*, 1993). Whilst these proximal elements appear important in directing the general level of *c-kit* expression, more recent studies have suggested that the distal 5' DNA region is more important in the regulation of cell-specific *c-kit* expression. Using promoter-deletion reporter constructs, the 5' flanking DNA between -4100 and -5500 bp was identified as important in the repression of *c-kit* expression in *c-kit* negative cells. This region suppresses most of the transcription from the proximal promoter region but complete repression requires the complete sequence of the promoter DNA. Additionally, two Myb elements were identified at -900 bp (Myb2) and -1329 bp (Myb1). Myb2 is a promoter which appears to be essential for *c-kit* expression, whilst Myb1 acts as a partial repressor (Vandenbark *et al.*, 1996). The complexity of *cis* acting sequences within the *c-kit* promoter region implicates different mechanisms as important in the transcriptional control of *c-kit* in different cell types.

1.4.3 INTRACELLULAR SIGNALLING PATHWAYS ACTIVATED BY *C-KIT*

The coupling of receptor-ligand binding to modification of nuclear gene expression by the SCF receptor is relatively poorly understood. However, it is becoming increasingly clear that receptors within both the receptor kinase family and the haemopoietin/interferon family utilise many common signalling pathways. Signalling by the SCF-R will therefore be discussed within this broader framework.

Typical of the receptors within the tyrosine kinase family, ligand binding induces SCF receptor dimerisation (Blume-Jensen *et al.*, 1991; Gordon, 1994). SCF is thought to promote the formation of SCF-R dimers by inducing a conformational change in the receptor, following ligand binding (Blechman *et al.*, 1995). The SCF ligand exists as a non-covalently associated dimer in its soluble form (Arakawa *et al.*, 1991). It has been proposed that receptor dimerisation may be mediated by the dimeric ligand bringing two receptor chains into apposition (Williams, 1989). However, dimer formation appears to require direct interaction between two receptor molecules with

the fourth immunoglobulin domain of the extracellular portion of the receptor identified as a putative dimerisation site. A monoclonal antibody, which inhibits receptor dimerisation binds to this domain. Furthermore, receptor mutants lacking the fourth immunoglobulin domain do not form dimers. Abolition of dimer formation by either mechanism abrogates signal transduction (Lev *et al.*, 1992; Blechman *et al.*, 1995).

Receptor dimerisation leads to receptor transphosphorylation and enhanced tyrosine kinase function (Yarden *et al.*, 1987; Blume-Jensen *et al.*, 1991; Herbst *et al.*, 1992; Heldin, 1995). Additionally, the phosphorylation of tyrosine residues outwith the kinase domain serves to create docking sites that enable interaction of the receptor with downstream signal transduction molecules. Interactions may occur with a number of proteins containing a *src* homology 2 (SH2) domain (reviewed by Koch *et al.*, 1991, Pawson, 1994, Schlessinger, 1994). These include growth factor receptor-bound protein 2 (GRB2) (Lowenstein *et al.*, 1992), phospholipase-C-γ (Anderson *et al.*, 1990), the p85 subunit of phosphatidylinositol-3' kinase (McGlade *et al.*, 1992; Rameh *et al.*, 1995) and Shc (Pelicci *et al.*, 1992). GRB2 is a key signalling protein for many receptors, within and without the receptor kinase family (Chardin *et al.*, 1995). GRB2 may bind either directly to the receptor kinase or may utilise an additional adaptor protein such as Shc (RozakisAdcock *et al.*, 1992; Skolnik *et al.*, 1993b). GRB2 binds with high affinity to a second protein, son of sevenless (sos) via its SH3 domain to form a stable complex (Chardin *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; RozakisAdcock *et al.*, 1993; Skolnik *et al.*, 1993a). Sos interacts with membrane-anchored Ras which is activated by the exchange of GTP for GDP (Baltensperger *et al.*, 1993; Egan *et al.*, 1993). When activated, Ras initiates a kinase cascade involving Raf-1 (Moodie *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993) and mitogen-activated protein (MAP) kinase kinase (Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Hughes *et al.*, 1993). Ultimately the activation of MAP kinase leads to modulation of transcription by the phosphorylation of transcription factors (Hunter and Karin, 1992; Nakajima *et al.*, 1993). This signalling pathway is depicted in Figure 1.6.

SCF-R activation has been associated with activation of Ras (Duronio *et al.*, 1992; O'Farrell *et al.*, 1996), Raf1 (Miyazawa *et al.*, 1991; O'Farrell *et al.*, 1996) and MAP kinase (Miyazawa *et al.*, 1991; Okuda *et al.*, 1992; O'Farrell *et al.*, 1996). Furthermore the stimulation of a mast cell line with a combination of SCF and IL-3 produces a synergistic increase in cell proliferation that is correlated with a synergistic increase in MAP kinase activity (O'Farrell *et al.*, 1996). This suggests a possible mechanism for the synergy between these two cytokines. Activation of MAP kinase by the Ras pathway prevents apoptotic cell death in haemopoietic cells and may provide the mechanism for SCF mediated haemopoietic cell survival (Kinoshita *et al.*, 1995).

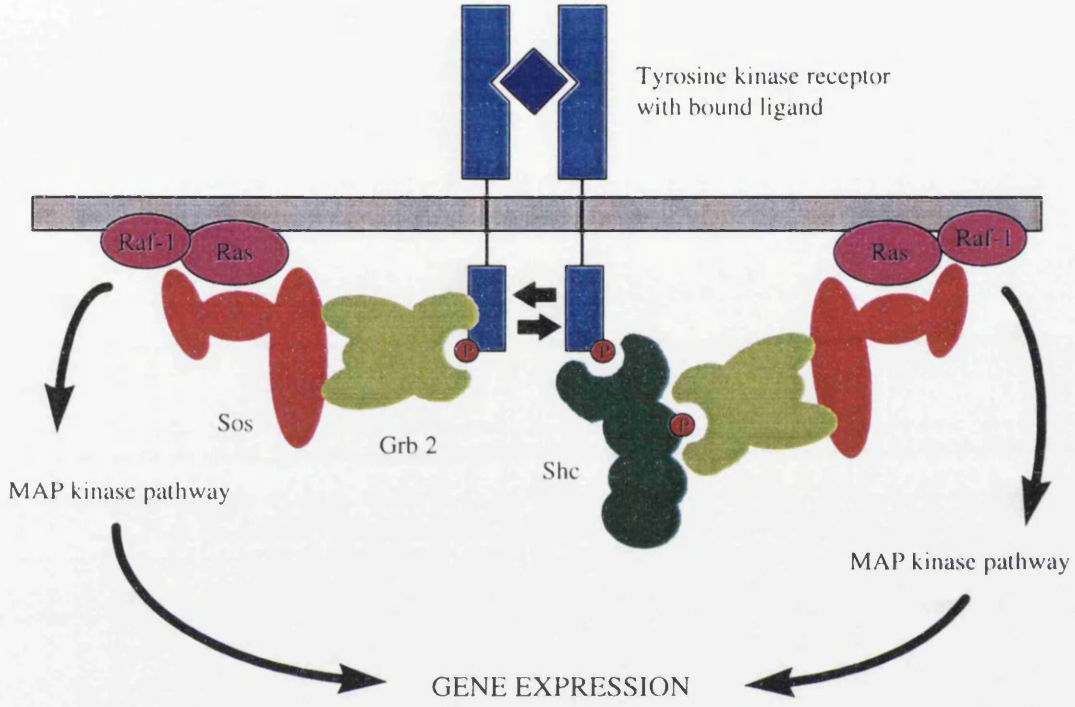


Figure 1.6: A simplified overview of the Ras signalling pathway. Activation of the tyrosine kinase receptor by its associated ligand mediates receptor dimerisation and phosphorylation of cytoplasmic tyrosine residue(s). The phosphorylated receptor binds to controllers of Ras exchange factors such as Grb2, either directly or via intermediates such as Shc. In turn, exchange factors such as Sos are recruited which convert inactive Ras-GDP to active Ras-GTP. Downstream signalling continues, principally through Raf, which once activated initiates a MAP kinase cascade. This ultimately leads the phosphorylation of transcription factors and the transduction of the signal to the cell nucleus, with a consequent modulation of gene expression. Adapted from (Bonfini *et al.*, 1996) and (Egan and Weinberg, 1993).

SCF also appears to mediate intracellular signalling, at least in part, by the JAK-STAT pathway. This signalling pathway was initially described for the interferon receptor family and has been reviewed by Darnell *et al.* (1994), Ihle and Kerr (1995), Schindler and Darnell (1995) and Heim, (1996). Subsequently it has been shown that a large number of cytokines utilise this pathway. The term JAK is an acronym for 'just another kinase' (reflecting the discovery of JAKs at a time when a number of novel tyrosine kinases were cloned); it is also an abbreviation for *Janus* kinase (*Janus* refers to an ancient two faced Roman god of gates and doorways). Four members of the JAK family of tyrosine kinases have been identified in mammals: Jak1, Jak2, Jak3 and Tyk2. The JAKs are believed to interact with the membrane proximal region of the cytoplasmic domain of numerous cytokine receptor chains. Mutagenesis studies using the EPO receptor (Witthuhn *et al.*, 1993; Miura *et al.*, 1994), prolactin receptor (DaSilva *et al.*, 1994), gp130 (Narazaki *et al.*, 1994; Tanner *et al.*, 1995) or GH (Tanner *et al.*, 1995) have implicated the box 1/box 2 motifs as the site of interaction. Deletion or mutations of residues in those regions abrogates tyrosine phosphorylation of JAK and abolishes receptor mediated effects (e.g. mitogenesis).

Signalling via the JAK kinases is thought to involve their apposition in association with ligand mediated receptor chain dimerisation/oligomerisation (Ihle, 1995; Schindler and Darnell, 1995). Once apposed the JAK kinases are thought to activate each other by reciprocal transphosphorylation, in much the same manner that the activation of receptor tyrosine kinases is proposed to occur. JAK activation may occur homodimerically where both receptor chains associate with a single class of JAK (e.g. the EPO receptor) or heterodimeric activation of JAK may occur. The obligate requirement for two different JAKs is illustrated in the case of signalling by the interferons. Studies using a series of cell lines that were unable to respond to interferon-γ established that both Jak1 and Jak2 are required for IFN-γ signalling. A U4 mutant (Muller *et al.*, 1993) lacking functional Jak1, shows no tyrosine phosphorylation of Jak2, in response to IFN-γ. Conversely, the γ-1 mutant (Watling *et al.*, 1993) lacking functional Jak2 shows no phosphorylation of Jak1, when similarly stimulated. The reconstitution of either mutant results in phosphorylation of

both kinases in response to IFN-γ. A similar requirement for both Jak1 and Tyk2 in IFN-α/β signalling has also been demonstrated (Muller *et al.*, 1993).

The unravelling of signalling pathways activated by the interferon receptor family led to the discovery of a novel family of proteins, termed STATS (signal transducers and activators of transcription). All known members of the STAT family are 750 - 850 amino acids in size. Conserved features include an SH2 domain between amino acids 600 - 700, and a single tyrosine residue in the carboxyl terminal region of the SH2 domain which becomes phosphorylated during activation [reviewed by Shuai *et al.*, (1993a), Shuai *et al.* (1994) and Schindler and Darnell (1995)]. Currently, six members of the STAT family are recognised, but more may exist (Schindler and Darnell, 1995). All are able to bind to sequence specific elements of nuclear DNA upon activation; by binding to the promotors of specific genes they are able to stimulate the transcription of these genes by RNA polymerase II (Heim, 1996).

The recruitment of specific STATs as signalling proteins by cytokine receptors is thought to be regulated by the interaction of specific receptor docking sites with the STAT protein. It has been shown that the SH2 domains of a number of STATs interact with receptor docking sites containing phosphorylated tyrosine residues. Examples include the interaction of Stat5 with the IL-2 receptor β chain (Fujii *et al.*, 1995) and the EPO receptor (Quelle *et al.*, 1996) and Stat6 with the IL-4 α-chain (Quelle *et al.*, 1995). Following recruitment to a specific cytokine receptor, the STAT is believed to be phosphorylated by a receptor associated JAK kinase (Shuai *et al.*, 1993b; Darnell, Jr. *et al.*, 1994). Once phosphorylated STATs are thought to form dimers via association of the SH2 domain of one molecule with the phosphotyrosine residue of a second molecule. (Shuai *et al.*, 1992; Shuai *et al.*, 1993a; Shuai *et al.*, 1994; Gupta *et al.*, 1996).

The modification of gene transcription by the STAT proteins involves translocation of the protein to the cell nucleus followed by binding of the proteins to specific DNA sequence elements. An overview of the JAK-STAT signalling pathway is shown in relation to IFN-α and IFN-γ in Figure 1.7.

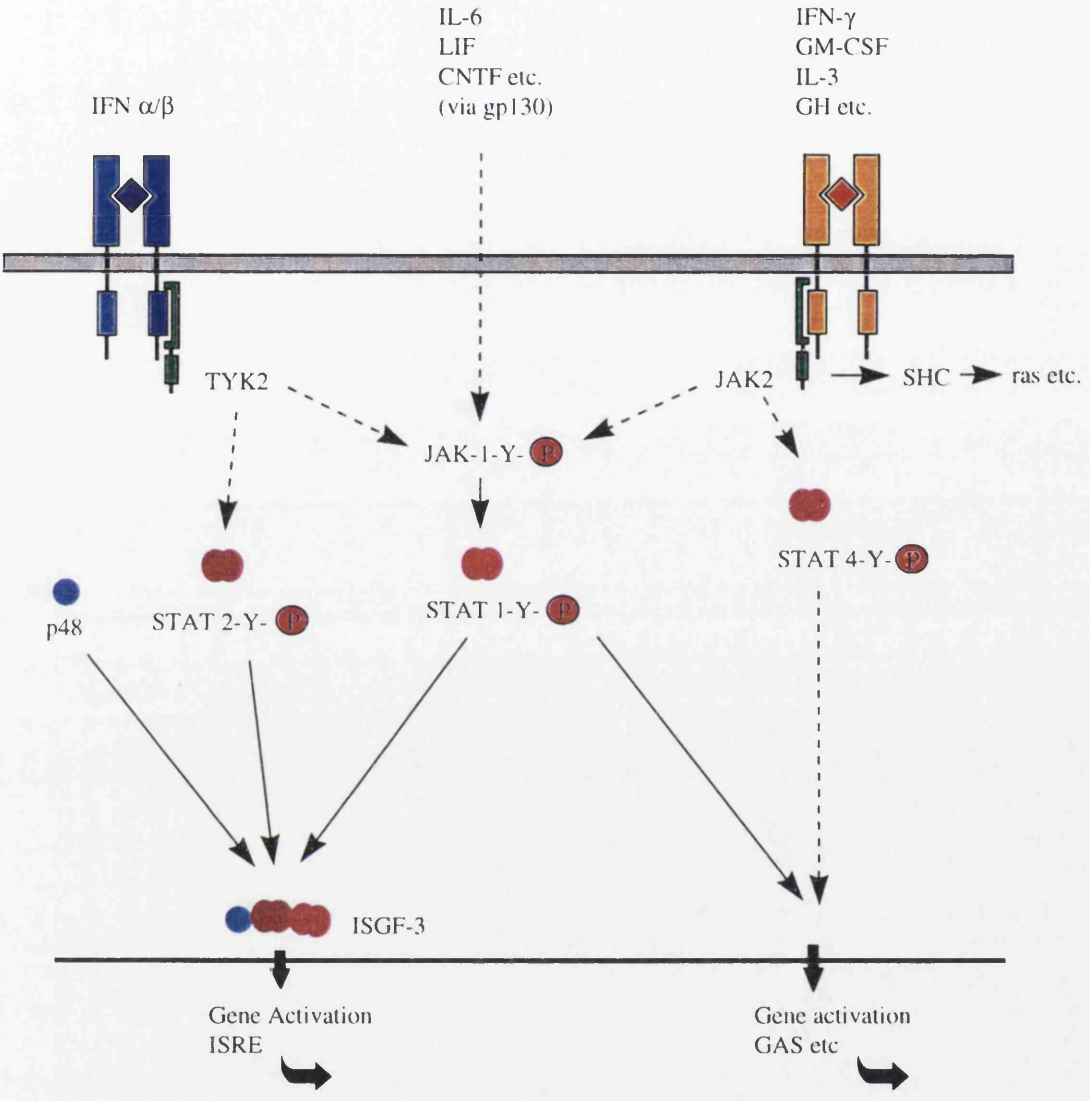


Figure 1.7: Overview of JAK-STAT pathway of signal transduction. Signal transduction by the IFN-α and IFN-γ receptors are principally shown. The modulation of gene transcription relies upon the production of an active transcription factor following a complex series of protein interactions and kinase mediated transphoshorylations. The primary transcription factor which controls expression of IFN-α induced genes is IFN-α stimulated gene factor 3 (ISGF3). ISGF3 is a complex of a Stat1/Stat2 heterodimer and a 48 kDa protein (p48) related to the interferon regulatory factor (IRF) family of binding proteins. After translocation to the nucleus, the STAT complex binds to specific DNA response elements (ISREs) and stimulates gene transcription (Fu *et al.*, 1990; Kessler *et al.*, 1990). Similarly, IFN-γ signalling results in the production of a transcription factor consisting of a Jak1/Jak2 heterodimer. This initiates the transcription of a family of genes containing the GAS (IFN-γ activation site) DNA sequence element in their promoter region (Shuai *et al.*, 1992). Adapted from Hilton, (1994).

The SCF-R is constitutively associated with Jak2 and upon SCF binding Jak2 is rapidly and transiently phosphorylated. Incubation of cells with Jak2 antisense oligonucleotides decreases SCF induced cell proliferation by approximately 50% (Linnekin *et al.*, 1996; Weiler *et al.*, 1996). The substrate for activated Jak2 is not yet known, however other members of this receptor family (CSF-1, EGF) have been shown to activate STAT1 (Silvennoinen *et al.*, 1993). Additionally Jak2 may couple SCF-R signalling to the Ras pathway. SCF also induces serine phosphorylation of STAT3 in human myeloid cells. Serine phosphorylation alone is not sufficient to induce nuclear translocation or DNA binding activity of STAT3. This requires additional tyrosine phosphorylation of STAT3, which SCF does not induce (Gotoh *et al.*, 1996). Tyrosine phosphorylation of STAT3 is, however, induced by other cytokines such as IL-6 or IL-9. Maximal activation of transcription requires both serine and tyrosine phosphorylation of STAT3 (Wen *et al.*, 1995). Therefore the production of a hyperphosphorylated STAT3 following stimulation by a combination of SCF and IL-6 or IL-9, may provide a mechanism for synergy between these cytokines (Gotoh *et al.*, 1996).

A number of other signalling proteins, implicated in SCF receptor signal transduction are depicted in Figure 1.8.

The downregulation of SCF receptor signalling involves other classes of proteins including tyrosine phosphatases and serine/threonine kinases. Protein tyrosine phosphatases mediate the dephosphorylation of proteins at specific tyrosine residues in direct antagonism to the tyrosine kinases. The protein tyrosine phosphatase Shp1 may negatively regulate SCF-R signalling by reducing the phosphorylation of downstream substrate(s), including Shc (Paulson *et al.*, 1996).

The autophosphorylation of SCF-R tyrosine residues is inhibited by the serine/threonine kinase, protein kinase C (PKC). This protein mediates phosphorylation of serine residues in the kinase insert of the SCF-R (Blume-Jensen *et al.*, 1995). Inhibition of PKC activity decreases serine phosphorylation of the SCF-R and increases its association with and activation of PI3K (Blume-Jensen *et al.*, 1994). Specific inhibition of PI3K with wortmannin inhibits SCF induced mitogenicity

(Blume-Jensen *et al.*, 1994). PKC therefore acts as a negative regulator of mitogenic signalling mediated by SCF. However, PKC activity is required for the generation of SCF generated changes associated with cell motility, such as actin reorganisation or chemotaxis (Blume-Jensen *et al.*, 1993).

The specific transcription factors induced by SCF, and the consequent pattern of gene induction remain largely unknown. It has, however, been shown that SCF stimulation can lead to a sustained induction of *c-myc* expression. A stimulatory role for Myc in cell cycle progression has been demonstrated in a number of haemopoietic cell lines (O'Farrell *et al.*, 1996 and references therein).

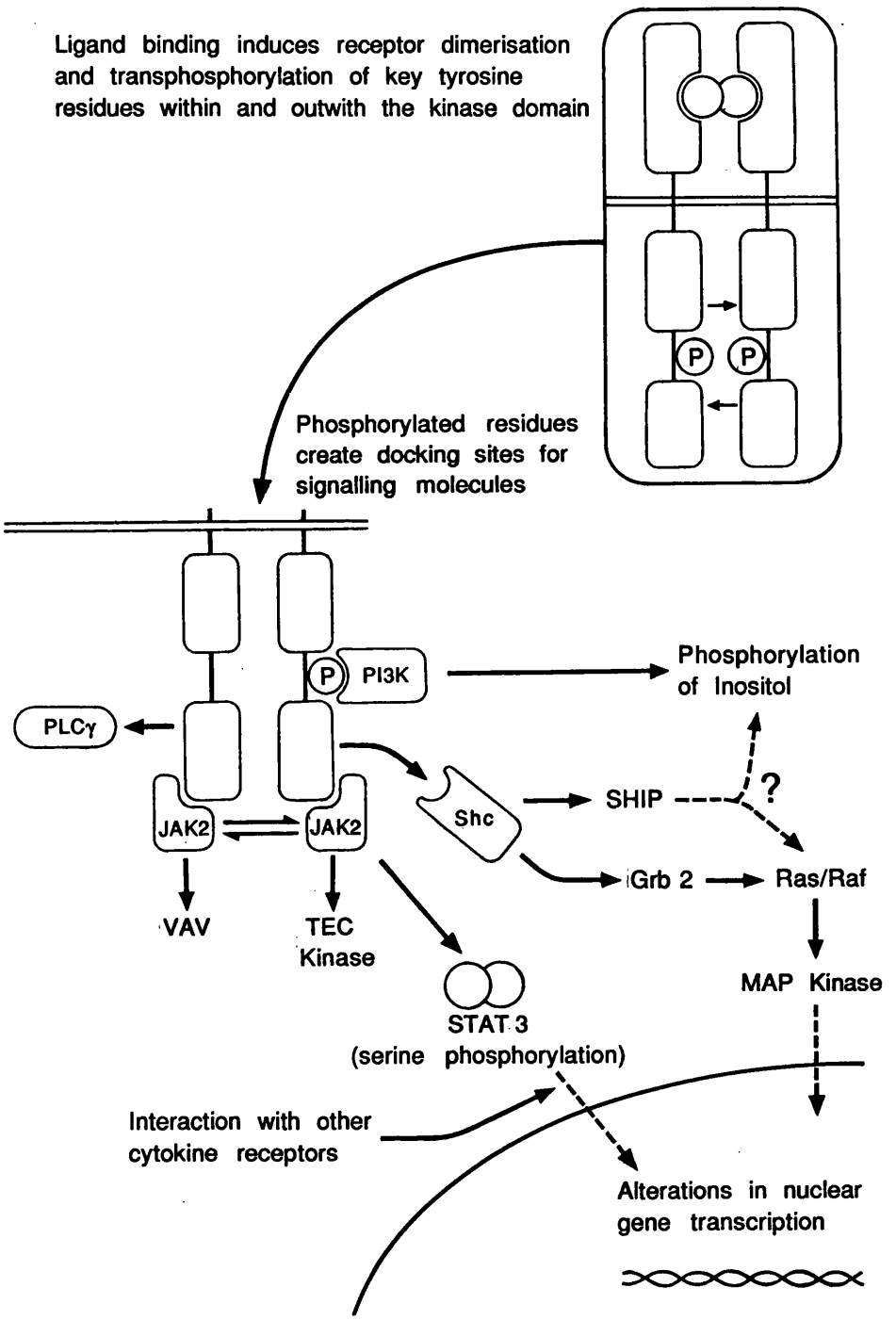


Figure 1.8: Overview of the SCF receptor signalling pathway. Receptor binding leads to signalling via the Ras/Raf/MAP kinase, JAK/STAT and inositol pathways as discussed within the text. Other signalling proteins include *Vav* and TEC kinase. *Vav* is selectively expressed in haemopoietic cells and is tyrosine phosphorylated following SCF stimulation (Matsuguchi *et al.*, 1995). Tec is constitutively associated with the SCF-R and is phosphorylated in SCF stimulated human Mo7e cells (Tang *et al.*, 1994).

1.4.4 HAEMOPOIETIC EFFECTS OF STEM CELL FACTOR

The aforementioned studies have provided much information about the important biological roles of SCF and its receptor. The production of recombinant SCF (rSCF) has enabled further characterisation of its potential functions. Due to interest in its potential clinical applications, discussed later, most of the studies have been directed towards its effects upon the haemopoietic system.

1.4.4.1 Erythropoiesis and myelopoiesis

A number of studies have shown the ability of SCF to support the survival of primitive stem cells *in vitro* and in concert with other cytokines, to promote their subsequent differentiation into cells of the erythroid, myeloid or megakaryocytic lineages (reviewed by Metcalf, 1993a, Galli *et al.*, 1994, McNiece and Briddell, 1995).

SCF is able to promote the survival *in vitro* of murine bone marrow cells with both short and long term *in vivo* repopulating potential. Furthermore this effect is not dependent on the induction of cell proliferation as cell survival is unaffected by the addition of mitotic inhibitors (Keller *et al.*, 1995). However it is apparent that SCF is just one of a number of factors affecting the survival of more primitive stem cells. Studies on *Sl/Sl* mutant mice, which do not express SCF, have shown that the number of stem cells in the fetal liver is reduced to 30% of normal levels at days 13 -15 post conception. However, subsequently the number of stem cells increases, presumably indicating the presence of alternative growth factors affecting their survival and proliferation (Ikuta and Weissman, 1992). *In vitro*, the ability of the preadipose cell line PA 6, to support the survival of stem cells (capable of long term reconstitution of erythropoiesis in W/W^v mice) is not affected by the addition of an antagonistic anti-SCF-R monoclonal antibody (ACK-2) (Kodama *et al.*, 1992).

The effects of rSCF on the growth of haemopoietic progenitor cells has been determined in a number of species using *in vitro* colony forming cell assays. As a single agent the effects of rSCF are generally limited. Murine SCF promotes the formation of low numbers of small colonies in agar; the majority of colonies consist of

granulocytes while approximately 20% of colonies are composed of blast cells (Metcalf and Nicola, 1991). Human and canine rSCF show little stimulatory effect alone on colony formation from bone marrow cells in agar or methylcellulose cultures (McNiece *et al.*, 1991; Shull *et al.*, 1992). However, SCF shows marked co-stimulatory or synergistic activity when combined with a number of other cytokines including erythropoietin, G-CSF, GM-CSF, thrombopoietin and IL-3. The addition of SCF, in most cases results in an increase in the number and/or size of colonies formed in a given assay, with the lineage of the colonies determined by the interacting cytokine. For example, the addition of rSCF to erythropoietin results in an increase in number and size of erythroid (BFU-E) and multipotential (CFU-GEMM) colonies formed in both murine and human systems (Anderson *et al.*, 1990; Nocka *et al.*, 1990a; Broxmeyer *et al.*, 1991c; McNiece *et al.*, 1991). Table 1.3 summarises some of the synergistic effects of SCF on colony formation *in vitro*.

Cytokine	*Effect of the addition of rSCF to CFC-Assays*	*Reference*
EPO	Enhances erythroid and multipotential colony formation.	Anderson *et al.*, 1990; Nocka *et al.*, 1990a; Broxmeyer *et al.*, 1991c; McNiece *et al.*, 1991.
IL-3	Increased size and number of mixed colonies consisting of neutrophils/macrophages and megakaryocytes.	Martin *et al.*, 1990; McNiece *et al.*, 1991.
G-CSF	Increased size and number of neutrophil colonies	Martin *et al.*, 1990; McNiece *et al.*, 1991.
GM-CSF	Increased size and number of neutrophil/macrophage colonies	Martin *et al.*, 1990; McNiece *et al.*, 1991.
TPO	Increased numbers of megakaryocyte colonies.	Broudy *et al.*, 1995.
IL-7	Enhances formation of mainly granulocytic colonies from primitive murine progenitors.	Fahlman *et al.*, 1994.
IL-6	Increased colony formation.	Zsebo *et al.*, 1990b.

Table 1.3: Interaction of SCF with other cytokines in *in vitro* colony forming assays.

Another line of evidence for the importance of SCF in the maintenance and proliferation of progenitor cell populations comes from studies by Ogawa *et al.* (1991). Administration of an anti-SCF-R antibody (ACK-2) to mice results in the near complete disappearance of haemopoietic progenitor cells from the bone marrow,

just two days after injection. Subsequently, mature cells of the myeloid and erythroid series disappear from the bone marrow.

1.4.4.2 Lymphopoiesis

Analysis of *Sl* mutant mice suggests that SCF can influence the development of lymphocytes. Although *Sl/Sl*^{d} mice have normal levels of circulating peripheral blood (PB) lymphocytes, the thymus of 12 week old mutant mice shows reduced cellularity when compared to congenic +/+ mice (Medlock *et al.*, 1992). Additionally, a number of stromal cell lines derived from thymic tissue express SCF (Williams *et al.*, 1992). Soluble SCF is able to synergistically increase the proliferation of a subset of normal murine immature B lymphocytes (B220+ pre-B cells) *in vitro* in combination with IL-7 (Funk *et al.*, 1993). Mature human circulating peripheral blood lymphocytes do not express significant levels of the SCF-R (Matos *et al.*, 1993) and SCF has no proliferative effect upon mouse peripheral blood B or T-lymphocytes *in vitro* (Williams *et al.*, 1992). However a subset of human natural killer cells, expressing high levels of CD56, show expression of the SCF-R and SCF enhances their proliferation *in vitro*, in response to IL-2 (Matos *et al.*, 1993).

1.4.4.3 Mature haemopoietic cells

1.4.4.3.1 Mast Cells

Mast cells are widely distributed throughout mammalian vascularised tissues, occurring particularly at sites in proximity to the external environment (skin, respiratory, gastrointestinal and urogenital tracts). They are important effector cells in the innate immune system, capable of producing a diverse array of biologically active mediators including histamine, proteoglycans, cytokines, chemotactic factors, leukotrienes and prostaglandins. Mast cells express the FcεR1 receptor on their cell surface; crosslinking of IgE antibodies bound to the mast cell triggers mediator release from the cell. Mast cells therefore have an important role in the generation of IgE dependent host responses including anaphylaxis and immunity to parasites.

SCF has multiple regulatory effects upon mast cells, at various developmental stages, affecting their proliferation, maturation, migration and function. Mast cell survival *in*

vitro is promoted by SCF, which suppresses cell death due to apoptosis (Iemura *et al.*, 1994). *In vitro*, soluble rrSCF induces the proliferation of cloned IL-3 dependent mouse mast cells, purified peritoneal mast cells and the proliferation and maturation of bone marrow-derived mast cells (BMDMC). After four weeks in culture BMDMC acquire multiple characteristics of mature connective tissue mast cells, such as increased histamine content and heparin synthesis (Tsai *et al.*, 1991b). SCF induces the outgrowth of mast cells from cultured murine (Ulich *et al.*, 1991) or human bone marrow progenitor cells (Valent *et al.*, 1992), human fetal liver cells (Irani *et al.*, 1992) or human umbilical cord blood cells (Mitsui *et al.*, 1993). However, human mast cells that develop in SCF supplemented cultures do not develop an identical phenotype to that expressed by mature mast cells *in vivo*. Additionally, the growth of human mast cells *in vitro*, from primitive progenitors is not supported by SCF alone if cultures are depleted of accessory cells. In such conditions additional growth factors, including IL-3, IL-4 and IL-10 are required for optimal cell growth and differentiation. It is likely that the growth and maturation of mast cells *in vivo* involves the interaction of a number of factors, which have yet to be fully defined (Rennick *et al.*, 1995).

SCF also affects the function of mature mast cells. SCF promotes secretion and mediator release from murine mast cells both *in vitro* (Galli *et al.*, 1991) and *in vivo* (Wershil *et al.*, 1992). This effect is mediated specifically via the SCF receptor; *W/W^v* derived cloned mast cells are unresponsive to SCF (but are activated by IgE and antigen) (Wershil *et al.*, 1992). SCF also augments mediator release in response to activation by IgE (Coleman *et al.*, 1993). SCF acts as a potent chemoattractant for human (Nilsson *et al.*, 1994) and murine (Meininger *et al.*, 1992) mast cells *in vitro.*

The membrane associated form of SCF is able to mediate the attachment of mast cells to stromal fibroblasts via specific interaction with the SCF receptor (Flanagan *et al.*, 1991; Adachi *et al.*, 1992). SCF also enhances the attachment of mast cells to fibronectin, a component of the extracellular matrix. This attachment is mediated by a mast cell integrin receptor; SCF may upregulate integrin receptor expression or its affinity for fibronectin (Dastych and Metcalfe, 1994).

The physiological role of SCF in the regulation of mast cells is likely to be complex. Although many of the documented functions of SCF are stimulatory, in some circumstances SCF administration may be associated with suppression of mast cell secretory activity (reviewed by Galli *et al.*, 1994).

1.4.4.3.2 Platelets

A potential role for SCF in the control of haemostasis via modulation of platelet function has been proposed. The SCF receptor can be detected on human platelets, following stimulation with ADP, by specific binding of ^{125}I-labelled SCF. SCF stimulation of platelets *in vitro* results in acceleration of platelet aggregation and increased serotonin secretion in response to adrenaline/ADP (Grabarek *et al.*, 1994).

1.4.5 Extra-Haemopoietic Effects of Stem Cell Factor

1.4.5.1 Germ cell development

SCF and its receptor are necessary for the proliferation of primitive germ cells and their subsequent migration to the gonads and differentiation into mature gametes. Primordial germ cells (PGCs) are first identifiable in normal mice at seven days post conception (p.c.) The number of PGCs increases from day 8 - 12 p.c., from approximately 10 - 100 to 2,500 - 5,000 cells. During this period the cells also migrate from the hindgut to the gonadal ridge, which by 12.5 days p.c. has undergone differentiation into the male or female gonad. In mice homozygous for severe (lethal) *Sl* or *W* mutations, germ cells can be identified at day 8 p.c., but they do not subsequently proliferate and few or none reach the developing gonad (reviewed by Galli *et al.*, 1994). In mice with less severe mutations some germ cells may populate the gonad but defects can be seen later in germ cell development, often preferentially affecting the male or female gonad (Geissler *et al.*, 1981). The expression patterns of SCF and SCF-R mRNA supports their attributed functions in germ cell proliferation and differentiation. SCF-R mRNA is expressed in primordial germ cells from day 7.5 to 13.5; it then decreases to undetectable levels as male germ cells enter mitotic arrest and female germ cells the prophase of meiosis (Manova and Bachvarova, 1991). SCF mRNA is expressed along the pathway of migration of the PGCs and in the gonadal

ridge and fetal gonads (Matsui *et al.*, 1990; Keshet *et al.*, 1991). SCF-R mRNA is also expressed during the second phase of germ cell development in both male and female gonads. In the adult male mouse, SCF-R mRNA is expressed in developing spermatogonia and Leydig cells, whilst SCF mRNA is expressed by Sertoli cells. In the murine ovary increasing levels of SCF mRNA are seen in follicle cells associated with growing oocytes, whilst the oocytes themselves express mRNA for the SCF-R (Manova *et al.*, 1993).

1.4.5.2 The nervous system

The detection of SCF and SCF-R mRNAs in tissues of the central nervous system (CNS) was an unexpected finding; amongst the variety of phenotypic abnormalities reported in *Sl* or *W* mutant mice, none were attributable to the CNS. The role of SCF in CNS development and function remains poorly understood. However, SCF and its receptor may be involved in the formation of certain synaptic connections within the CNS. In a number of cases, pairs of neurones which form synaptic connections express either the mRNA for SCF or its receptor (Hirota *et al.*, 1992). If SCF and its receptor do have such a role, then a number of possible explanations may be considered for the lack of CNS abnormalities in *Sl* or *W* mutant mice. These effects may require an intact extracellular domain of either SCF or its receptor (mutants lacking this domain in either the receptor or its ligand do not survive to adulthood therefore CNS abnormalities may not be apparent). The function(s) of SCF in the CNS may be redundant to a degree that other molecules may compensate adequately for its absence. Alternatively *Sl* or *W* mutant mice may have relatively subtle CNS abnormalities that have yet to be recognised (Galli *et al.*, 1994).

1.4.5.3 Melanocytes

The SCF-R and its ligand are important for the migration of primitive melanocyte precursors from the embryonic neural crest to sites such as the dermis of the skin or the ectoderm of whisker follicles. Melanocyte precursors and melanoblasts can be shown, by *in situ* hybridisation, to express SCF-R mRNA *in vivo* (Orr-Urtreger et al., 1990; Besmer et al., 1993). Expression is seen initially in presumptive melanoblasts in the cervical region of 10 day murine embryos and continues as the cells migrate to sites in the epidermis and differentiate into hair follicles after birth (Manova and

Bachvarova, 1991). Conversely SCF mRNA expression is highest at sites to which the melanoblasts migrate, seen in the dorsal region of the somites of 9.5 - 10.5 day murine embryos and at later intervals in the dermis and developing hair follicles (Matsui et al., 1990). Furthermore, using a monoclonal antibody that blocked activation of the murine SCF receptor, Nishikawa *et al.* (1991) demonstrated that SCF and its receptor are also required for the proliferation of murine melanocyte precursors *in vivo*. Mutations of the SCF receptor have also been shown to be causal for piebaldism in humans. Piebaldism is an autosomal dominant disorder of melanocyte development characterised by congenital white patches of skin and hair from which melanocytes are absent. Missense mutations or a single base pair substitution within the tyrosine kinase domain of the receptor have been associated with the disease (Giebel and Spritz, 1991; Spritz et al., 1992). Interestingly, it is not associated with disorders of haemopoiesis or fertility.

1.5 SUMMARY

The observation of white spotting on the fur of mice with mutations at either the steel (*Sl*) or dominant white spotting (*W*) loci was the first important step in the pathway to characterising a receptor and associated ligand that have key roles in the migration and development not only of melanocytes but also cells of the haemopoietic, reproductive and nervous systems. SCF is a particularly pleiotropic cytokine. It influences a broad range of target cells, at a number of stages of development, both in the developing embryo and in the adult and also exerts a wide range of actions upon these cells. SCF is able to affect the survival, proliferation and differentiation of immature cells and also modulate the function of mature cells.

1.6 AIMS OF THE PROJECT

As inferred above and discussed elsewhere within this thesis, recombinant SCF has a number of potential applications both with regard to the support of haemopoietic cell growth *in vitro* and the manipulation of haemopoiesis *in vivo*. The availability of feline recombinant SCF would therefore facilitate further studies regarding haemopoiesis in this species both in normal and diseased states and also provide a potentially valuable therapeutic agent.

The aim of this project was to clone and express the cDNA encoding feline SCF and examine the biological properties of the recombinant protein, both *in vitro* and *in vivo*. This work forms part of a larger project within the Department of Veterinary Pathology at Glasgow, which aims to isolate a number of haemopoietic and immunomodulatory cytokines within this species. The availability of a number of feline specific cytokines should enable the development of new methods for prevention and treatment of various feline diseases and assist the understanding of the pathogenesis of diseases of the cat.

More specifically, the aims of the project were:

- To amplify, by the polymerase chain reaction, cDNA encoding feline stem cell factor from mRNA derived from feline fibroblast cells (FEA cell line).
- To sub-clone the PCR product into a plasmid vector and sequence the recombinant DNA.
- To express the feline SCF protein in *Escherichia coli* as a fusion protein with glutathione S-transferase.
- To purify the resultant protein using Fast Protein Liquid Chromatography (FPLC).
- To develop a biological assay for feline SCF to enable monitoring of its specific activity during expression and subsequent purification.
- To investigate the effects of recombinant feline SCF on haemopoiesis both *in vitro* and *in vivo*.
- To develop reagents to enable identification of a marker of feline haemopoietic stem and progenitor cells, namely CD34.

The following chapters will detail the experimental techniques used to pursue these objectives and the results that were obtained.

CHAPTER TWO - MATERIALS AND METHODS

2.1 MATERIALS

Materials in regular use such as equipment, general reagents and solutions are detailed in appendix A 1, whilst those used infrequently are detailed in the appropriate methods section.

2.2 METHODS

Methods used throughout the thesis are described within this chapter, whilst techniques specific to one area are described in later chapters. Many of the methods described herein are based on standard techniques, which are detailed in several laboratory manuals (Ausubel *et al.*, 1994; Maniatis *et al.*, 1982).

2.2.1 GROWTH AND MANIPULATION OF MAMMALIAN CELLS

2.2.1.1 Basic Techniques

All procedures involving manipulation of mammalian cells were carried out using standard aseptic procedures. Where possible all procedures were performed in a laminar flow hood.

2.2.1.1.1 Cryopreservation of cells

In order to preserve stocks of cell lines for long term use, cells were stored over liquid nitrogen. Cells to be frozen were grown to mid-log phase (as described below) and removed into a sterile 50 ml centrifuge tube (using trypsin-EDTA where necessary). The cells were centrifuged at 400 x g for five minutes, the supernatant discarded and the cells resuspended in freezing medium (appropriate culture medium supplemented with FCS to 20% and 10% DMSO) to a concentration of approximately 2 x 10^6 cells/ml. The cell suspension was transferred in one millilitre aliquots, to labelled cryovials and brought to -70°C in a controlled rate cell freezer (Kryo 10 - Planer Products Ltd., Sunbury on Thames, UK). The vials were then transferred to a liquid nitrogen freezer. Cell stocks were revived by rapid thawing in a 37°C water bath and subsequently cultured using standard techniques (described below).

2.2.1.1.2 Cell counting

Cells were counted using a haemocytometer (Weber Scientific International), as follows. Cell were diluted in PBS to give an approximate concentration of 5 x 10^5 to 1 x 10^6 cells/ml. The cell suspension was introduced to the haemocytometer chamber and cells counted under an inverted microscope with 4 x or 10 x objective; cells lying on the top and right hand perimeter of each large (one millimetre) square were included, those on the bottom or left hand were excluded. Cell concentration (cells/ml) was calculated by multiplying the mean number of cells per large square by 10^4 and correcting for the dilution factor. Where an estimate of live cell numbers was required 0.4% trypan blue (Gibco BRL) was added to the cell suspension (1:1) and allowed to incubate for five minutes at room temperature prior to counting; dead cells take up the stain thus appearing blue.

2.2.1.2 FEA cell line

The FEA cell line is a fibroblast cell line derived from whole feline embryos (Jarrett *et al.*, 1973); the cell line used had been transformed with FeLV subgroup A (Glasgow A). The cells grow as an adherent monolayer in culture. Cells were cultured in 20 - 30 ml DMEM-10 (DMEM containing 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 100 iu/ml penicillin, 100 μg/ml streptomycin) in 75 cm^2 tissue cultures flasks at 37°C, 5% CO_2. Cultures were split, typically 1:3 to 1:4, every three to four days, when sub-confluent. The medium was decanted from the cell monolayer, the cells washed with trypsin-EDTA and then incubated at 37°C with approximately one millilitre of fresh trypsin-EDTA for three to five minutes. The detached cells were then washed in fresh medium, pelleted by centrifugation at 400 x g for five minutes, prior to resuspending in fresh medium and seeding new tissue culture flasks.

2.2.1.3 MC/9 cell line

MC/9 is a murine mast cell line (Nabel *et al.*, 1981; Galli *et al.*, 1982) that responds to a number of cytokines including low levels of mouse and rat SCF. It is maintained in suspension in rat spleen cell conditioned medium (prepared as described below). Cells were cultured in MC/9 growth medium (DMEM with 10% FBS, 2 mM L-glutamine,

50 μM 2-ME, 1% NEAA, 100 iu/ml penicillin, 100 μg/ml streptomycin, additional 32 mg/L L-arginine, 36 mg/L L-asparagine and 2 mg/L folic acid, and 20 - 45% concanavalin-A activated rat spleen cell supernatant) at a cell density of 1 - 5 x 10^5 cells/ml in 75 cm^2 or 162 cm^2 tissue culture flasks at 37°C, 5% CO_2. The cultures were passaged on Monday, Wednesday and Friday to a final concentration of 1 - 2 x 10^5 cells/ml.

2.2.1.4 TF-1 cell line

The TF-1 cell line is a human erythroleukaemia cell line which proliferates in response to a number of cytokines, including GM-CSF, SCF and erythropoietin (Kitamura *et al.*, 1989). Cells were cultured in suspension in RPMI 1640 medium supplemented with 5% FBS, 2 mM glutamine, penicillin (100 iu/ml), streptomycin (100 μg/ml) and hGM-CSF (2 ng/ml) in upright 75 cm^2 tissue culture flasks. Cultures were maintained at 37°C in a humidified incubator with 5% CO_2. Cultures were split every two to three days, typically 1:5 to 1:7, when the cell density reached approximately 5 x 10^5 cells/ml; after passaging they were refed GM-CSF.

2.2.1.5 Production of rat spleen cell conditioned medium

Rats were killed by cervical dislocation and the spleens collected aseptically into 10 - 20 ml DMEM. The spleens were placed in a petri dish containing modified DMEM-4 (DMEM with 4% FBS, 2 mM L-glutamine, 50 μM 2-ME, 1% NEAA, 100 iu/ml penicillin, 100 μg/ml streptomycin, and additional 32 mg/L L-arginine, 36 mg/L L-asparagine and 2 mg/L folic acid) and teased apart using sterile scalpels. To break up the spleen further the tissue was forced through a 230 μm pore size wire mesh (Sigma) using a 50 ml syringe barrel. The cells were washed in medium, centrifuged in 50 ml conical tubes at 200 x g for 10 minutes and the supernatant discarded. The cells were then resuspended in ACK lysing buffer, using approximately five millilitres per spleen. The cell suspension was incubated at room temperature, with occasional shaking, for five minutes, to lyse the red blood cells. The tube was then filled with fresh medium, the cells pelleted by centrifugation at 200 x g for 10 minutes and the supernatant discarded. The cells were washed once in DMEM-4 and then resuspended in medium; the cells were counted and their concentration adjusted to

1.25 x 10^6 cells/ml. Concanavalin A (Con-A) was added to a final concentration of 2 μg/ml and the cells cultured for 48 hours at 37°C, 5% CO_2. The supernatant was collected following removal of cells and debris by centrifugation at 800 x g for 10 minutes. Residual Con-A was neutralised by adding α-methylmannosidase to a final concentration of 0.1 M. The medium was then filter sterilised and stored in aliquots at -70 °C until use.

2.2.1.6 Production of cat lymphocyte conditioned medium

Cats used for this procedure were SPF and between one and four years of age. The cats were anaesthetised with a mixture of ketamine (Ketaset - Willows Francis Veterinary, Crawley, West Sussex, UK) and xylazine (Rompun - Bayer plc, Bury St Edmunds, Suffolk, UK) given by intramuscular injection. After collection of blood (for unrelated procedures) by the intracardiac route they were then euthanased with pentobarbitone by intracardiac injection. The abdominal cavity was opened and the mesenteric lymph nodes removed, using aseptic technique, into 50 ml of RPMI-5 culture medium (RPMI medium containing 5% FBS, 2 mM L-glutamine, 100 iu/ml penicillin, 100 μg/ml streptomycin). The tissue was teased apart using two scalpel blades in a 90 mm petri dish, overlain with medium. The cells were then resuspended in 10 - 15 ml RPMI-5. Mononuclear cells (predominantly lymphocytes) were isolated by centrifugation at 1500 x g for 15 minutes over Ficoll-diatrizoate solution (Ficoll-Hypaque - Pharmacia Biotech). Cells at the interface layer were carefully removed, washed with an equal volume of fresh medium and then centrifuged at 400 x g for 10 minutes. The supernatant was discarded and the cells resuspended in approximately five millilitres of RPMI-5 medium. The cells were counted and further medium added to give a cell concentration of 2.5 x 10^6 cells/ml. Replicate 20 ml cultures were set up with phytohaemagglutinin (PHA) added to a final concentration of 1 μg/ml. Cultures were incubated for 48 hours at 37°C, 5% CO_2. Cells and debris were then removed by centrifugation at 800 x g for 10 minutes; the supernatant was collected and stored in aliquots at -20°C.

2.2.1.7 Marrow culture techniques

2.2.1.7.1 Collection of Bone Marrow

Cats were anaesthetised using 1.5 - 2 ml of alphaxelone/alphadolone acetate (Saffan, Pitman-Moore Ltd., Crewe, UK) given by intravenous injection. The left or right gluteal area was clipped and prepared aseptically, aspirates of marrow were then collected from the femur via the inter-trochanteric fossa using a disposable 18 G Jamshidi sternal/iliac aspiration needle (Baxter Healthcare, Glasgow, UK). Aspirates were transferred to two millilitres of IMDM containing 100 iu/ml heparin (Monoparin, CP Pharmaceuticals Ltd., Wrexham, UK). Bone marrow aspirates were also collected from cats following euthanasia; aspirates were collected immediately after euthanasia, with the femur being removed and opened with bone cutting forceps, if necessary.

2.2.1.7.2 Preparation of agar

Agar for GM-CFC assays was prepared by adding 33 g of agar (Agar Noble, DIFCO Laboratories, Michigan, USA) to 1000 ml of tissue culture grade distilled water. The mixture was heated to boiling point, stirring all the time. When the agar had dissolved, the solution was autoclaved at 121°C for 20 minutes, then aliquoted into 50 ml centrifuge tubes, cooled and stored at room temperature.

2.2.1.7.3 Cell preparation

Marrow aspirates were layered onto a Ficoll-Diatrizoate gradient (Ficoll-Paque, Pharmacia LKB Biotechnology Inc., New Jersey, USA) and centrifuged for 15 minutes at 1,500 x g. Interface cells were then aspirated and washed in an equal volume of IMDM, centrifuged at 400 x g for 10 minutes and resuspended in one to five millilitres of IMDM, depending on pellet size. Cells were then counted using a haemocytometer.

2.2.2 RECOMBINANT DNA TECHNIQUES

2.2.2.1 Storage and growth of bacteria

Plasmids were maintained in *E.coli* strains DH5α, JM105, or INVαF'. To enable storage of these *E.coli* host strains and of transformants obtained during this work, glycerol stocks were prepared. The desired bacterial culture was streaked onto a 1.5% agar plate (1.5% agar in LB medium); in cases where the bacterial stock contained a plasmid conferring ampicillin resistance (all vector strains used in this project) the medium was supplemented with 50 - 100 μg/ml ampicillin. The plate was incubated overnight at 37°C and the following day single colonies were picked using a pipette tip, into 10 ml LB medium (supplemented as appropriate with 50 - 100 μg/ml ampicillin) in a sterile universal. The culture were incubated at 37°C overnight in an orbital incubator. Confirmation that the overnight culture was derived from bacteria containing the desired recombinant plasmid was achieved by DNA extraction and restriction digest (section 2.2.2.4). Glycerol stocks were prepared by the addition of 200 μl of 80% glycerol to one millilitre of culture; stocks were stored at -70°C. Bacterial stocks were revived for subsequent work by using a sterile platinum wire to scratch the surface of the stock, following which it was streaked onto an agar plate as outlined above.

2.2.2.2 Extraction and purification of plasmid DNA

Plasmid DNA was isolated using a modification of the alkali lysis technique described by Birnboim And Doly (1979).

2.2.2.2.1 Large Scale Plasmid Preparations.

A 10 ml overnight culture of the desired transformant was grown and used to seed a 500 ml culture which was further grown overnight at 37°C, with shaking. Bacterial pellets were obtained by centrifugation at 3,300 x g for 20 minutes at 4°C. The pellet was resuspended in 20 ml lysis buffer and left on ice for 30 minutes. The bacteria were lysed by addition of 40 ml 1% SDS/0.2 M NaOH and the preparation left on ice for a further five minutes. High molecular weight RNA, chromosomal DNA and protein/membrane complexes were precipitated on ice for 30 minutes following the

addition of 30 ml 3M KoAc. The precipitate was separated from the supernatant by centrifugation at 3,300 x g for five minutes at 4°C, and subsequent filtration through sterile gauze. DNA was precipitated at room temperature for 30 minutes by the addition of 0.6 volumes of isopropanol to the supernatant. The DNA was pelletted by centrifugation at 3,300 x g for ten minutes, the supernatant discarded and the DNA pellet vacuum dried for one hour then resuspended in eight millilitres of TE buffer (pH 8.0). Following the addition of 9.6 g CsCl and 600 µl of ethidium bromide (3mg/ml) the refractive index of the resultant solution was measured using a refractometer and adjusted to 1.388 - 1.390. The mixture was transferred to two Quick-Seal tubes (Beckman) which were balanced to within 0.05 g and heat sealed. The tubes were spun overnight in a Vti65 rotor at 55,000 rpm, 20°C in a Beckman model L8M ultracentrifuge. The closed circular plasmid DNA (lower band) was collected using a sterile two millilitre syringe and 19 G needle and the ethidium bromide removed by four sequential extractions using equal volumes of isoamyl alcohol. The aqueous phase was transferred to a collodion bag (Sartorius) that had been pre-soaked in dH_2O for one hour and the CsCl removed by dialysis against TE buffer (pH 8.0). The DNA solution was concentrated by butan-2-ol extraction, then precipitated at -20°C following the addition of 0.1 volumes of 3M NaAc and 2.5 volumes of ethanol. The DNA was pelleted by centrifugation at 13,000 rpm for 10 minutes, then washed with 70% ethanol, spun at 13,000 rpm for 10 minutes, and vacuum dried. The DNA was resuspended in 0.5 - 1.0 ml of dH_2O, its concentration determined by spectrophotometry (see section 2.2.2.3.1), diluted to a final concentration of 1 µg/µl and stored at -20°C.

2.2.2.2.2 Small-Scale Preparations.

One and a half millilitres of an overnight culture of the desired transformant was removed to an eppendorf and spun at 13,000 rpm in a microcentrifuge for two minutes. The supernatant was discarded and the tubes inverted for two minutes to ensure that the pellet was media free. The pellet was resuspended in 150 µl of lysis buffer containing approximately 0.5 mg of lysozyme. Lysis was effected by addition of 300 µl of 1% SDS/0.2 M NaOH to the resuspended cells; following gentle mixing the tubes were left on ice for five minutes. Protein, bacterial chromosomal DNA and cellular debris were precipitated by addition of 225 µl of 3M KoAc; following gentle

mixing the tubes were left on ice for five minutes. After centrifugation at 13K for five minutes the supernatant was removed into a clean eppendorf. The plasmid DNA was recovered by addition of 630 µl of 100% ethanol followed by centrifugation at 13,000 rpm for 10 minutes. The pellet washed with one millilitre of 70% ethanol and again centrifuged (13,000 rpm, two minutes). The ethanol was removed and the pellet dried in a vacuum desiccator for one minute. The DNA was then resuspended in 20 - 40 µl dH_2O.

2.2.2.2.3 Preparation of DNA for cycle sequencing

Preparation of pure DNA template for use in cycle sequencing reactions was facilitated by use of the Wizard™ Minipreps DNA Purification System. This system utilises a modified alkali lysis procedure followed by further purification using a resin that binds plasmid DNA.

Three millilitres of overnight culture was removed to an eppendorf and spun at 13,000 rpm in a microcentrifuge for two minutes. The supernatant was discarded and the tubes inverted for two minutes to ensure that the pellet was media free. The pellet was resuspended in 200 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/ml RNAse A). Lysis was effected by addition of 200 µl of 1% SDS/0.2 M NaOH to the re-suspended cells; following gentle mixing, protein, bacterial chromosomal DNA and cellular debris were precipitated by addition of 200 µl of neutralisation solution (2.55 M KoAc). The tube was inverted several times to mix and after centrifugation at 13K for five minutes the supernatant was removed into a clean eppendorf. One millilitre of DNA purification resin was added to the supernatant, mixed and then loaded onto a minicolumn using a two millilitre syringe. The column was washed twice with two millilitres of column wash solution (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 42.5% ethanol). The column containing the resin was then dried by centrifugation at 13,000 rpm for one minute. Plasmid DNA was recovered by applying 50 µl of dH_2O to the column; after a delay of one minute, the DNA was eluted into a clean eppendorf by centrifugation at 13,000 rpm for one minute.

2.2.2.3 Determination of nucleic acid concentration

2.2.2.3.1 Determination by spectrophotometry

The nucleic acid sample was diluted 1:100 by addition of 4 µl of nucleic acid to 396 µl of dH_2O. The optical density was measured at 260 nm and 280 nm, in comparison to a blank of dH_2O. An OD reading of 1.0 at 260 nm corresponds to an approximate nucleic acid concentration of 50 µg/ml for double stranded DNA, 40 µg/ml for RNA, or 33 µg/ml for single stranded oligonucleotides. The ratio of the OD readings at 260 nm and 280 nm (OD_{260}/OD_{280}) was used to estimate the purity of the nucleic acid. Pure preparations of DNA and RNA have an OD_{260}/OD_{280} of 1.8 and 2.0, respectively; a lower value suggests possible protein or phenol contamination.

2.2.2.3.2 Estimation of double stranded DNA concentration via gel electrophoresis

In cases where there was insufficient sample to permit quantification via spectrophotometry or where it was desired to verify the purity of DNA fragments of a certain size, the concentration of dsDNA was determined by running the sample on a polyacrylamide or agarose gel (see section 2.2.2.5) and comparing the intensity of the fluorescence of the unknown DNA to that of a known quantity of the appropriate size marker (ϕX174 RF DNA/Hae III fragments or λ DNA/HindIII fragments), following staining with ethidium bromide and visualisation by UV transillumination.

2.2.2.4 Restriction endonuclease digestion

Typically, 1 - 2 µg of DNA was digested in a 20 µl reaction mix containing the appropriate buffer, 5 mM spermidine and 5 - 10 units of the desired restriction enzyme. The reactions were incubated at 37°C for a minimum of one hour. Where the isolation of restriction fragments was required, larger quantities of DNA, generally 5 - 10 µg, were digested, with the reaction volume and components being increased proportionally. When digesting plasmid DNA prepared by the small scale procedure, likely to contain significant RNA contamination, this was followed by the addition of 10 µg of RNAse A with a further 15 minutes incubation at 37°C.

2.2.2.5 Electrophoresis of DNA

2.2.2.5.1 Agarose gel electrophoresis

DNA fragments of 1.0 - 10 kb were separated and identified by agarose gel electrophoresis using a submarine agarose gel kit (Mini the Gel Cicle - Hoefer Scientific Instruments, San Francisco, CA). Typically, 0.5 - 0.75 g agarose was added to 50 ml of TAE buffer, melted in a microwave and mixed to produce a 1 - 1.5% gel. Once the gel mix had cooled to 55°C, the gel was poured into a 100 x 65 mm gel support in its casting tray and an appropriate gel comb (eight or twelve well) inserted. The gel was allowed to solidify before transferring to an electrophoresis tank; the gel was immersed in TAE buffer and the comb carefully removed. DNA samples were prepared by the addition of an appropriate volume of 10 x gel loading buffer; molecular size standard DNA was prepared similarly and the samples loaded into the wells using a micropipette. Gels were run at 40 - 50 volts for 60 - 120 minutes, then removed from the gel apparatus and stained in buffer solution containing 0.5 μg/ml ethidium bromide for 30 minutes. Following destaining for 30 minutes in dH_2O, gels were visualised on a UV transilluminator and photographed using black and white Polaroid film (Type 667 - Polaroid UK Ltd., St Albans, Herts, UK).

2.2.2.5.2 Polyacrylamide gel electrophoresis

In order to separate, visualise and determine the size of DNA fragments under approximately 1.2 kb (including PCR products and products of restriction digests), non-denaturing polyacrylamide gel electrophoresis was employed. Glass plates of 16 cm x 16 cm size were assembled with a 0.75 mm spacer in a casting stand (Atto). Five to six percent gels were prepared with five to six millilitres of 30%:0.8% acrylamide/bisacrylamide solution (Scotlab, Strathclyde, Scotland), three millilitres of 10 x TBE buffer and dH_2O added to 30 ml total volume. Following the addition of 25 μl of TEMED and 250 μl 10% APS, the gel solution was poured between the assembled gel plates and a comb (12 or 20 well) inserted. After polymerisation, the gel plates were removed from the casting apparatus, the spacer removed and the plates transferred to the gel electrophoresis apparatus. The apparatus was filled with 1 x TBE buffer, the gel comb removed and the wells flushed with buffer. Samples

were prepared as described in 2.2.2.5.1 and loaded onto the gel using 0.4 mm flat ended gel loading tips; ϕX174 RF DNA/Hae III fragments were used as a molecular size standard. Gels were electrophoresed at 220 V for 45 - 90 minutes then removed and stained in buffer solution containing 0.5 μg/ml ethidium bromide for 15 minutes. Following destaining for 30 minutes in dH_2O, gels were visualised on a UV transilluminator and photographed using black and white Polaroid film.

2.2.2.6 Purification of restriction enzyme fragments

Where purification of DNA fragments over 500 bp in size was required for construction of recombinant plasmids, DNA was purified from agarose gels using the GenecleanII Kit (Bio 101 Inc.), which utilises a silica matrix (glassmilk) to bind single and double stranded DNA without binding contaminants. The DNA was separated by electrophoresis through a 0.8 - 1.5% agarose gel prepared using TEA buffer. Following staining with ethidium bromide the gel was visualised by UV illumination and the desired band excised using a sterile scalpel. Three volumes of NaI were added and the gel slice incubated at 55°C for five minutes or until all of the agarose was dissolved. Five microlitres of glassmilk suspension was added to the resultant DNA solution, with an additional one microlitre added for each 0.5 μg of DNA above 5 μg. The DNA was allowed to bind to the silica matrix on ice for 10 minutes. The glassmilk/DNA complex was pelleted by brief centrifugation in a microcentrifuge and then washed by resuspending in 200 μl new wash (Tris buffered NaCl, ethanol and water solution) after which the glassmilk/DNA complex was again pelleted by brief centrifugation; this wash step was repeated three further times. After the final wash, the pellet was dried briefly in an vacuum dessicator. The DNA was eluted by resuspending the pellet in 20 μl of dH_2O and incubating at 55°C for 10 minutes. The matrix was pelleted in a microcentrifuge and the supernatant containing the DNA removed to a clean eppendorf.

2.2.2.7 Ligation of vector and target DNA

Vector and insert DNA were mixed at a molar ratio of 1:1 to 1:5 (typically using 50 - 100 ng vector DNA), with an appropriate volume of ligation buffer and 4 units DNA ligase, in a volume of 10 - 20 μl. Reactions were allowed to proceed overnight at

14°C and stored thereafter at -20°C if not used immediately. A control ligation, omitting insert DNA was generally set up in parallel to the above, in order to check for 'background' when performing subsequent bacterial transformations.

2.2.2.8 Transformation of bacteria with plasmid DNA

When transforming bacteria, by either of the methods described below, in addition to the transformation with recombinant plasmid, the bacteria were also transformed with a control plasmid (as a positive control) and a ligation reaction from which the insert DNA had been omitted (as a negative control).

2.2.2.8.1 Use of ready-competent bacteria

Transformation of DH5α cells: Cells were thawed on wet ice, gently mixed and 20 µl of cells aliquoted to a chilled microcentrifuge tube for each transformation required. Unused cells were refrozen in 20 µl aliquots in a dry ice/ethanol bath for five minutes before returning them to the -70 °C freezer. One microlitre of ligation reaction or control plasmid (pUC18) was added to the cells (1 - 10 ng DNA), moving the pipette through the cells while dispensing in order to facilitate mixing. Cells were left on ice for 30 minutes then heat shocked in a 42 °C water bath for 40 seconds. Cells were then placed on ice for 2 minutes, 80 µl of SOC medium added and the tubes incubated at 37°C for 1 hour with shaking at 225 rpm. Cells were plated onto LB plates (containing 50 µg/ml ampicillin; 25 µl of X-Gal stock solution was spread on the plate one hour prior to use if blue-white colour selection was used) and incubated at 37 °C overnight.

Transformation of INVαF' cells: The transformation procedure for these cells was essentially as described for DH5α cells with minor modifications: one vial of cells (50 µl) was used for each transformation; 2 µl of 0.5 M 2-ME was added to the cells prior to incubation on ice; cells were heat shocked for 30 seconds; 450 µl of SOC medium was added to the cells; typically 100 µl of cells was spread on each LB agar plate.

2.2.2.8.2 Preparation of freshly competent bacteria and subsequent transformation

JM 105 and BL21 *E.coli*, used for procedures involving the pGEX plasmid were made competent using a modification of the procedure described by Chung *et al.* (1989). A single colony was picked from an LB agar plate after overnight growth and used to inoculate 50 ml of LB broth. Bacteria were grown at 37°C, with shaking at 250 rpm, to an OD_{600} of 0.4 - 0.5. The cells were then sedimented at 2500 x g for 15 minutes at 4°C, the supernatant discarded, and then resuspended in five millilitres of ice-cold TSS buffer. Cells were kept on ice until used for transformations; within two to three hours of preparation.

To 100 µl of freshly competent E.coli was added two microlitres of ligation reaction, containing approximately 1 - 10 ng of plasmid DNA. After chilling briefly on ice, 900 µl of LBG medium (LB medium supplemented with 20 mM glucose) was added and the tubes incubated at 37°C for one hour with shaking at 250 rpm. Cells (50 - 200 µl) were plated onto LBAG plates (LB plates containing 50 µg/ml ampicillin; 20 mM glucose) and incubated at 37 °C overnight.

2.2.2.9 Screening of transformants for desired recombinant plasmids

All plasmid strains used in this project conferred ampicillin resistance upon host bacteria, allowing selection and maintenance of transformed bacteria with ampicillin supplemented media.

2.2.2.9.1 α-complementation

The pCR™II plasmid contains genes encoding the *lacZα* fragment of β-galactosidase and the *lac* promoter and is thus capable of complementation with the φ fragment encoded by the *E.coli* host strains DH5α and INVαF', giving active β-galactosidase. The incorporation of X-gal into LB agar plates allows the selection of transformants based on blue-white screening. Disruption of *lacZα* expression occurs with the cloning of fragments into the multiple cloning site of this vector, hence recombinants with plasmid containing insert DNA appear white whilst non-recombinants, expressing a functional β-galactosidase, appear blue.

2.2.2.9.2 Restriction analysis of small-scale plasmid preparations

Plasmid DNA, isolated as described in 2.2.2.2.2 , was subjected to restriction digest with the appropriate enzyme(s), and the resulting products of digestion run on a polyacrylamide or agarose gel. Bacteria with plasmids containing inserts of the desired size were stored as glycerol stocks as detailed in section 2.2.2.1.

2.2.3 PREPARATION OF NUCLEIC ACIDS

2.2.3.1 Preparation of mRNA

For procedures involving RNA preparation and subsequent manipulation, care was taken to avoid degradation by ribonucleases. All plasticware used was either new or was treated by soaking overnight in DEPC treated water, followed by autoclaving twice at 121°C for 15 minutes. All solutions were prepared using DEPC treated water. Gloves were worn and changed frequently.

The preparation of high quality mRNA was facilitated by the use of the Quickprep® mRNA kit (Pharmacia). Tissue is disrupted in guanidinium isothiocyanate to ensure rapid inactivation of endogenous RNAse activity and dissociation of cell components from the mRNA (based on the method of Chirgwin *et al.*, 1979). After adjustment of the buffer concentration and pelleting of cellular debris and insoluble proteins by centrifugation, polyadenylated mRNA is extracted by binding to oligo(dT) cellulose column (Aviv and Leder, 1972.). Finally, following washes to remove DNA, protein and non-poly A+ RNA, the mRNA is eluted from the column.

The manufacturer's instructions were followed, briefly, as follows. Cultured cells (< 5 x 10^7 cells) were pelleted by centrifugation at 400 x g for five minutes. The cells were resuspended in 1.5 ml extraction buffer (aqueous solution containing guanidinium thiocyanate and N-lauroyl sarcosine) and homogenised by passing through a 21 G needle attached to a syringe. To the sample was added three millilitres of elution buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]; after brief homogenisation the sample was transferred to a sterile polypropylene centrifuge tube and centrifuged at 10,000 rpm in a Beckman JA-20 rotor for 10 minutes. An

oligo(dT)-cellulose spun column was prepared by centrifugation (all centrifuge steps involving the spin column were at 350 x g for two minutes) with both top and bottom closures removed; the supernatant was then applied to the column and mixed gently for 15 minutes. The column was centrifuged with both closures on and the supernatant discarded. The column was then washed (followed each time by centrifugation) three times with high-salt buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl] and twice with low-salt buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl]. The mRNA was then eluted with three sequential washes of 0.25 ml elution buffer warmed to 65°C. The mRNA was precipitated overnight at -70°C following the addition of 10 µl glycogen solution (5 -10 mg/ml glycogen in DEPC treated water), 75 µl 2.5 M KAc solution and 1.5 ml of 95% ethanol. The mRNA was then pelleted by centrifugation at 13 K for 15 minutes, the ethanol removed, and the mRNA dried for one minute in a vacuum desiccator. The mRNA was then dissolved in 20 - 40 µl DEPC treated water and its concentration determined by spectrophotometry (section 2.2.2.3.1).

2.2.3.2 First-strand cDNA synthesis

In order to maximise the likelihood of obtaining full-length cDNA copies of mRNA, a commercial cDNA synthesis kit was employed (First-strand cDNA synthesis kit - Pharmacia Biotech). The kit contains all components required for first strand cDNA synthesis, including a preassembled reaction mix containing Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase (Roth *et al.*, 1985), RNAguard (an RNAse inhibitor), RNAse/DNAse free BSA, and dNTPs in an aqueous buffer. An oligo-dT primer (Not I-d$(T)_{18}$ primer) supplied with the kit was used to prime cDNA synthesis; sequence as follows:

5'-d[AAC TGG AAG AAT TCG CGG CCG CAG GAA T_{18}]-3'

Typically, 200 ng mRNA was placed in a microcentrifuge tube and brought to 20 µl with RNAse free water. The RNA was denatured at 65°C for 10 minutes then chilled on ice. To the RNA was added 11 µl bulk first strand reaction mix, 1 µl of 200 mM DTT and 1 µl (0.2 µg) Not I-d$(T)_{18}$ primer. The reaction mix was incubated for one hour at 37°C, then kept on ice (or stored at -70°C) prior to amplification by the polymerase chain reaction.

2.2.4 AMPLIFICATION OF DNA BY THE POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is a powerful technique for amplification of specific DNA sequences from a complex mixture of DNA. The procedure was developed by Mullis and co-workers in the mid 1980s (Mullis *et al.*, 1986; Mullis and Faloona, 1987), enabling large amounts of a single copy gene to be generated from genomic (Saiki *et al.*, 1985; Saiki *et al.*, 1986) or viral DNA (Kwok *et al.*, 1987). The initial method used the Klenow fragment of DNA polymerase I, which had to be replenished during each cycle as it is readily denatured by the amplification conditions used. The substitution of thermostable *Taq* polymerase, isolated from *Thermus aquaticus*, circumvented this problem and allowed the automation of thermal cycling (Saiki *et al.*, 1988).

PCR enables the amplification of unknown DNA sequence by the simultaneous extension of a pair of primers, flanking the unknown sequence, each complimentary to opposite strands of the DNA. The uses of PCR are many and it has superseded more conventional molecular biological methods in many areas, including sequencing (Innis *et al.*, 1988), cloning (Scharf, 1990) and detection and analysis of RNA (Veres *et al.*, 1987). An extensive overview of PCR, its applications and detailed protocols are given in Innis *et al.* (1990). An overview of the procedure is given below, with more detail in the appropriate chapters.

2.2.4.1 Primer design

Primer design was aided by some basic guidelines as suggested by Innis and Gelfland (1990). Primers were generally 18 - 28 nucleotides in length, with a G + C composition of 50 - 60% where possible. For a given primer pair, the annealing temperatures (T_m), were balanced and complementary regions between and within primers were avoided; design of primers in this respect was aided by the Oligo primer analysis software program (Version 4.1 - Medprobe AS, Oslo, Norway).

2.2.4.2 Preparation of PCR reactions

As PCR is such a sensitive procedure it is essential to take stringent precautions to avoid PCR contamination from tube to tube or carry over of PCR products (Saiki *et*

al., 1988). PCR reactions were set up in a designated area, at a site distant from the main laboratory area, where the PCR products were handled. A set of micropippetes were kept for the sole purpose of setting up PCR reactions. Filter tip pipette tips were used to decrease the risk of carry over of reaction components from one tube to the next. A bulk reaction mix was used in order to minimise the number of pipetting steps. Reaction components (including primers) were aliquoted prior to use and aliquots stored at -20°C.

2.2.4.3 Reaction conditions

The use of high quality reagents is essential to the success of PCR; to facilitate this the Gene Amp Kit, containing all necessary reagents, was used. The manufacturer's instructions were followed. Typically a reaction mix was set up in 50 µl volume in 0.5 µl tubes containing 125 µM each dNTP, 1 x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 2.5 units *AmpliTaq*® DNA polymerase, 0.2 - 2.0 µM each primer and an appropriate volume of DNA or cDNA template, as determined experimentally; the reaction was overlain with mineral oil. Thermal cycling was carried out in a DNA thermal cycler (Perkin Elmer), with a typical cycle consisting of an initial denaturation of 94°C for five minutes, followed by 30 - 35 cycles of: denaturation at 94°C, for one minute; annealing at 45 - 60°C for one minute; extension at 72°C for one minute; with a final extension step of 72°C for five minutes. Reaction products were visualised by polyacrylamide gel electrophoresis as detailed in 2.2.2.5.2, generally using five microlitres of reaction product per well.

2.2.5 DNA SEQUENCE ANALYSIS

2.2.5.1 Manual sequencing by the chain termination method

2.2.5.1.1 Sequencing reactions

Manual sequencing used the Sequenase version 2.0 DNA sequencing kit, which is based on the chain termination method originally described by Sanger et al. (1977). The kit uses Sequenase 2.0 DNA polymerase (Tabor and Richardson, 1989), a

modified version of the original Sequenase enzyme, described by Tabor and Richardson (1987). The enzyme lacks 3' - 5' exonuclease activity, present in the wild type enzyme, and shows higher speed and processivity. The manufacturer's instructions were followed; the procedure is described briefly below, divided into four stages.

Denaturation of dsDNA template: Double-stranded plasmid DNA, prepared as described in 2.2.2.2.2, (approximately six to ten micrograms contained in 36 µl dH_2O) was denatured by the addition of four microlitres of 2 M NaOH followed by incubation at 37°C for 15 minutes. Eight microlitres of 5 M ammonium acetate was added to neutralise the mixture, and the DNA precipitated with 2 - 2.5 volumes ethanol at -70°C for 30 minutes. The DNA was pelleted by centrifugation at 13,000 rpm for 15 minutes, the supernatant discarded and the pellet washed in 500 µl 70% ethanol. After a brief centrifuge at 13,000 rpm, the pellet was dried in a vacuum desiccator, then dissolved in 14 µl dH_2O, providing sufficient DNA for two sequencing reactions.

Annealing step: Plasmid DNA, contained in seven microlitres of dH_2O, was mixed with two microlitres of reaction buffer (200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl) and one microlitre (0.5 - 2.0 pmol) of primer in a microcentrifuge tube. The mixture was brought to 65°C for two minutes, then removed to a beaker of water at 65°C and allowed to cool slowly to 35°C, at which point the reaction was considered complete and the tube placed on ice.

Labelling step: The primer annealed to the DNA template was extended using limiting concentrations of a mix of dNTPs, including radioactively labelled dATP. This step results in the generation of a mix of labelled DNA chains, varying from several to hundreds of nucleotides in length. To the above template-primer mix was added one microlitre 0.1 M DTT, 0.5 µl of [α-^{35}S]-dATP and two microlitres of labelling mix (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP) diluted five-fold with dH_2O. Finally Sequenase enzyme was diluted 1:8 with ice cold enzyme dilution buffer

(10 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.5 mg/ml BSA), two microlitres added to the labelling mix, and the reaction incubated for five minutes at room temperature.

Chain-termination step: DNA synthesis was continued using a mix of dNTPs and a dideoxynucleoside triphosphate; this results in the termination of DNA synthesis with a known ddNTP. Two and a half microlitres of each termination mix (80 µM each dNTP, 50 mM NaCl plus 8 µM appropriate ddNTP) was aliquoted into labelled screw-top microcentrifuge tubes and pre-warmed to 37°C for one minute. Three and a half microlitres of the above labelling reaction was then added to each tube, mixed by gentle pipetting, and incubated at 37°C for 15 minutes. The reactions were terminated by the addition of four microlitres of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Reactions were stored at -20°C for up to one week prior to gel electrophoresis.

2.2.5.1.2 Gel electrophoresis

The completed sequencing reactions were run on six percent denaturing polyacrylamide gels, incorporating Long Ranger™ polyacrylamide gel solution (AT Biochem, Malvern, USA). Long Ranger gel mix contains modified acrylamide monomers and a modified crosslinker that results in a gel that produces longer readable sequence, is stronger and more elastic than conventional gels, and does not require fixing or removal of urea prior to drying. Glass sequencing plates (50 x 22 cm) were cleaned with 1% SDS, rinsed thoroughly then cleaned with ethanol. Repelcote (BDH) was applied to the surface of one plate to ensure that the gel would not stick to the glass plate. The sequencing plates were assembled with 0.2 mm spacers and plastic adhesive tape. To 50 ml of sequencing gel mix was added 25 µl of TEMED and 250 µl of 10% APS; the gel was poured using a 50 ml syringe, a 24 well sharkstooth comb inserted in an inverted position and clamped in place using 'bulldog' clips. The gel was then allowed to polymerise in a near-horizontal position for at least one hour at room temperature. The tape at the bottom of the gel plates was removed, the plates mounted into the sequencing apparatus and the upper and lower gel tanks filled with 1 x TBE. The comb was removed, the surface of the gel rinsed with buffer and the comb reinserted in the correct orientation. The gel was

pre-electrophoresed for 15 minutes at 35 Watts. The DNA samples were denatured by heating to 75°C for two to five minutes immediately prior to loading two to three microlitres of sample per well. Aliquots of each reaction were run at 35 Watts for approximately two hours and four hours; this generally allowed 350 - 500 bp to be read from each sequencing reaction.

Following electrophoresis, the plates were removed from the sequencing apparatus, allowed to cool briefly then separated. The gel was transferred to Whatman 3 MM filter paper (Whatman International Ltd., Maidstone, England), covered with Saran wrap (Dow Chemical Co.) and dried, gel side uppermost, under vacuum in a gel drier for 30 - 60 minutes at 80°C. The plastic wrap was then removed and the top of the gel trimmed to fit an autoradiography cassette. A sheet of autoradiography film (Biomax HR single sided emulsion film or X-omat AR double sided emulsion film (both 35 x 43 cm) - IBI Limited, A Kodak Company, Cambridge, England) was placed in contact with the gel and exposed overnight, at room temperature, prior to developing in an automated processor. The sequence was read manually over a light box, and the data stored on a UNIX computer system. Sequence data was managed and analysed using the University of Wisconsin Genetics Computer Group (UWGCG) software (notably SeqEd, Bestfit, Lineup and Pileup programs; more detailed descriptions follow in Chapter 3).

2.2.5.2 Automated sequencing

During the later stages of this project a Licor model 4000 automated sequencer became available, which considerably increased the ease and throughput of sequencing. The sequencer utilises an infrared detection system, whereby DNA fragments are detected following labelling with IRD41 labelled primers, as they run through the denaturing polyacrylamide gel. In addition sequencing reactions were performed using a thermostable DNA polymerase (SequiTherm DNA polymerase), allowing reactions to be performed in a thermal cycler. This variation on the original method of chain termination sequencing, known as 'cycle sequencing', was first described by Innis *et al.* (1988). The method allows direct sequencing of dsDNA without alkali denaturation, requires less template and is more efficient at sequencing templates that are G/C rich or have high secondary structure.

2.2.5.2.1 Sequencing reactions

Protocols for cycle sequencing using SequiTherm DNA polymerase were provided by Licor. The following were combined in a 0.5 ml microcentrifuge tube: two microlitres IRD41 labelled primer, 2.5 µl 10 x sequencing buffer (0.5 M Tris-HCl (pH 9.3), 25 mM $MgCl_2$), one microlitre SequiTherm DNA polymerase, 1 - 2 µg dsDNA template, dH_2O to 17 µl. Into each of four labelled 0.5 µl microcentrifuge tube was aliquoted 2.0 µl of A, T, G, or C long read termination mix (45 µM each of dATP, dCTP, dTTP and 7-deaza-dGTP and either 0.03 mM ddGTP, 0.3 mM ddCTP, 0.45 mM ddATP or 0.45 mM ddTTP as appropriate); four microlitres of the template/primer/enzyme mix was then added to each of these tubes and a drop of mineral oil placed on top of the complete reaction mix. The tubes were placed in a thermal cycler (Perkin Elmer) and subjected to an initial denaturation step of 95°C for two minutes followed by 30 cycles of 95°C for 30 seconds; 60°C for 30 seconds; 70°C for 30 seconds; terminating in 4°C soak. After cycling was complete, four microlitres of stop solution (95% (w/v) formamide, 10 mM EDTA (pH 7.6), 0.1% xylene cyanol and 0.1% bromophenol blue) was injected into each of the reaction mixes, under the mineral oil. Reactions were stored at +4°C for short periods or -20°C for up to one week prior to gel electrophoresis; exposure to light was avoided.

2.2.5.2.2 Gel electrophoresis

Sequencing reactions were run on 6% denaturing polyacrylamide gels, as detailed in the manufacturer's instructions, outlined as below. Standard size plates (18 x 33 cm) were used in conjunction with 28 well rectangular toothed combs. Glass plates were cleaned thoroughly before applying bind-silane to the notched glass plate over the area to be in contact with the comb; 170 µl of a solution containing five microlitres of 10% acetic acid and 165 µl silane solution (50 µl γ-methacryloxy-propyltrimethoxysilane in 10 ml 100% ethanol) was applied using a pasteur pipette in a fume hood. After this had dried, the glass plates were assembled and placed in a casting stand. A gel mix was prepared before use with 21 g urea, six millilitres Long Ranger™ polyacrylamide gel mix (AT Biochem.), six millilitres 10 x 'long run' TBE buffer (162 g tris base, 27.5 g boric acid, 9.3 g EDTA; to 1 L with dH_2O) and dH_2O added to 50 ml total volume. To the gel mix was added 270 µl 10% APS. Three

millilitres of the gel mix was removed to a bijou and 32 µl 10% APS and four microlitres of TEMED added; a pasteur pipette was then used to inject the mix between the bottom of the glass plates to form a plug. After the plug had polymerised, 25 µl TEMED was added to the remaining gel solution, mixed and the gel poured. A comb was inserted and clamped in place using two 'bulldog' clips; the gel was then inclined at approximately 30° from horizontal, and allowed to polymerise for a minimum of 90 minutes. The gel was then transferred to the electrophoresis apparatus, both buffer tanks filled with 1 x TBE, the comb removed and wells flushed. The gel was pre-electrophoresed for 30 - 45 minutes, during which time the scanning microscope was focused and the gain controls adjusted. Sequencing reactions were denatured at 95°C for three minutes prior to loading; generally 0.8 - 1.5 µl was loaded per well. The gel was run at 31.5 W, with the scanner programmed to collect 25 frames (approximately 800 bp). Sequence data was read automatically, after manually defining the lanes; base ambiguities were checked by visual inspection of the gel image.

2.2.6 ANALYSIS OF RECOMBINANT PROTEINS

2.2.6.1 Estimation of protein concentrations

In order to estimate protein yields at a number of stages during protein expression and purification, a modified Bradford assay (Bradford, 1976) was used. This assay utilises the fact that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm following binding to protein. This shift in absorbance is linear over a relatively broad range of protein concentrations so allows accurate protein quantification within a sample.

A protein standard (bovine serum albumin or bovine gamma globulin) was diluted with dH_2O to concentrations ranging from 0.2 to 1.4 mg/ml to enable the production of a standard curve each time the assay was performed. Samples of protein for quantification were diluted in order to give a concentration in the above range. Into sterile 15 ml Falcon tubes, 0.1 ml of each solution was aliquoted; 0.1 ml of dH_2O was added to one tube as a sample blank. The dye reagent was prepared by addition of 4

volumes of dH_2O to 1 volume of dye and filtration through a 0.4 μm syringe filter (Acrodisc, Gelman Sciences); five millilitres of this reagent was added to each tube. The tubes were vortexed, incubated at room temperature for 5 minutes to 1 hour, and the OD_{595} subsequently measured against the reagent blank. The absorbance of the protein standards was used to construct a standard curve from which an approximate concentration of the unknown samples could be read.

2.2.6.2 SDS - polyacrylamide gel electrophoresis of proteins

The separation and analysis of proteins was facilitated by one dimensional denaturing discontinuous gel electrophoresis, as originally described by Laemmli (1970). Proteins are denatured by boiling in the presence of SDS and β-mercaptoethanol. The sample is then loaded onto a discontinuous gel consisting of a stacking buffer which concentrates the loaded protein sample into a narrow band and a separating gel which separates proteins on the basis of molecular size, with smaller proteins migrating faster towards the anode.

Minigels (8.0 x 7.3 cm) were formed and run using the Mini-PROTEAN II electrophoresis system (Biorad, Herts, UK) as recommended by the manufacturer. Glass plates were assembled with 0.75 mm spacers in a casting stand. The separating gel was poured to a depth of approximately 5 cm; consisting of 4 ml 30%:0.8% w/v acrylamide/bisacrylamide (giving a 12% gel), 3.35 ml dH_2O, 2.5 ml 1.5 M Tris-HCl (pH 8.8), 50 μl 20% SDS, 100 μl 10% APS and 10 μl TEMED. This was overlain with tris-saturated butanol and allowed to polymerise. The butanol was then poured off, the surface of the separating gel rinsed with dH_2O and the stacking gel poured; consisting of 650 μl 30%:0.8% w/v acrylamide/bisacrylamide, 3.0 ml dH_2O, 1.25 ml 0.5M Tris-HCl (pH 6.8), 25 μl 20% SDS, 25 μl 10% APS and 7 μl TEMED. A 10 well comb was inserted and the gel allowed to polymerise. The gel was then transferred to the electrophoresis tank, both buffer tanks filled with running buffer, the comb removed and the wells flushed. Protein samples (typically 5 - 30 μg of protein in 5 - 25 μl) were prepared by addition of an appropriate volume of 5 x protein sample loading buffer, followed by heating to 100°C for five minutes. Samples were then loaded onto the gel using 0.2 mm flat ended gel loading tips. A protein

molecular weight standard (5 -10 µl) was loaded in one or both outer wells to allow estimation of the size of sample proteins.

Gels were electrophoresed at 140V for 60 - 80 minutes until the bromophenol blue dye reached the bottom of the separating gel. The gel was then removed from the glass plates, the stacking gel discarded, and the protein bands detected either by staining with Coomassie blue or by immunodetection (section 2.2.6.3). Visualisation of protein bands with Coomassie blue involved staining for two hours in four to five gel volumes of protein fix-stain solution, followed by destaining for approximately 12 - 16 hours in destain solution (12% methanol, 7% glacial acetic acid, 81% dH_2O, with four to five changes of solution. Gels were then removed and preserved by drying for two to six hours, sandwiched between prewetted cellulose film, in a gel drying apparatus (Easy Breeze Air Gel Dryer - Hoefer Scientific Instruments, San Francisco, CA).

2.2.6.3 Detection of proteins by immunoblotting

The detection of proteins by immunoblotting (western blotting) is a rapid and sensitive technique that exploits the inherent specificity of antigen recognition by antibodies (Towbin *et al.*, 1979; Burnette, 1981). Proteins were transferred to PVDF membrane by electroblotting, following electrophoretic separation and detected using ECL reagents (Amersham Life Science). This detection system is based on the emission of light following the oxidation of luminol by horse radish peroxidase (HRP labelled antibodies), in the presence of chemical enhancers such as phenols (enhanced chemiluminescence) (Durrant, 1990) and is particularly sensitive (Gillespie and Hudspeth, 1991). The light emitted can be detected by a short exposure to blue-light sensitive film (Hyperfilm ECL, Amersham).

Following SDS-PAGE, as described in section 2.2.6.2, the gel was removed from the glass plates and rinsed in TBS. Hybond-ECL membrane was prewetted in 100% methanol for 15 seconds, with distilled water for 2 minutes and then allowed to equilibrate with transfer buffer for 10 minutes prior to blotting. Proteins were transferred to the membrane using a semi-dry electroblotting system (Transblot SD -

Biorad Laboratories, Hercules, CA) The gel and membrane were sandwiched in close apposition between two sheets of extra thick filter paper (Biorad) pre-soaked in transfer buffer, and transferred at 10 V for 45 minutes.

The membrane was then rinsed in TBS and non-specific binding sites blocked by immersing the membrane for 30 minutes in 10% low fat dried milk (Marvel - Premier Beverages, Stafford, UK) TBS-T (0.1% Tween in tris buffered saline) solution at room temperature on an orbital shaker. The membrane was rinsed briefly with TBS-T, washed once for 15 minutes then twice for five minutes, with shaking at room temperature. The membrane was then incubated with the primary antibody, at a pre-determined dilution in 5% low fat dried milk, TBS-T, for two hours at room temperature, with shaking. The membrane was then washed as detailed above prior to incubating with the secondary antibody (HRP labelled), appropriately diluted in 5% low fat dried milk, TBS-T, for one hour, at room temperature, with shaking.

The membrane was washed (as above) before detection by the ECL method, which was carried out in a darkroom. An equal volume of detection reagent A was mixed with reagent B (typically 0.5 ml each). Excess buffer was drained from the membrane and the detection solution was pipetted onto the surface of the membrane carrying the protein. After incubation for one minute at room temperature, excess reagent was drained from the membrane which was then wrapped in plastic film. The membrane was placed, protein side up, in a film cassette and a sheet of autoradiography film (Hyperfilm-ECL) placed on top, in the dark. The cassette was closed and the film exposed for 15 - 30 seconds, before developing in an automated processor. A second sheet of film was then exposed, generally for 2 - 20 minutes, the time being estimated from the appearance of the first autoradiograph.

In order to confirm that the bands seen on Western blots were not due to non-specific binding of the secondary antibody, the blots were 'stripped' by soaking in 10 ml stripping buffer (6.25 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM 2-ME) for 30 minutes, at 50°C, with agitation. The membrane was then reprobed, as above, however the primary antibody labelling step was omitted. Any non-specific binding of the secondary antibody was then detected by the ECL method, as outlined above.

CHAPTER THREE - CLONING AND SEQUENCE OF TWO ISOFORMS OF FELINE STEM CELL FACTOR

3.1 INTRODUCTION

3.1.1 FELINE CYTOKINES

At the time of writing the study of feline cytokines is in its infancy. Isolation and cloning of cytokines in this species enables determination of their nucleotide sequence permitting techniques such as quantitative RT-PCR, Northern assays or *in situ* hybridisation to be applied to the study of the role of cytokines in feline disease. Once cloned the cytokine can be expressed in one of a number of expression systems (see Chapter 4 for discussion) and antibodies to the cytokines may be produced; both the *in vitro* and *in vivo* effects of the cytokine may then be investigated. Ultimately a number of cytokines are likely to form a new array of therapeutic agents to treat feline disease. The current status of cloned feline cytokines with ascribed DNA database accession numbers is shown in Table 3.1.

Cytokine	*EMBL Accession Number*	*Reference*
Interferon-α	S62636	Nakamura *et al.*, 1992; Ueda *et al.*, 1993.
Interferon-γ	D30619	Argyle *et al.*, 1995.
TNF-α	M92061	Daniel *et al.*, 1992.
IL-1β	M92060	Daniel *et al.*, 1992.
IL-2	L19402	Cozzi *et al.*, 1993.
IL-4	X87408	Schijns *et al.*, 1995.
IL-6	D13227	Ohashi *et al.*, 1993.

Table 3.1: List of feline cytokines with assigned EMBL database accession numbers.

3.1.2 MOLECULAR BIOLOGY OF SCF

3.1.2.1 Identification as SCF as the *Sl* locus gene product

The establishment of the alleleism between the *W* locus and *c-kit* in the mouse and the finding that the *c-kit* gene encoded a receptor prompted a number of groups to investigate the possibility that the *Sl* locus encoded the SCF-R ligand. Nocka *et al.* (1990) used a mast cell proliferation assay to purify a 30 kDa protein from the

Addendum

3.1.2.1 Identification of SCF as the *Sl* locus gene product

A partial cDNA encoding ovine SCF has also been cloned (McInnes *et al.*, 1995; unpublished data, EMBL accession no. Z50743).

supernatant of Balb/3T3 fibroblast cultures. The purified protein, designated *kit*-ligand (KL), supported the proliferation of normal bone marrow mast cells (BMMCs) and peritoneal mast cells but not BMMCs derived from *W/W*^{v} mice. In addition KL promoted the formation of erythroid bursts (BFU-E) from fetal liver or spleen cells in conjunction with erythropoietin, increasing both the number and size of colonies grown following plating onto methylcellulose. These findings were soon followed by the cloning and characterisation of the product of the *Sl* locus, the *c-kit* ligand, reported simultaneously by Nocka *et al.* and two other groups (Anderson *et al.*, 1990; Copeland *et al.*, 1990; Huang *et al.*, 1990; Martin *et al.*, 1990; Zsebo *et al.*, 1990a; Zsebo *et al.*, 1990b).

Subsequent to the cloning of human and rodent SCFs (Anderson *et al.*, 1990; Martin *et al.*, 1990; Anderson *et al.*, 1991; Flanagan *et al.*, 1991), SCF cDNAs have been cloned in a number of other species including porcine (Zhang and Anthony, 1994), bovine (Zhou *et al.*, 1994), canine (Shull *et al.*, 1992), brushtail possum (Greenwood *et al.*, 1996), chicken (Zhou *et al.*, 1993) and quail (Petitte and Kulik, 1996). In the case of chicken and porcine SCF a single isoform is described and only a partial cDNA is characterised in the rat. All other species show two major SCF isoforms. The full length SCF mRNA encodes a predicted protein of 273 (human/murine), 274 (porcine/bovine/canine/possum) or 287 (chicken/quail) amino acids. The predicted protein has a signal peptide of 25 aa, an extracellular ligand domain, a transmembrane domain and a short intracellular domain (Figure 3.1). The shorter isoform has a deletion of 84 bp in the extracellular coding sequence, resulting in a protein truncated by 28 aa. The deleted nucleotides correspond exactly to exon 6 of the characterised human and rat SCF genes (Martin *et al.*, 1990). An additional isoform is described for murine SCF, which has a deletion of 48 aa, the 5' boundary of which is the same for the 84 bp truncated isoform (Anderson *et al.*, 1990). Each of these isoforms encodes a membrane associated form of SCF.

The soluble form of SCF, initially isolated as described above, was further characterised by Zsebo *et al.* (1990), who isolated the protein from Buffalo rat liver-conditioned medium. The soluble growth factor consists of the first 164 or 165 amino acids of the extracellular domain of the longer, cell membrane associated, SCF

isoform (Lu *et al.*, 1991) and is generated by proteolytic cleavage at a site that lies within the extracellular region encoded by exon 6. This process is analogous to the generation of soluble isoform of the structurally related cytokine, CSF-1 from its respective membrane associated precursor (Bazan, 1991a). The shorter membrane associated SCF protein lacks the proteolytic cleavage site of the longer isoform, however the shorter isoform of murine, but not human SCF is cleaved, albeit less efficiently, at an alternative site, encoded by exon 7 (Huang *et al.*, 1992; Majumdar *et al.*, 1994). The rate of proteolysis of both murine isoforms may be increased *in vitro* by stimulation with phorbol 12-myristate 13-acetate or calcium ionophore A23187 (Huang *et al.*, 1992). The cleavage of each murine isoform is inhibited by a different panel of protease inhibitors, suggesting distinct regulatory mechanisms for each (Pandiella *et al.*, 1992).

Regulation of the expression of each isoform may provide a mechanism allowing control of the relative amounts of soluble or membrane associated ligand. The respective biological functions of soluble and membrane associated SCF have yet to be fully elucidated, however a number of lines of evidence support the concept that they have different, yet complimentary roles. Using RNAse protection assays, expression of the two major SCF isoforms in a number of murine tissues has been shown to be tissue specific. In brain and thymus, the longer isoform predominates, with the shorter isoform barely detectable. Other tissues express both isoforms in variable ratios (e.g. testis 1:2.6, bone marrow 3:1; longer:shorter isoform) (Huang *et al.*, 1992). The transfection of a human cell line *Sl/Sl*^{d} with expression vectors for the shorter (hSCF^{220}) or longer isoform (hSCF^{248}) of human SCF showed that hSCF^{220} was mainly membrane associated, whilst hSCF^{248} expression led predominately to the production of soluble SCF. Stable transfectants expressing either isoform are able to support the growth of human haemopoietic progenitor cells *in vitro*. However, haemopoietic progenitor cells are maintained in culture for 1 - 2 weeks longer when cultured over stromal cells expressing the membrane associated receptor (hSCF^{220}) as opposed to hSCF^{248} expressing stromal cells (or cultures to which soluble recombinant hSCF had been added) (Toksoz *et al.*, 1992). Further evidence for the importance of membrane associated SCF *in vivo* is derived from studies on *Sl* mutant mice. The *Sl*^{d} mutant has a 4.0 kb intragenic deletion of SCF genomic DNA (Brannan

et al., 1991). This deletion results in the production of a truncated soluble protein which lacks both the transmembrane and cytoplasmic domains. Expression of the SCF-Sl^d gene product *in vitro* in either yeast or mammalian COS cells produces a biologically active protein that supports mast cell proliferation (Brannan *et al.*, 1991; Flanagan *et al.*, 1991; Huang *et al.*, 1992). Despite this activity *in vitro*, Sl^d mutant mice have a relatively severe phenotype; although they are viable, they have a severe anaemia, lack skin pigment and are sterile. There are a number of possible explanations for the different effects mediated by the soluble and membrane bound isoforms, speculated upon by Flanagan *et al.* (1991). Expression of a 'fixed' membrane associated cytokine may serve to maintain local levels of the growth factor above a critical level. The membrane associated isoform may act as an adhesion factor; this has been demonstrated *in vitro* for mast cells which adhere to COS cells expressing membrane associated SCF (Flanagan *et al.*, 1991). More recently, it has been demonstrated that membrane bound SCF induces more persistent tyrosine kinase activation of the SCF receptor in murine MO7e cells than soluble SCF. Furthermore, stimulation with soluble SCF is associated with a more rapid downmodulation of cell surface SCF-R levels, mediated by receptor-ligand endocytosis, than occurs in response to membrane associated SCF (Miyazawa *et al.*, 1995).

Most studies of SCF function have concentrated on the biological properties of the extracellular portion of the membrane associated receptor or the soluble protein. There is, however, some evidence that the intracellular domain of the membrane associated receptor may have important biological functions. The *Steel* mutant mouse, Sl^{17H} produces a splicing defect which leads to a predicted SCF protein with a truncated cytoplasmic domain of 28 aa (c.f. 36 aa), with only the first amino acid read in the correct frame. The mutation does not affect the level of SCF expression, nor the ability of cells transfected with constructs for Sl^{17H} SCF to support mast cell proliferation. However, mutant homozygous mice show sterility in male but not female mice, a mild anaemia and dilution of coat colour (Brannan *et al.*, 1992). In order to clarify the role of the cytoplasmic domain of SCF, further studies are required to confirm that tissue specific changes in expression or impaired intra-

membrane stability of the truncated receptor are not responsible for the phenotypic effects seen in Sl^{17H} mutant mice.

Soluble SCF, isolated from Buffalo rat liver conditioned medium, is a heavily glycosylated protein with a molecular weight of 28 - 35 kDa, as determined by SDS-PAGE. The variability in molecular weight reflects the heterogeneity of *N*-linked glycosylation; removal of *N*-linked sugars leads to a more homogenous molecular weight of 26 kDa. Further removal of *O*-linked sugar residues leads to a molecular weight (on reducing gels) of 18 - 19 kDa (Zsebo *et al.*, 1990b). Purified rat SCF and recombinant SCF expressed in either *Escherichia coli* or Chinese hamster ovary (CHO) cells exists as a non-covalently associated dimer under non-denaturing conditions (Zsebo *et al.*, 1990b; Arakawa *et al.*, 1991). Consequently, molecular weight determination by equilibrium sedimentation reveals molecular weights of 36 kDa and 53 kDa for *E. coli* and CHO expressed SCF, respectively (Arakawa *et al.*, 1991).

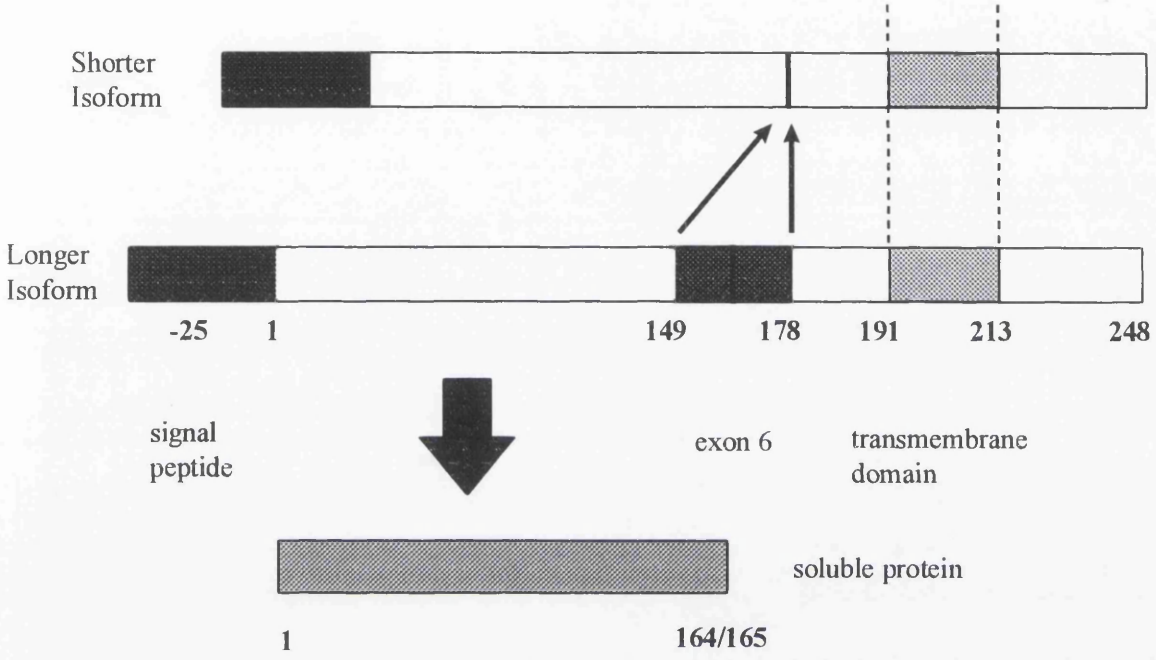


Figure 3.1: Structure of the two isoforms of human SCF. The longer isoform can be cleaved at a proteolytic cleavage site which lies within the region of the extracellular domain of the protein encoded by exon 6. This results in the release of biologically active soluble SCF. The shorter isoform lacks exon 6 and is therefore found mainly as a membrane bound protein. The signal peptide serves to target the protein to the endoplasmic reticulum and is removed during post translational processing.

3.1.2.2 Genomic organisation of the SCF gene

The SCF gene maps to mouse chromosome 10 and human chromosome 12 (between 12q22 and 12q24) (Anderson *et al.*, 1991; Geissler *et al.*, 1991). Human and rat SCF genes are composed of at least eight exons, with the intron locations conserved between the species. Exon 1 encodes approximately 200 bp of 5' untranslated sequence and the first five amino acids of the predicted signal peptide. Exons 2 - 7 encode the extracellular domain, with exon 7 also encoding the transmembrane region. Exon 8 encodes the intracellular region and part or all of the long (approximately 4 kb) 3' untranslated region (Martin *et al.*, 1990). There is very little known regarding the control of SCF gene transcription; the transcription initiation site and promotor regions have yet to be characterised. A potential role for the transcription factor c-Myb as a positive regulator of SCF gene transcription has been identified. Incubation of stromal fibroblasts with c-Myb antisense oligonucleotides downregulates c-Myb expression and both SCF and GM-CSF mRNA expression (Szczylik *et al.*, 1993).

At the cellular level a number of factors have been shown to modulate the expression of SCF. Exposure of bone marrow stromal cells to TNF-α, IL-1α, and TGF-β1 *in vitro* leads to down regulation SCF mRNA levels (Andrews *et al.*, 1991; Heinrich *et al.*, 1995). Further, in the case of TGF-β1 this is mediated at the level of gene transcription (rather than decreased mRNA stability) and results in decreased SCF protein expression (Heinrich *et al.*, 1995).

3.1.3 CLONING OF NOVEL CYTOKINES

The earliest of human cytokines to be described and studied in detail were the interferons; in particular interferon-α. Studies were initially limited by the availability of sufficient quantities of pure preparations of interferon-α, which had to be purified from the supernatants of human leukocyte cultures. Interferon-α has subsequently been produced in large quantities from Sendi virus infected mammalian cell cultures. However, for most cytokines such large scale production was not possible, until the advent of recombinant DNA technology. The ability to clone and manipulate genes of

interest has enabled the production of many cytokines in sufficient quantities to enable characterisation of their biologically activities and subsequent advancement to the clinic.

The isolation of novel genetic sequences using recombinant DNA technology has traditionally relied upon the generation of a genomic or cDNA library followed by screening using one of a number of potential techniques. The isolation of a given genetic sequence is particularly difficult when the gene exists only rarely, as a single copy gene in a complex genome (a 3 kb fragment will comprise only 1 part in 10^6 of a preparation of haploid mammalian DNA) or as a rare mRNA species in an mRNA population (Seidman, 1994). Where an mRNA of interest is strongly inducible it may represent a much higher proportion of the total mRNA population and isolation may be possible using techniques such as subtractive hybridisation. Many of the chemokines have been identified in this way; for example RANTES (**r**egulated upon **a**ctivation, **n**ormal **T** cell **e**xpressed and **s**ecreted) was initially discovered by subtractive hybridisation as a T-lymphocyte specific sequence (Schall *et al.*, 1988). If one is interested in the regulatory sequences and non-coding (intron) sequences then a genomic library must be utilised to isolate the gene of interest whereas if the predominant concern is in the transcribed protein, its amino acid sequence and potential biological function then a cDNA library will generally be utilised. Whilst rare mRNAs can be obtained by screening cDNA libraries, ideally the cDNA library should utilise mRNA that contains the mRNA sequence of interest at a high level in order simplify the screening procedure and to maximise the chance of obtaining a full length clone.

The isolation of a specific gene requires an effective screening procedure that should ideally be rapid and simple. The first cytokine genes were isolated using a system of hybrid release selection. A population of recombinant cDNA clones is screened by hybridisation of mRNA to the recombinant plasmids bound to a solid support (e.g. nitrocellulose powder or disks). The hybridised mRNA is then eluted and translated *in vivo* by injection into *Xenopus laevis* oocytes and the biological activity of the translated protein determined. Human β interferon (Derynck *et al.*, 1980) and interleukin-2 (Taniguchi *et al.*, 1983) are amongst the cytokines to have been isolated

using this technique. This method is however technically demanding and requires an abundant source of highly active mRNA.

An alternative procedure was used in the cloning of murine IL-4 (Noma *et al.*, 1986) and IL-5 (Kinashi *et al.*, 1986); mRNA was synthesised *in vitro* from a linearised pSP6K cDNA library using SP6 DNA polymerase, translated by injection into *Xenopus laevis* oocytes and screened for specific biological activity. This modification reduced the need for a high quantity of biologically active mRNA but still relied upon translation in *Xenopus laevis* oocytes. Okayama and Berg (1982) introduced the pCD mammalian expression vector which permitted the direct expression of cDNAs in a mammalian cell line; this system was first employed to isolate the murine IL-3 cDNA by transient expression in COS-7 monkey cells (Yokota *et al.*, 1984). The technique of direct expression in mammalian cells has also been used to isolate human GM-CSF (Wong *et al.*, 1985) and IL-3 (Yang *et al.*, 1986). This technique was particularly important in the isolation of hIL-3, as attempts to isolate the gene by screening human cDNA libraries with a murine IL-3 probe had been unsuccessful due to the low interspecies homology (Cohen *et al.*, 1986).

Advancements in protein sequencing techniques have permitted the determination of *N*-terminal amino acid sequence from increasingly smaller quantities of protein. The availability of such amino acid sequence data permits the design of degenerate oligonucleotide probes which may then be used to screen a cDNA library; this technique was used to isolate human G-CSF (Nagata *et al.*, 1986). With the advent of the polymerase chain reaction (Mullis *et al.*, 1986), peptide sequence data could be used to design degenerate primers for PCR amplification of specific oligonucleotides with complete homology to the desired gene; these oligonucleotides may then be used as hybridisation probes to screen cDNA libraries. Murine (Anderson *et al.*, 1990), rat and human (Martin *et al.*, 1990) stem cell factor cDNAs were isolated in this way.

If a cDNA of interest has already been cloned and sequenced in a limited number of species, it is possible to use a probe derived from known sequence data to screen a cDNA library of another species under reduced stringency conditions. This technique has been widely used, for example in the isolation of chicken SCF (Zhou *et al.*, 1993).

Use of the polymerase chain reaction may enable the direct amplification of cDNA clones from mRNA using the reverse transcriptase - polymerase chain reaction (RT-PCR); this technique avoids the often difficult and laborious process of cDNA library generation and screening and significantly reduces the quantity of mRNA that is required to successfully clone a gene of interest. This approach was used to isolate porcine SCF (Zhang and Anthony, 1994) and has recently been used in this department to isolate a number of cytokine cDNAs (Curran *et al.*, 1994; Argyle *et al.*, 1995; Dunham *et al.*, 1995).

Other methods which have been used to isolate cytokine genes include chromosome walking, which was used to isolate the ovine IL-3 gene. IL-3 was known to be closely linked to GM-CSF in mouse and man, separated by approximately 10 kb on the human chromosome 5 (Frolova *et al.*, 1991). Ovine IL-3 was isolated from a 40 kb cosmid clone following isolation of the ovine GM-CSF gene by 'walking' along the DNA to find the IL-3 gene, which was identified by homology to human IL-3 (McInnes *et al.*, 1993). The development of techniques such as representational difference analysis, which uses a PCR based approach to analyse the difference between two genomes (Lisitsyn *et al.*, 1993) or populations of cDNA (Hubank and Schatz, 1994) may lend itself to the isolation of novel cytokine genes in the future.

3.1.4 THE CLONING OF FELINE STEM CELL FACTOR

It was decided to attempt isolation of feline SCF by RT-PCR for the following reasons:

i. As one of the ultimate aims of this project was to express a recombinant protein for feline SCF it was necessary to obtain only the protein coding sequence (i.e. DNA sequence data for introns or non-coding 5' and 3' flanking mRNA was not required).
ii. SCF shows high sequence homology between species, therefore the use of PCR primers, designed from conserved regions flanking the protein coding sequence, was considered to have a good chance of success.

iii. Experience with RT-PCR had already been gained during a previous project involving the isolation, cloning and sequencing of canine interleukin-2 (Dunham *et al.*, 1995).

iv. It offered a simpler potentially faster approach than techniques involving the generation and screening of cDNA libraries.

An important consideration when attempting the isolation of any gene, as mentioned above, is the choice of a suitable cell line or tissue. A feline fibroblast cell line (FEA cells) infected with FeLV strain A was used as a substrate for the isolation of feline SCF by RT-PCR. This choice was based on the knowledge that fibroblasts are a potential source of SCF (Fujita *et al.*, 1989) and also that this cell line is known to promote the growth of erythroid colonies (BFU-E) in culture (Abkowitz *et al.*, 1986), implying the production of haemopoietic growth factor(s). In addition, the use of a cell line, in preference to freshly isolated tissue, provides a virtually limitless source of mRNA without the requirement for experimental animals.

This chapter describes the cloning and sequencing of both the long and shorter isoforms of feline stem cell factor using an RT-PCR based technique. An overview of the experimental procedure is shown in Figure 3.2, overleaf.

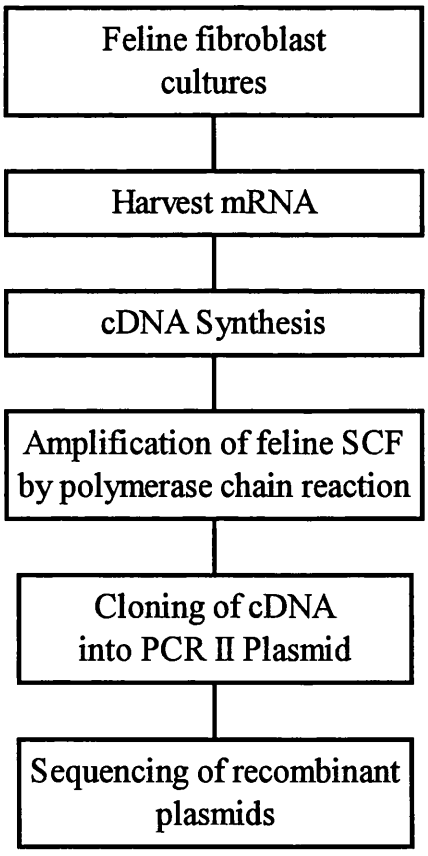


Figure 3.2: Overview of experimental procedure used to clone cDNAs for feline stem cell factor.

3.2 MATERIALS AND METHODS

3.2.1 ISOLATION OF mRNA FROM FEA CELLS

Messenger RNA was isolated from the feline cell line FEA using a Quickprep mRNA isolation kit as described in section 2.2.3.1. Cells were harvested from a 162 cm^2 tissue culture flask by applying 1.5 ml extraction buffer directly to the cell monolayer following removal of culture medium. Upon completion of mRNA extraction the yield and purity of the RNA was measured by spectrophotometry. The harvested mRNA was then used as a template for cDNA synthesis.

3.2.2 SYNTHESIS OF cDNA

cDNA was synthesised from FEA mRNA using a First-strand cDNA synthesis kit as described in section 2.2.3.2. The completed first strand reaction was heated to 90°C for five minutes then chilled on ice immediately prior to use as a PCR template; this was performed to denature the RNA-cDNA duplex.

3.2.3 PCR AMPLIFICATION OF FELINE STEM CELL FACTOR

3.2.3.1 Design of Stem Cell Factor PCR primers

In order to amplify feline SCF it was necessary to design oligonucleotide primers which flanked the protein coding sequence. The sequences of porcine, canine, human and murine cDNAs were aligned using the 'Lineup' and 'Pileup' programs (UWGCG software) as shown in Figure 3.3. Primers were chosen from conserved areas at the 5' and 3' ends of the protein coding sequence:

5' primer:	5'-CCA-GAA-CAG-CTA-AAC-GGA-GT-3'	T_m 61.3°C
3' primer:	5'-ATG-AAG-CAA-ACA-TGA-ACT-GT-3'	T_m 56.8°C

These were expected to amplify a stem cell factor cDNA of approximately 950 nucleotides in size, containing the complete protein coding sequence.

Figure 3.3: Sequence comparison of human, murine, porcine and canine stem cell factor sequences.

```
                1                                                   50
 canine scf     .......... .......... .......... .......... ..........
porcine scf     .......... .......... .......... .......... ..........
  human scf     CCGCCTCGCG CCGAGACTAG AAGCGCTGCG GGAAGCAGGG ACAGTGGAGA
 murine scf     .......... .......... .......... ....GCAACG GCCAAGGACG

                51                                                  100
 canine scf     .......... .......... .......... .......... ..........
porcine scf     .......... .......... .......... .......... ..........
  human scf     GGGCGCTGCG CTCGGGCTAC CCAATGCGTG GACTATCTGC CGCCGCTGTT
 murine scf     GGGCGCTGCG TTCGAGCTAC CCAATGCTGG GACTATCTGC AGCCGCTGCT

                101                                                 150
 canine scf     .......... .......... .......... .......... ..........
porcine scf     .......... ....GAGCTC CAGAACAGCT AAACGGAGTT GCCACACCGC
  human scf     CGTGCAATAT GCTGGAGCTC CAGAACAGCT AAACGGAGTC GCCACACCAC
 murine scf     GGTGCAATAT GCTGGAGCTC CAGAACAGCT AAACGGAGTC GCCACACCGC

                151                                                 200
 canine scf     .......... .......... .......... ...ATGAAGA AGACACAAAC
porcine scf     TGCCTGGGCT GGATCACAGC GCTGCCTTTC CTTATGAAGA AGACACAAAC
  human scf     TGTTTGTGCT GGATCGCAGC GCTGCCTTTC CTTATGAAGA AGACACAAAC
 murine scf     TGCCTGGGCT GGATCGCAGC GCTGCCTTTC CTTATGAAGA AGACACAAAC

                201                                                 250
 canine scf     TTGGATTATC ACTTGCATTT ATCTTCAGCT GCTCCTATTT AATCCTCTGG
porcine scf     TTGGATTATC ACTTGCATTT ATCTTCAACT GCTCCTATTT AATCCTCTCG
  human scf     TTGGATTCTC ACTTGCATTT ATCTTCAGCT GCTCCTATTT AATCCTCTCG
 murine scf     TTGGATTATC ACTTGCATTT ATCTTCAACT GCTCCTATTT AATCCTCTTG

                251                                                 300
 canine scf     TCAAAACTAA AGGGATCTGC GGGAAACGTG TGACTGATGA TGTGAAGGAC
porcine scf     TCAGAACTCA AGGGATCTGC AGGAACCGTG TGACTGATGA TGTGAAAGAC
  human scf     TCAAAACTGA AGGGATCTGC AGGAATCGTG TGACTAATAA TGTAAAAGAC
 murine scf     TCAAAACCAA GGAGATCTGC GGGAATCCTG TGACTGATAA TGTAAAAGAC

                301                                                 350
 canine scf     GTTACAAAAT TGGTGGCAAA TCTTCCAAAA GACTATAAGA TAGCCCTCAA
porcine scf     GTTACAAAAT TGGTGGCAAA TCTTCCAAAA GACTATAAGA TAACCCTCAA
  human scf     GTCACTAAAT TGGTGGCAAA TCTTCCAAAA GACTACATGA TAACCCTCAA
 murine scf     ATTACAAAAC TGGTGGCAAA TCTTCCAAAT GACTATATGA TAACCCTCAA

                351                                                 400
 canine scf     ATATGTCCCC GGGATGGATG TTTTGCCTAG TCATTGTTGG ATAAGCGTGA
porcine scf     ATATGTCCCC GGGATGGACG TTTTGCCTAG TCATTGTTGG ATAAGCGAAA
  human scf     ATATGTCCCC GGGATGGATG TTTTGCCAAG TCATTGTTGG ATAAGCGAGA
 murine scf     CTATGTCGCC GGGATGGATG TTTTGCCTAG TCATTGTTGG CTACGAGATA

                401                                                 450
 canine scf     TGGTGGAACA GTTGTCAGTC AGCTTGACTG ATCTTCTGGA CAAGTTTTCA
porcine scf     TGGTGGAACA ACTGTCAGTC AGCTTGACTG ATCTTCTGGA CAAGTTTTCC
  human scf     TGGTAGTACA ATTGTCAGAC AGCTTGACTG ATCTTCTGGA CAAGTTTTCA
 murine scf     TGGTAATACA ATTATCACTC AGCTTGACTA CTCTTCTGGA CAAGTTCTCA
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Figure 3.3 (continued): Sequence comparison of human, murine, porcine and canine stem cell factor sequences

```
                451                                                 500
 canine scf     AATATTTCTG AAGGCCTGAG TAATTATTCT ATCATAGACA AACTTGTGAA
porcine scf     AATATTTCTG AAGGCTTGAG TAATTATTCT ATCATAGACA AACTTGTGAA
  human scf     AATATTTCTG AAGGCTTGAG TAATTATTCC ATCATAGACA AACTTGTGAA
 murine scf     AATATTTCTG AAGGCTTGAG TAATTACTCC ATCATAGACA AACTTGGGAA

                501                                                 550
 canine scf     AATAGTGGAT GATCTTGTGG AGTGCACAGA AGGATACTCA TTTGAGAATG
porcine scf     AATTGTTGAT GACCTCGTGG AATGCATGGA AGAACACTCA TTTGAGAATG
  human scf     TATAGTCGAT GACCTTGTGG AGTGCGTCAA AGAAAACTCA TCTAAGGATC
 murine scf     AATAGTGGAT GACCTCGTGT TATGCATGGA AGAAAACGCA CCGAAGAATA

                551                                                 600
 canine scf     TAAAAAAAGC ACCTAAGAGC CCAGAACTCA GGCTTTTTAC TCCTGAAGAA
porcine scf     TAAAAAAATC ATCTAAGAGC CCAGAACCCA GGCTGTTTAC TCCTGAAAAA
  human scf     TAAAAAAATC ATTCAAGAGC CCAGAACCCA GGCTCTTTAC TCCTGAAGAA
 murine scf     TAAAAGAATC TCCGAAGAGG CCAGAAACTA GATCCTTTAC TCCTGAAGAA

                601                                                 650
 canine scf     TTCTTTAGAA TTTTTAATAG ATCCATCGAT GCCTTTAAGG ACTTGGAGAC
porcine scf     TTCTTTGGGA TTTTTAATAG ATCCATCGAT GCCTTCAAGG ATTTGGAGAT
  human scf     TTCTTTAGAA TTTTTAATAG ATCCATTGAT GCCTTCAAGG ACTTTG...T
 murine scf     TTCTTTAGTA TTTTCAATAG ATCCATTGAT GCCTTTAAGG ACTTT...AT

                651                                                 700
 canine scf     GGTGGCATCT AAAAGTAGTG AATGTGTGGT TTCTTCAACC TTAAGTCCTG
porcine scf     GGTGGCACCT AAAACTAGTG AATGTGTGAT TTCTTCAACA TTAACTCCTG
  human scf     AGTGGCATCT GAAACTAGTG ATTGTGTGGT TTCTTCAACA TTAAGTCCTG
 murine scf     GGTGGCATCT GACACTAGTG ACTGTGTGCT CTCTTCAACA TTAGGTCCCG

                701                                                 750
 canine scf     ATAAAGATTC CAGAGTCAGT GTCACAAAAC CATTTATGTT ACCCCCTGTT
porcine scf     AAAAAGATTC CAGAGTCAGT GTCACAAAAC CATTTATGTT ACCCCCTGTT
  human scf     AGAAAGATTC CAGAGTCAGT GTCACAAAAC CATTTATGTT ACCCCCTGTT
 murine scf     AGAAAGATTC CAGAGTCAGT GTCACAAAAC CATTTATGTT ACCCCCTGTT

                751                                                 800
 canine scf     GCAGCCAGCT CCCTTAGGAA TGACAGCAGT AGCAGTAATA GGAAGGCCTC
porcine scf     GCAGCCAGCT CCCTTAGGAA TGACAGCAGT AGCAGTAATA GGAAGGCCTC
  human scf     GCAGCCAGCT CCCTTAGGAA TGACAGCAGT AGCAGTAATA GGAAGGCCAA
 murine scf     GCAGCCAGCT CCCTTAGGAA TGACAGCAGT AGCAGTAATA GGAAAGCCGC

                801                                                 850
 canine scf     AAATTCCATT GGAGACTCCA ACTTACAATG GGCAGCCATG GCATTGCCAG
porcine scf     AGATTCCATT GAAGACTCCA GCCTCCAGTG GGCAGCCGTA GCATTGCCAG
  human scf     AAATCCCCCT GGAGACTCCA GCCTACACTG GGCAGCCATG GCATTGCCAG
 murine scf     AAAGGCCCCT GAAGACTCGG GCCTACAATG GACAGCCATG GCATTGCCGG

                851                                                 900
 canine scf     CATTCTTTTC TCTTGTAATT GGGTTTGCTT TTGGAGCCTT ATACTGGAAG
porcine scf     CATTCTTCTC TCTTGTCATT GGGTTTGCTT TTGGAGCCTT ATACTGGAAG
  human scf     CATTGTTTTC TCTTATAATT GGCTTTGCTT TTGGAGCCTT ATACTGGAAG
 murine scf     CTCTCATTTC GCTTGTAATT GGCTTTGCTT TTGGAGCCTT ATACTGGAAG
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                901                                                  950
 canine scf     AAGAAACAAC CAAATCTCAC AAGGACAGTT GAAAATATAC AGATTAATGA
porcine scf     AAGAAACAAC CAAACCTTAC AAGGACAGTG GAAAATATAC AGATTAATGA
  human scf     AAGAGACAGC CAAGTCTTAC AAGGGCAGTT GAAAATATAC AAATTAATGA
 murine scf     AAGAAACAGT CAAGTCTTAC AAGGGCAGTT GAAAATATAC AGATTAATGA

                951                                                 1000
 canine scf     AGAGGATAAT GAAATAAGTA TGTTGCAAGA GAAAGAGAGG GAGTTTCAAG
porcine scf     AGAGGATAAT GAGATAAGTA TGTTGCAAGA AAAAGAGAGA GAGTTTCAAG
  human scf     AGAGGATAAT GAGATAAGTA TGTTGCAAGA GAAAGAGAGA GAGTTTCAAG
 murine scf     AGAGGATAAT GAGATAAGTA TGTTGCAACA GAAAGAGAGA GAATTTCAAG

                1001                                                1050
 canine scf     AGGTGT.AA. .......... .......... .......... ..........
porcine scf     AAGTGT.AAT TGTGGCGTGT ATCAACACTG TTGCTTTCGT ACATTGGGTG
  human scf     AAGTGTAAAT TGTGGCTTGT ATCAACACTG TTACTTTCGT ACATTGGCTG
 murine scf     AGGTGTAATT GTGGAC..GT ATCAACATTG TTACCTTCGC ACAGTGGCTG

                1051                                                1100
 canine scf     .......... .......... .......... .......... ..........
porcine scf     GTAACAGTTC ATGTTTG... .......... .......... ..........
  human scf     GTAACAGTTC ATGTTTGCTT CATAAATGAA GCAGCTTTAA ACAAATTCAT
 murine scf     GTAACAGTTC ATGTTTGCTT CATAAATGAA GCAGCCTTAA ACAAATTCCC

                1101                                                1150
 canine scf     .......... .......... .......... .......... ..........
porcine scf     .......... .......... .......... .......... ..........
  human scf     ATTCTGTCTG GAGTGACAGA CCACATCTTT ATCTGTTCTT GCTACCCATG
 murine scf     ATTCTGTCTC AAGTGACAGA CCTCATCCTT ACCTGTTCTT GCTACCCGTG

                1151                                                1200
 canine scf     .......... .......... .......... .......... ..........
porcine scf     .......... .......... .......... .......... ..........
  human scf     ACTTTATATG GATGATTCAG AAATTGGAAC AGAATGTTTT ACTGTGAAAC
 murine scf     ACCTTGTGTG GATGATTCAG TTGTTGGAGC AGAGTGCTTC GCTGTGAACC
```

Figure 3.3 (continued): Sequence comparison of human, murine, porcine and canine stem cell factor sequences. Sequences were obtained from the EMBL database with the accession numbers M59964, M57647, L07786 and S53329 respectively. Sequence alignments were made using LineUp and PileUp programs (GCG software). The conserved regions used to design PCR primers for the amplification of feline stem cell factor are shown in bold type. The start codons (ATG) and stop codons (TAA) are underlined.

3.2.3.2 Positive controls

In order to assess the efficiency of the PCR process a number of positive controls were set up each time PCR amplification of feline stem cell factor was attempted.

β-actin primers:

β-actin is a gene that is constitutively expressed at high levels in many cell and tissue types, making it a suitable choice for a positive control (Nakajima-Iijima., *et al.*, 1985). The gene is highly conserved between species, making it possible to amplify feline β-actin using primers designed for the amplification of human β-actin (Clontech Laboratories Inc., Palo Alto, CA). The following primers were provided at a concentration of 20 μM.

5' primer: 5'-ATC-TGG-CAC-CAC-ACC-TTC-TAC-AAT-GAG-CTG-CG-3'

3' primer: 5'-CGT-CAT-ACT-CCT-GCT-TGC-TGA-TCC-ACA-TCT-GC-3'

These primers amplify a PCR fragment of 838 bp in size when used to amplify human cDNA. In addition a positive cDNA template (human β-actin) was also supplied with these primers in order to verify that they were amplifying efficiently under a given set of reaction conditions.

Lambda DNA primers:

A further positive control is included with the GeneAmp PCR reagent kit; whole bacteriophage lambda is used as a template for PCR amplification using the primer pair:

Primer 1: 5'-GAT-GAG-TTC-GTG-TCC-GTA-CAA-CTG-G-3'

Primer 2: 5'-GGT-TAT-CGA-AAT-CAG-CCA-CAG-CGC-C-3'

These primers amplify a 500 bp fragment (nucleotides 7131 to 7630) of the lambda target DNA.

These "internal controls" ensured that should any PCR failures occur, then the cause of failure could be localised further:

i. Should both positive controls fail to amplify efficiently this would suggest a problem involving the bulk reaction mix (e.g. *Taq* polymerase, dNTPs or PCR buffer) or the PCR cycler.

ii. Failure of the β-actin positive control alone (with FEA cDNA template) would suggest either that the starting mRNA was of poor quality or had been inefficiently reverse transcribed to cDNA (e.g. due to inefficient denaturation of the mRNA template or failure of the reverse transcriptase enzyme).

iii. If both positive controls worked efficiently but there was no or poor amplification of cDNA using the fSCF primers this would suggest that the reaction conditions were inappropriate (e.g. annealing temperature too high/low or PCR primer concentration too low), that fSCF was expressed at low levels in the starting mRNA or that the PCR primers did not anneal efficiently to feline SCF due to mismatches.

3.2.3.3 Negative controls

Each time a series of PCR reactions was set up a number of negative controls was also included:

Reverse transcriptase negative control:

In order to determine that the product generated by the SCF primers was derived from cDNA and not contaminating genomic DNA a reverse transcriptase negative control was included. The template for this control was provided by following the protocol for cDNA synthesis without the addition of the bulk first strand reaction mix (containing M-MuLV reverse transcriptase); in effect therefore the mRNA was simply incubated with DTT and Not I-d$(T)_{18}$ primer in a final volume of 33 µl.

Reagent control:

A reaction mix was set up containing all PCR components (SCF primers, dNTPs, PCR buffer and *Taq* polymerase) except template. This control was included to check that there was no contamination of the PCR reactions with extraneous DNA that might serve as a template for PCR amplification.

3.2.3.4 Reaction conditions

All reactions were performed in 50 µl volume in 0.5 ml tubes. A master reaction mix was prepared by combining 2.5 units *Taq* polymerase, five microlitres 10 x PCR buffer, five microlitres of dNTP mix (1.25 mM each dNTP) and 4.5 µl dH_2O for each reaction; 15 µl was then pipetted into each reaction tube containing the primers and appropriate DNA template and the volume was made up to 50 µl by the addition of

dH_2O. The use of such a master mix minimises losses and inaccuracies associated with pipetting and ensures consistency from tube to tube. Table 3.2 shows the reactions that were typically set up each time a PCR amplification was performed. The complete reaction mix was overlain with mineral oil and the tubes were transferred to the thermal cycler.

Primer Pair	*Primer Concentration*	*DNA template*
fSCF	0.5 - 2.0 μM	10 μl FEA cDNA
fSCF	0.5 - 2.0 μM	10 μl RT negative "cDNA"
β-actin	0.4 μM	10 μl FEA cDNA
β-actin	0.4 μM	100 attomoles control cDNA template
Lambda	1.0 μM	1 ng lambda control template
Reagent control	None	None

Table 3.2: Constituents of each PCR reaction. To each template/primer mix was added 15 μl of PCR master mix containing *Taq* polymerase, dNTPs and PCR buffer; dH_2O was then added to a final volume of 50 μl.

The optimum annealing temperature for the SCF primer pair, as predicted by the Oligo primer analysis software (Medprobe AS) was 51.3°C. The thermal cycler was initially programmed therefore, to give a PCR cycle consisting of denaturation at 94°C for one minute, primer annealing at 51.3°C for one minute and extension at 72°C for one minute, repeated for a total of 30 cycles; followed by a 4°C 'soak'. PCR reaction products were visualised by polyacrylamide gel electrophoresis as detailed in 2.2.5.2, using five microlitres of reaction product per well.

A series of optimisation experiments was carried out investigating the effect of primer concentration and annealing temperature on the amplification of feline SCF. It was found that a greater yield of a single DNA band of the appropriate size was found using an annealing temperature of 48°C. A primer concentration of 1.0 μM was associated with consistent amplification of a specific product of the expected size (results not shown).

3.2.4 CLONING OF FELINE SCF PCR PRODUCTS

Products of PCR reactions using the SCF primer pair were cloned into the pCR™ II vector. One microlitre of PCR product was added to 50 ng (two microlitres) vector, one microlitre 10 x ligation buffer, five microlitres dH_2O and four units (one microlitre) T4 DNA ligase. The ligation reaction was incubated overnight at 14°C. The ligated vector was then cloned into INVαF' *E.coli* cells as detailed in section 2.2.2.8.1. Colonies were selected on LB agar plates containing 50 μg/ml ampicillin; white colonies were picked and small scale DNA preparations made. Miniprep DNA was subjected to restriction endonuclease digestion using ECoRI and products of digestion were run on a 5% polyacrylamide gel. No inserts of the desired size were seen following this procedure. It was postulated that the amplified cDNA sequence of feline stem cell factor contained an ECoRI restriction enzyme site and was thus being digested by the enzyme. The restriction digest was therefore repeated using the enzymes ECoRV and BamHI. The digest was initially set up in 15 μl volume using ReactII buffer and ECoRV. After digestion for one hour at 37°C the buffer was changed by the addition of two microlitres of ReactIII, and the DNA was digested with BamHI in 20 μl volume. The products of digestion were then run on a 5% polyacrylamide gel; following this alternative procedure inserts of the desired size could be seen. Bacterial stocks were made from the isolates that contained inserts of the appropriate size.

3.2.5 SEQUENCING OF FELINE SCF

DNA for sequencing was prepared by the small scale procedure as detailed in 2.2.2.2.2. The DNA was sequenced using a Sequenase version 2.0 sequencing kit as outlined in section 2.2.5.1.1 Initial sequencing reactions used M13 universal primers with the following sequence:

M13(-40) Forward Primer	5'-GTT-TTC-CCA-GTC-ACG-AC-3'
M13 Reverse Primer	5'-TTC-ACA-CAG-GAA-ACA-G-3'

Sequencing reactions were run on six percent denaturing polyacrylamide gels as described in section 2.2.5.1.2. After the acquisition of initial sequence data it was possible to design internal primers in order to accurately sequence both strands of the

insert DNA. Primer design was aided by use of Oligo primer analysis software (Version 4.1 - Medprobe A.S.); in particular primers were chosen to be stable at their 5' ends but less stable at their 3' end to reduce false priming. Primers were 18 - 20 nucleotides in length and were chosen to anneal 30 - 60 bp upstream (5') to the sequence of interest so that a sufficient overlap of sequence generated by adjacent sequencing primers was seen. The following primers were used (the annealing site is given in parentheses after the primer sequence):

Sense Primers:

SCFU#2: 5'-GAT-AAG-CGT-GAT-GGT-GGA-AC-3' (nt 271 - 290)

SCFU#3: 5'-CTA-GTG-AAT-GTG-TGG-TTT-C-3' (nt 546 - 564)

Antisense Primers:

SCFD#2: 5'-GCC-TTC-CTA-TTA-CTG-CTA-3' (nt 678 - 661)

SCFD#3 5'-AGT-TTG-TCT-ATG-ATA-GAA-TA-3' (nt 375 - 356)

In addition it was necessary to design a further antisense primer as it was found that the primer SCFD#2 did not anneal to the DNA template in one of the clones because of the deletion of a number of nucleotides in that clone (see results for further details).

SCFD#4: 5'-TAT-GCT-GGA-GTC-TTC-TAT-3' (nt 706 - 689)

Sequencing reactions and gel electrophoresis using these primers were carried out as described for the universal primers.

3.2.6 VERIFICATION OF INITIAL SEQUENCE

Initial analysis of the sequence data revealed a consensus sequence with ambiguity at one nucleotide only (nt 261); in order to confirm the likely identity of this base, RT-PCR was repeated and two further clones sequenced from each of two separate RT-PCR reactions. To minimise the amount of sequencing work required and to simplify cloning of PCR products it was decided to amplify a short (230 bp) cDNA fragment spanning the region of interest. The primers used were:

Sense primer: 5'-TGG-CAA-ATC-TTC-CAA-AAG-ACT-ATA-AGA-3' (nt 195-221).

Antisense primer: 5'-AGA-TGA-GTG-TCC-TTC-CAC-GCA-CTC-3' (nt 424-401).

The procedure and conditions used were identical to those described above except for the following modification: the PCR thermal cycler was programmed to give an initial

denaturation step of 94°C for five minutes, followed by 30 cycles of 94°C for one minute, 50°C for one minute, 72°C for one minute, and a final extension step of 72°C for 10 minutes. Plasmid DNA was sequenced using a Sequitherm long-read cycle-sequencing kit as described in section 2.2.5.2.1. Due to the short length of the DNA insert it was possible to sequence both strands completely using M13 universal IRD41 labelled primers:

M13 forward (-29) Primer: 5'-CAC-GAC-GTT-GTA-AAA-CGA-C-3'

M13 reverse Primer: 5'-GGA-TAA-CAA-TTT-CAC-ACA-GG-3'

The reaction products were run on a Licor Model 4000 automated sequencer as described in section 2.2.5.2.2. The sequence data was collected and read automatically; nucleotide 261 was checked by inspection of the gel image.

3.2.7 SEQUENCE DATA ANALYSIS

Sequence data was stored and managed on a UNIX computer system using GCG software (University of Wisconsin). 'Raw' sequence data was handled and edited using the 'SeqEd' program. The sequences of stem cell factor for other species were downloaded from the EMBL database using the 'Fetch' command. Sequence (nucleotide and amino acid) comparisons were performed using 'BestFit' which aligns sequences using the algorithm of Smith and Waterman (1981). The protein sequence of feline stem cell factor was predicted using 'Translate'. Predictions of protein secondary structure were made using 'PepPlot', 'PeptideStructure' and 'PlotStructure'. The signal sequence was predicted using 'SigSeq' (Rockefeller University) which predicts signal sequence cleavage sites based on the method of von Heijne (1986).

3.3 RESULTS

3.3.1 RT-PCR AMPLIFICATION FELINE SCF CDNA

The synthesis of mRNA gave yields of seven to nine micrograms per 162cm^2 flask; purity was generally very good (OD_{260}/OD_{280} = 2.0). Figure 3.4 shows a polyacrylamide gel with the PCR products from a typical series of reactions (i.e. amplification of feline stem cell factor cDNA and appropriate controls). The product

Addendum

3.3.2 NUCLEOTIDE SEQUENCE OF FSCF ISOFORMS

The full length fSCF cDNA shows 93% homology to the ovine SCF partial cDNA sequence.

amplified using fSCF primers is approximately 950 bp in size, in agreement with the the predicted size. The lambda and β-actin primers both generate strong products of the expected sizes whilst no products are seen with the negative controls.

3.3.2 NUCLEOTIDE SEQUENCE OF FSCF ISOFORMS

Manual sequencing was carried out on three separate clones, taken from two different PCR reactions; these were identical except for base changes at nucleotides 16 (A to G) and 261 (A to G) in clone 3. A further clone was sequenced and found to be identical to clone three except for a deletion of 84 nucleotides (nt. 588 - 671 inclusive). Due to the concern that a change of nucleotide 261 from A to G would lead to a change in the deduced amino-acid sequence of asparagine to serine, four further clones were sequenced, as described above; all four clones showed the nucleotide G at position 261. Since the change seen at nucleotide 16 was within the 5' PCR primer and non-coding, it was felt unnecessary to investigate this ambiguity any further; the PCR primer sequence is given in the consensus fSCF sequence. The different sequence obtained for each of the two isoforms can be seen by inspection of the autoradiographs shown in Figure 3.5A and 3.5B.

The consensus feline stem cell factor sequence and deduced amino acid sequence is shown in Figure 3.6; this also shows the deletion of 84 nucleotides seen in one clone. Comparison of the full-length fSCF cDNA to published sequences in other species (as cited above) shows homology of 95% to canine, 93% to bovine, 93% to porcine, 92% to human, 87% to murine and 71% to chicken SCFs.

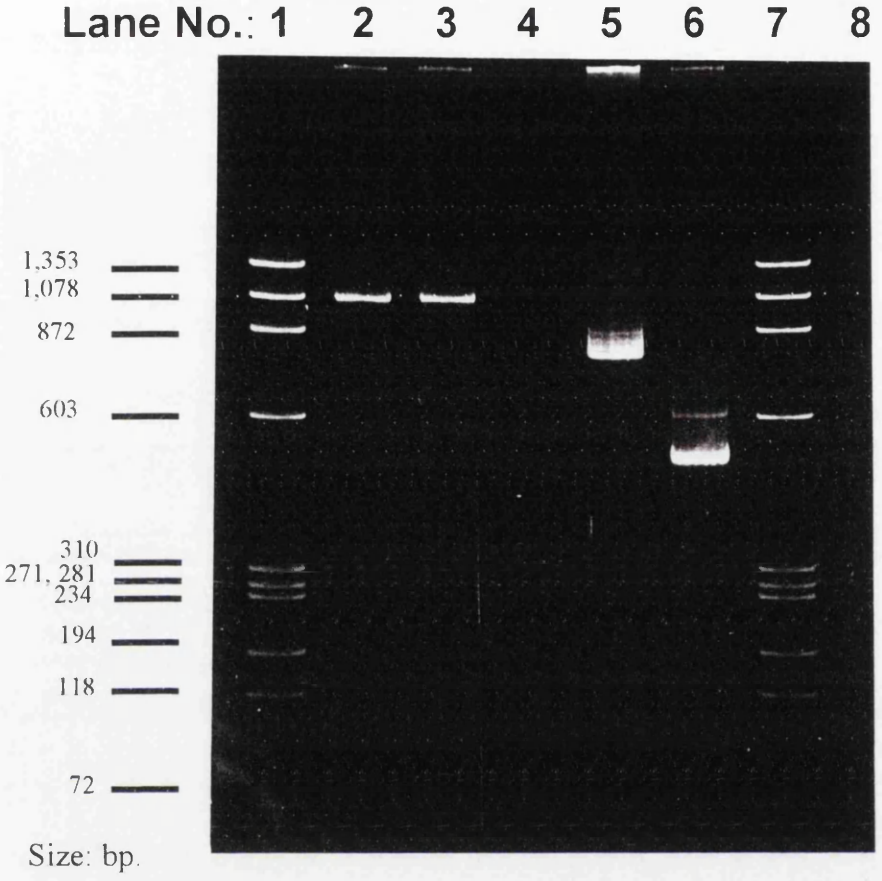


Figure 3.4: 5% polyacrylamide gel showing the reaction products of a typical PCR reaction used to amplify feline stem cell factor cDNA and the control reactions carried out concurrently. Lanes 1 and 7: molecular size markers (ϕX174 RF DNA/Hae III fragments); Lane2: fSCF primers (1.0 μM); Lane 3: fSCF primers (2.0 μM); Lane 4: fSCF primers (1.0 μM) - RT negative; Lane 5: β-actin primers - FEA cDNA template; Lane 6: lambda primers - lambda DNA template; Lane 8: Reagent control (no added template or primers).

3.3.3 PREDICTED POLYPEPTIDE SEQUENCE

3.3.3.1 Homology and predicted features of feline SCF protein

The predicted amino acid sequence of feline SCF is shown in alignment with those of other species in Figure 3.7. The derived protein shows identity of 92% to canine and porcine, 91% to bovine, 88% to human, 80% to murine and 53% to chicken homologues. The output from the 'SigSeq' program is shown in Figure 3.8. This clearly shows a predicted signal peptide of 25 amino acids with the predicted mature protein beginning KGLCR etc. Feline SCF has four predicted *N*-glycosylation sites (NXT or NXS), at Asn 65, 72, 120 and 171, which are shown in Figure 3.7. The outputs from the programs 'PepPlot' and 'PlotStructure', showing predictions of secondary protein structure, are shown in Figure 3.9, Figure 3.10 and Figure 3.11. Structural features of interest shown in Figures 3.7 to 3.11 are discussed below.

Figure 3.5: Autoradiograph of fSCF sequencing reactions. Reaction mixes were loaded in the order G, A, T, C, G, A, T, C, etc. from left to right. The longer isoform (52C) and shorter isoform (8C) are shown; the nucleotides deleted in the shorter isoform are delineated by the solid line next to the sequence of the longer isoform and by an arrow on the sequence of the shorter isoform.

```
   1                  CCAGAACAGCTAAACGGAGTCGCCACACCGCTGCCTGGACTGGATCACAGCGCTGCCTTTCCTT  64

  65 ATG AAG AAG ACA CAA ACT TGG ATT GTC ACT TGC ATT TAT CTT CAG CTG CTC CTA TTT AAC 124
   1  M   K   K   T   Q   T   W   I   V   T   C   I   Y   L   Q   L   L   L   F   N  20

 125 CCT CTG GTC AAA ACT AAA GGG CTC TGC AGG AAC CGT GTG ACT GAT GAT GTG AAA GAC GTT 184
  21  P   L   V   K   T   K   G   L   C   R   N   R   V   T   D   D   V   K   D   V  40

 185 ACA AAA TTG GTG GCA AAT CTT CCA AAA GAC TAT AAG ATA GCC CTC AAA TAT GTC CCC GGG 244
  41  T   K   L   V   A   N   L   P   K   D   Y   K   I   A   L   K   Y   V   P   G  60

 245 ATG GAT GTT TTG CCT AGT CAT TGT TGG ATA AGC GTG ATG GTG GAA CAG TTG TCA GTC AGT 304
  61  M   D   V   L   P   S   H   C   W   I   S   V   M   V   E   Q   L   S   V   S  80

 305 TTG ACT GAT CTT CTG GAC AAG TTT TCG AAT ATT TCT GAA GGC TTG AGT AAT TAT TCT ATC 364
  81  L   T   D   L   L   D   K   F   S   N   I   S   E   G   L   S   N   Y   S   I 100

 365 ATA GAC AAA CTT GTG AAA ATA GTG GAT GAC CTT GTG GAG TGC GTG GAA GGA CAC TCA TCT 424
 101  I   D   K   L   V   K   I   V   D   D   L   V   E   C   V   E   G   H   S   S 120

 425 GAG AAT GTA AAA AAA TCA TCT AAG AGC CCA GAA CCC AGG CTT TTT ACT CCT GAA GAA TTC 484
 121  E   N   V   K   K   S   S   K   S   P   E   P   R   L   F   T   P   E   E   F 140

 485 TTT AGA ATT TTT AAT AGA TCC ATT GAT GCC TTC AAG GAC TTG GAG ATG GTG GCA TCT AAA 544
 141  F   R   I   F   N   R   S   I   D   A   F   K   D   L   E   M   V   A   S   K 160

 545 ACT AGT GAA TGT GTG GTT TCT TCA ACA TTA AGT CCT GAA AAA GAT TCC AGG GTC AGT GTC 604
 161  T   S   E   C   V   V   S   S   T   L   S   P   E   K   D   S   R   V   S   V 180

 605 ACA AAA CCA TTT ATG TTA CCC CCT GTT GCA GCC AGC TCC CTT AGG AAT GAC AGC AGT AGC 664
 181  T   K   P   F   M   L   P   P   V   A   A   S   S   L   R   N   D   S   S   S 200

 665 AGT AAT AGG AAG GCC ACA AAT CCC ATA GAA GAC TCC AGC ATA CAA TGG GCA GTC ATG GCA 744
 201  S   N   R   K   A   T   N   P   I   E   D   S   S   I   Q   W   A   V   M   A 220

 725 TTA CCA GCG TGC TTT TCT CTT GTA ATC GGA TTT GCT TTT GGA GCC TTC TAC TGG AAG AAG 804
 221  L   P   A   C   F   S   L   V   I   G   F   A   F   G   A   F   K   W   K   K 240

 785 AAA CAA CCG AAT CTC ACA AGG ACA GTT GAA AAT ATA CAG ATT AAC GAA GAG GAT AAT GAG 864
 241  K   Q   P   N   L   T   R   T   V   E   N   I   Q   I   N   E   E   D   N   E 260

 845 ATA AGT ATG CTG CAA GAA AAA GAG AGA GAG TTT CAA GAG GTG TAA TTGTGGCTTATATCAACAC 908
 261  I   S   M   L   Q   E   K   E   R   E   F   Q   E   V   *                     274

 909 TGTTACTTTTGTGCCTTGGCGGGTAACAGTTCATGTTTGCTTCAT                                 953
```

Figure 3.6: Nucleotide sequence and deduced amino-acid sequence of feline stem cell factor cDNA. The nucleotides depicted in bold type and overlined are those deleted in the shorter isoform of fSCF, which results in a removal of amino-acids 150-178 and the insertion of a glycine residue at this site. Primers used in the PCR are underlined.

```
     _____S-P_____♦ •
-25 MKKTQTWIVT CIYLQLLLFN PLVKTKGLCR NRVTDDVKDV TKLVANLPKD YKIALKYVPG
-25 --------I- ---------- ---R-Q-I-- ---------- ---------- ---T------
-25 --------I- ---------- -------I-G K--------- ---------- ----------
-25 --------I- ---------- ---H-Q-I-S ---------- ---------- -M-T------
-25 --------L- ---------- -----E-I-- ----NN---- ---------- -M-T------
-25 --------I- ---------- ------EI-G -P---N---I --------N- -M-T-N--A-
-25 ---A----I- -FC-----L- ----AQSS-G -P-----N-I A---G---N- -L-T-----K

         •                          *         *                      •
36  MDVLPSHCWI SVMVEQLSVS LTDLLDKFSN I...SEGLSN YSIIDKLVKI VDDLVECVE.
36  ---------- -E-------- ---------- ---------- ---------- -------M--
36  ---------- ---------- ---------- ---------- ---------- -------T--
36  ---------- -E-------- ---------- ---------- -C-------- -------M--
36  ---------- -E--V---D- ---------- ---------- --------N- --------K-
36  ---------L RD--I---L- --T------- ---------- -------G-- -----L-M--
36  --S--N---L HL--PEF-R- -HN--Q---D -SDM-DV--- ----NN-TR- IN--MA-LAF

                                     *                    •       →
92  GHSSENVKKS SKSPEPRLFT PEEFFRIFNR SIDAFKDLEM VASKTSECVV SSTL.SPEKD
92  E--F------ ---------- --K--G---- ---------- --P------I -----T----
92  -Y-F-----A P----L---- ---------- ---------T ----S----- -------D--
92  E--------- -------Q-- --K--G---K ---------I ----M----I ---S------
92  EN--KDL--- F--------- ---------- -------F.V ---E--D--- ----------
92  ENAPK-I-E- P-R--T-S-- -----S---- -------F.- ---D--D--L -----G----
96  DKNKDFI-EN GHLY-EDR-I --N---L--S T-EVY-EFAD SLD-.ND-IM P--VET--N-

                    ↓↓    *          ←                  _____T-M_____
151 SRVSVTKPFM LPPVAASSLR ND......SS SSNRKATNPI EDSSIQWAVM ALPACFSLVI
151 ---------- ---------- ---------- ------SDS- ----L---AV ----F-----
151 ---------- ---------- ---------- ------S-S- G--NL---A- ----F-----
151 ---------- ---------- ---------- ------S-S- ----L---AV ----F-----
150 ---------- ---------- ---------- ------K--P G---LH--A- ----L---I-
150 ---------- ---------- ---------- ------AKAP ---GL--TA- ----LI----
155 ---A---TIS F--------- --SIGSNT-- N--KE-LGF- SS--L-GISI --TSLL--L-

205 GFAFGAFYWK KKQPN.LTRT VENIQIN..E EDNEISMLQE KEREFQEV 249 FELINE
205 ------L--- ---------- ---------- ---------- -------- 249 PORCINE
205 ------L--- ---------- ---------- ---------- -------- 249 CANINE
205 ---------- ---------- ---R------ ---------- -------- 249 BOVINE
204 ------L--- -R--S----A ---------- ---------- -------- 248 HUMAN
204 ------L--- ---SS----A ---------- ---------Q -------- 248 MURINE
215 --IL--I--- -TH-KSRPES N-T--CHGCQ -E-------Q --K-HLQ- 262 CHICKEN
```

Figure 3.7: Alignment of the amino-acid sequences of feline, porcine, canine, bovine, human, murine and chicken stem cell factor polypeptides. Identical amino-acids are indicated by dashes (-) and dots indicate gaps introduced to maintain optimal sequence alignment. The likely signal peptide and transmembrane domains are indicated by _S-P_ and _T-M_ respectively. The predicted amino terminus is marked "♦". Conserved cysteine residues are depicted by "•" and potential N-glycosylation sites by "*". The amino acids shown in bold type between the horizontal arrows (→ ←) are those deleted in the shorter isoforms of feline, canine, human, murine and bovine SCF; in each case this deletion results in the replacement of the 29 deleted amino-acids by a glycine residue. The major proteolytic cleavage sites, responsible for generation of soluble SCF are indicated by "↓".

PREDICTION OF SIGNAL SEQUENCE CLEAVAGE SITES BASED ON THE METHOD OF G. VON HEIJNE, N.A.R., 14, 4683(1986)

SEQUENCE NAME: scfpep.txt LENGTH: 274
SEARCH RANGE: 50 SEQUENCE TYPE: Eukaryotic

WINDOW	SUBSEQUENCE	NORMALIZED PROBABILITY P[i]/Pmax
1 - 15	MKKTQTWIVTCIY^LQ	0.000000
2 - 16	KKTQTWIVTCIYL^QL	0.000008
3 - 17	KTQTWIVTCIYLQ^LL	0.000000
4 - 18	TQTWIVTCIYLQL^LL	0.000019
5 - 19	QTWIVTCIYLQLL^LF	0.000007
6 - 20	TWIVTCIYLQLLL^FN	0.000906
7 - 21	WIVTCIYLQLLLF^NP	0.024491
8 - 22	IVTCIYLQLLLFN^PL	0.000053
9 - 23	VTCIYLQLLLFNP^LV	0.000704
10 - 24	TCIYLQLLLFNPL^VK	0.000020
11 - 25	CIYLQLLLFNPLV^KT	0.000149
12 - 26	IYLQLLLFNPLVK^TK	0.002134
13 - 27	YLQLLLFNPLVKT^KG	1.000000
14 - 28	LQLLLFNPLVKTK^GL	0.000033
15 - 29	QLLLFNPLVKTKG^LC	0.003902
16 - 30	LLLFNPLVKTKGL^CR	0.001254
17 - 31	LLFNPLVKTKGLC^RN	0.036330
19 - 33	FNPLVKTKGLCRN^RV	0.000138
20 - 34	NPLVKTKGLCRNR^VT	0.000000
21 - 35	PLVKTKGLCRNRV^TD	0.000000
22 - 36	LVKTKGLCRNRVT^DD	0.000023
23 - 37	VKTKGLCRNRVTD^DV	0.000001
24 - 38	KTKGLCRNRVTDD^VK	0.000001
25 - 39	TKGLCRNRVTDDV^KD	0.000000
26 - 40	KGLCRNRVTDDVK^DV	0.000000
27 - 41	GLCRNRVTDDVKD^VT	0.000000
28 - 42	LCRNRVTDDVKDV^TK	0.000000
29 - 43	CRNRVTDDVKDVT^KL	0.000000
30 - 44	RNRVTDDVKDVTK^LV	0.000000
31 - 45	NRVTDDVKDVTKL^VA	0.000000
32 - 46	RVTDDVKDVTKLV^AN	0.000000
33 - 47	VTDDVKDVTKLVA^NL	0.000005
34 - 48	TDDVKDVTKLVAN^LP	0.000000
35 - 49	DDVKDVTKLVANL^PK	0.000000
36 - 50	DVKDVTKLVANLP^KD	0.000000

Figure 3.8: Prediction of fSCF signal peptide using the 'Sigseq' program (Rockefeller University). The predicted signal peptide cleavage site is between Thr[25] and Lys[26].

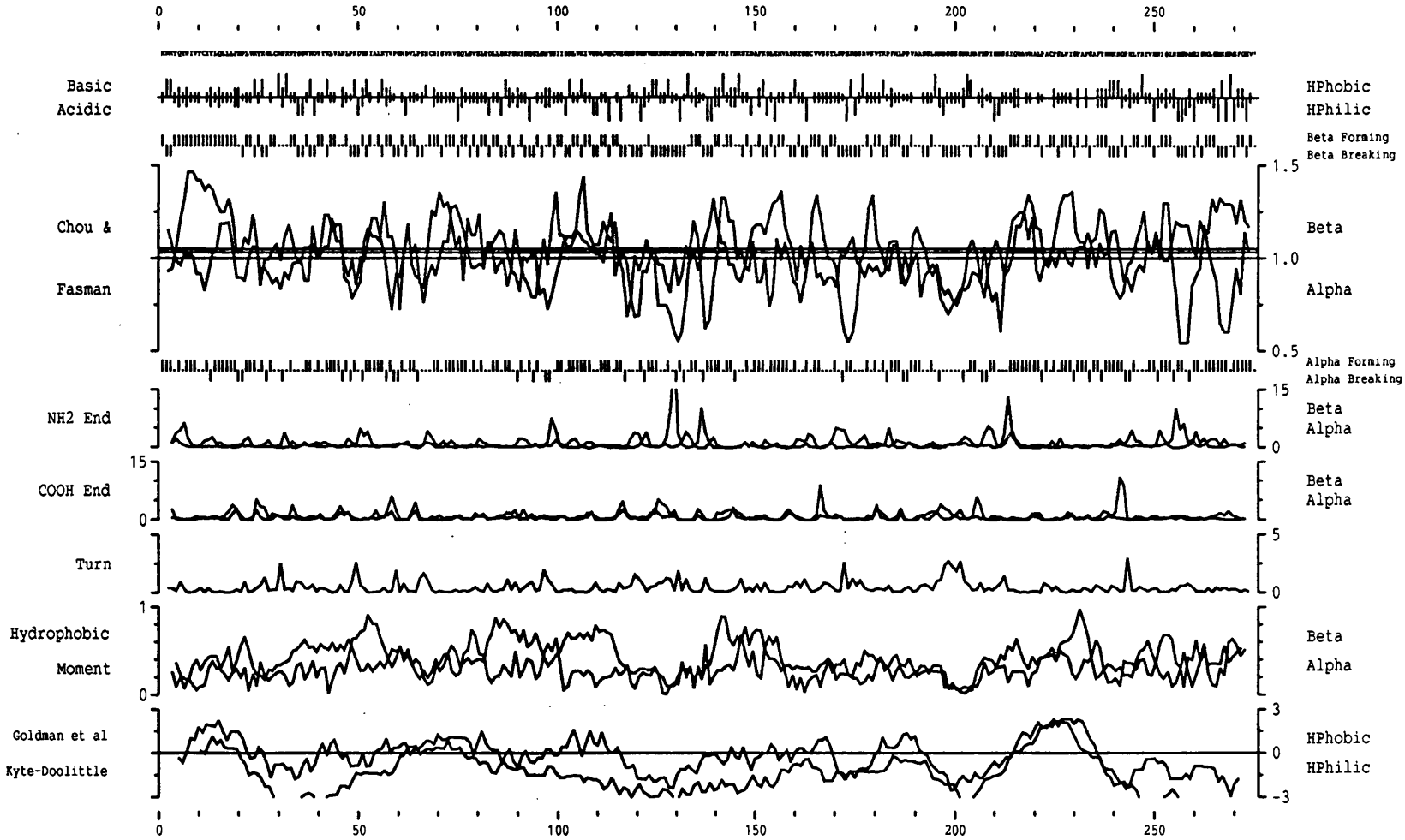


Figure 3.9: Measures of fSCF protein secondary structure and hydrophobicity predicted using 'Pepplot' (GCG software - Gribskov *et al.*, 1986).

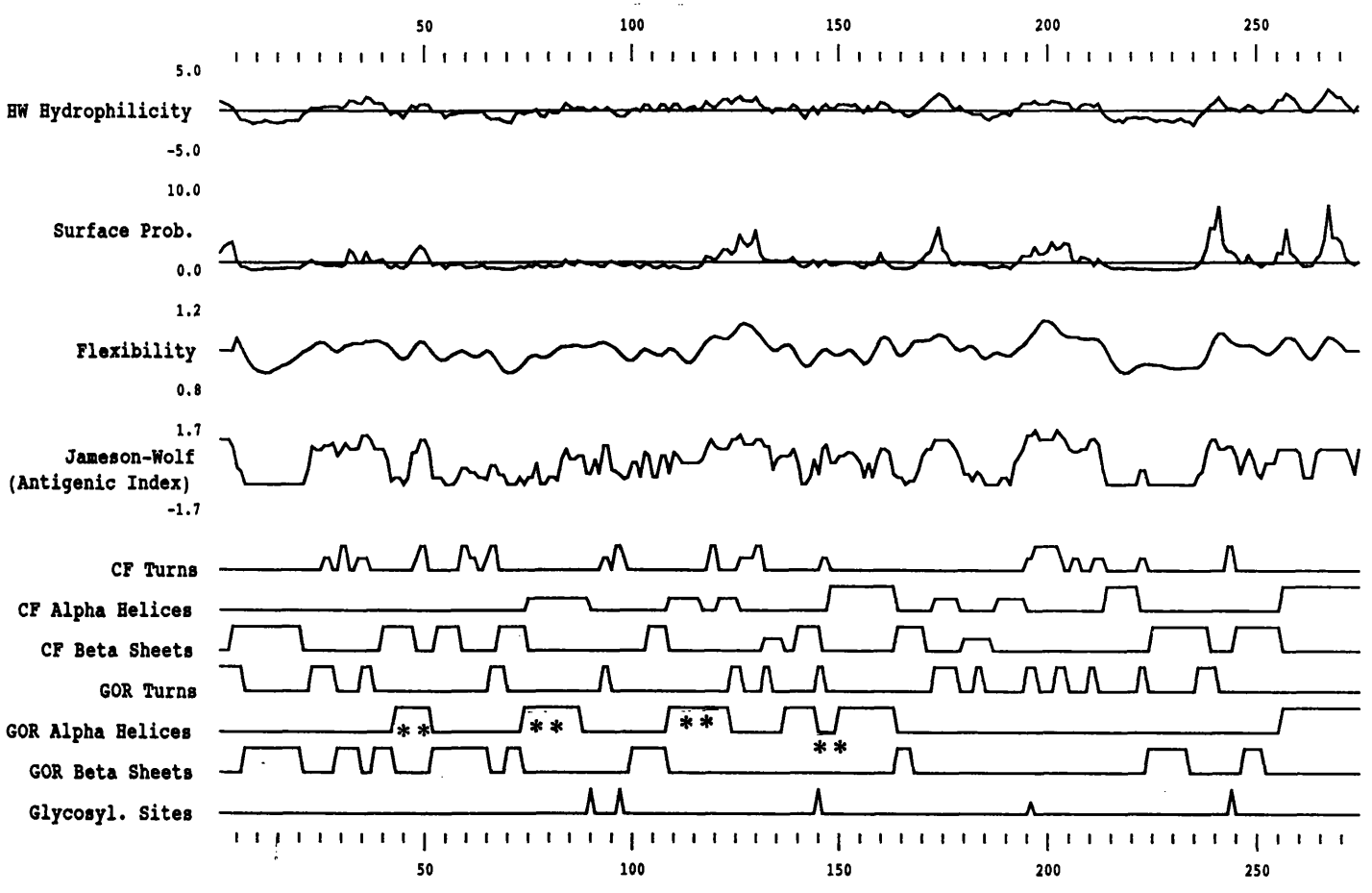


Figure 3.10: Hydropathy plot and measures of secondary structure of the predicted fSCF protein. This was produced using the GCG software programs PeptideStructure and PlotStructure (Wolf *et al.*, 1987). The likely positions of conserved alpha helices within the extracellular domain (in comparison to those predicted for human and murine SCF [Matous *et al.*, 1996]) are indicated by **.

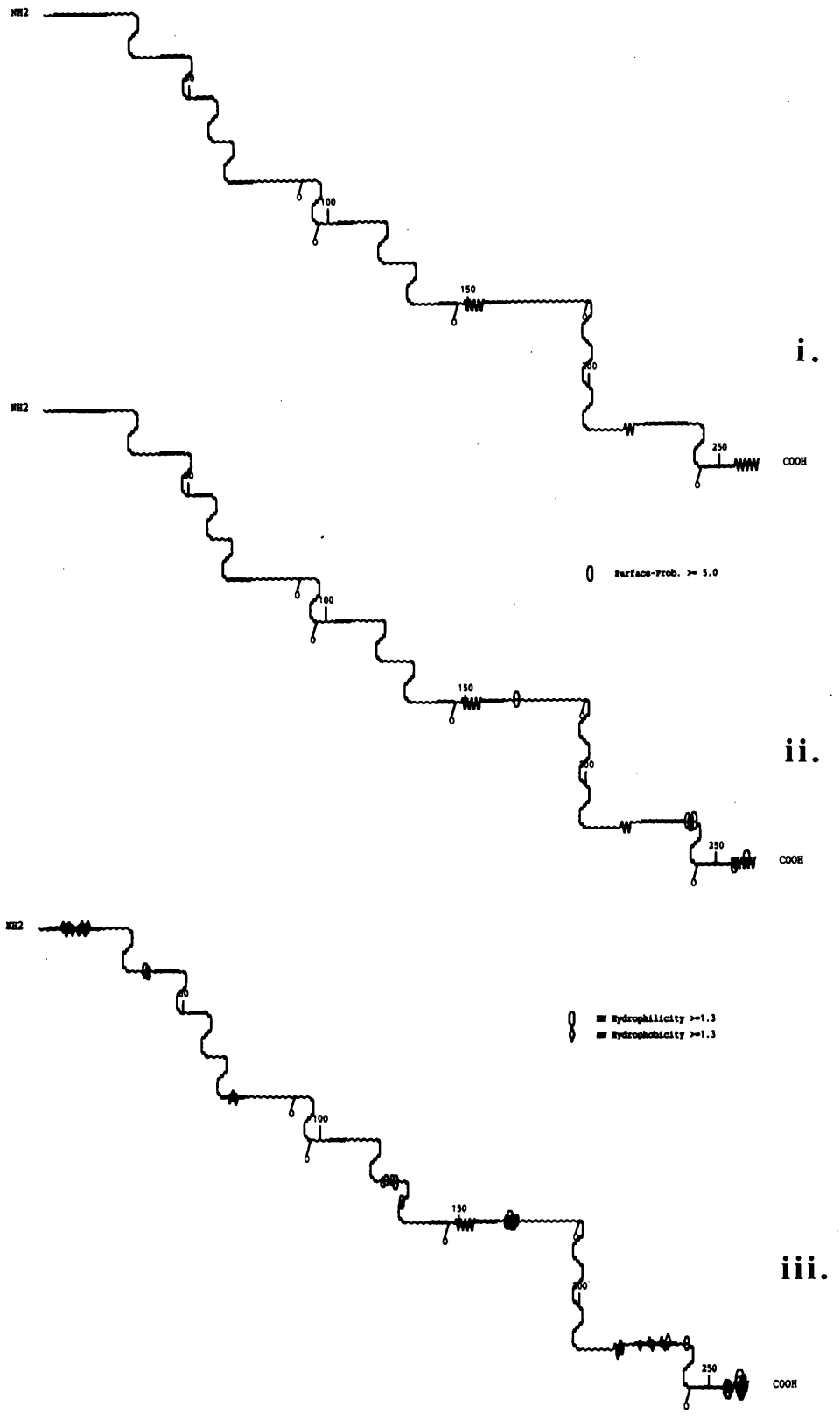


Figure 3.11: Analysis of the fSCF predicted protein using GCG software programs PeptideStructure and PlotStructure (Wolf *et al.*, 1987). Overlain on Chou-Fasman predictions of secondary structure (i) are motifs indicating regions with predicted high ii) surface probability iii) hydrophilicity or hydrophobicity scores.

3.4 DISCUSSION

3.4.1 AMPLIFICATION OF FSCF BY PCR

The polymerase chain reaction provides an elegant method of isolating novel gene sequences, where there is limited information regarding their nucleotide sequence. Its utilisation in this project enabled the isolation of feline stem cell factor in a fraction of the time that would have been taken to generate and screen a cDNA library. However, the use of PCR in this setting is not without potential disadvantages. The most significant of these is total failure to amplify the desired cDNA. This may occur despite attempts to optimise the PCR by variation of conditions including primer annealing temperature, number of PCR cycles and reaction conditions e.g. Mg^{2+}, primer concentrations (discussed in Innis and Gelfland, 1990). A number of potential causes of PCR failure can be considered:

i. The mRNA of interest may be absent (or degraded) in the starting material. The correct choice of starting cells or tissue, need for additional stimulation and optimal timing of mRNA harvest must be addressed.

ii. There may be a failure of reverse transcription to synthesise full length cDNA, either due to the reaction conditions or because of secondary structure in the mRNA. Treatment of mRNA with methylmercury hydroxide to remove secondary structure may be helpful in some circumstances.

iii. The oligonucleotide primers may not anneal efficiently to the cDNA due to mismatches. This is probably the most important consideration when attempting isolation of sequences where the exact sequence is unknown. The design of alternative primers or use of degenerate primers may prove successful in such circumstances.

iv. Non-specific primer binding may lead to production of multiple PCR products (seen as multiple bands or smears on PAGE) or poor yield of specific product. The use of 'hot-start' protocols (e.g. using anti *Taq* antibody or wax beads) may improve the yield of a specific product by reducing the extension of primers bound non-specifically.

If isolation by RT-PCR is successful, another inherent disadvantage which must be considered, illustrated in the isolation of fSCF, is the misincorporation of nucleotides during DNA synthesis by *Taq* DNA polymerase. The most common error is that of single base substitutions, generally occurring with low frequency (around one base substitution per 10^6 nucleotides). The error rate is, however, increased with higher than optimal nucleotide and magnesium concentrations, and may approach one per thousand nucleotides in unfavourable conditions. Given an error rate of 10^{-5} per nucleotide, after 30 cycles, when amplifying a 1 kb sequence, it may be estimated that 13% of fragments have a sequence differing from that of the correct (starting) sequence (for discussion see Hayashi, 1994). The nucleotide differences seen between different clones of fSCF were attributed to such errors; it is possible that they represented alternative cDNAs, however, given that the change in nt 261, from A to G, would have produced an amino acid change from serine to asparagine (where other mammalian SCFs show a serine at the same position) this was considered less likely. The sequencing of a number of cDNA clones, from different RT-PCR reactions enables a consensus sequence to be elucidated with a relatively high degree of confidence. It has been suggested that sequencing of between three to six clones will generally suffice in the determination of a consensus sequence at a given allele (Ennis *et al.*, 1996). The use of thermostable DNA polymerases with proof reading activity (e.g. *Pfu* polymerase, *Vent* polymerase) can result in an increase in fidelity over that seen with *Taq* polymerase; the use of such alternatives should be considered in future projects.

3.4.2 FEATURES OF THE PREDICTED FELINE SCF PROTEINS

Feline stem cell factor shares a high degree of homology at both the nucleic acid and protein level with other mammalian SCFs; this evolutionary conservation suggests a similar biological role for stem cell factor in the cat to that described for other species. The high sequence homology to the SCFs of other species allows the identification of an extracellular domain, a transmembrane domain and an intracellular domain within the longer fSCF isoform.

3.4.2.1 Signal Peptide (aa -25 to -1)

A signal peptide serves to direct ribosomal protein synthesis to the endoplasmic reticulum; the signal sequence is then removed on the luminal side of the endoplasmic reticulum (Walter and Lingappa, 1986). Signal sequences typically have an N-terminal basic region, a hydrophobic core and a polar C-terminal region followed by a proteolytic cleavage site (Perlman and Halvorson, 1983; von Heijne, 1983). Using the method developed by von Heijne (1986), feline SCF shows a predicted signal peptide of 25 amino acids, in common with SCF proteins of other species.

3.4.2.2 Extracellular domain (aa 1 to 190)

The extracellular domains of murine and human SCFs are predicted to form four alpha helices that are folded into an anti-parallel structure, stabilised by disulphide bridges. The helical cytokine domain is attached by a 12 to 24 amino acid spacer chain to a membrane 'tether' that is contiguous with the transmembrane domain (Bazan, 1991a). The approximate positions of the helical domains are amino acids 5 to 23, 48 to 62, 80 to 92 and 117 to 135 (Matous *et al.*, 1996). Predictions of fSCF secondary structure, using the methods of Chou and Fasman (1978) or Garnier *et al.* (1978) shows helices in the feline homologue at similar positions (Figure 3.9 to Figure 3.11). This is not unexpected given the overall high level of homology between fSCF and SCFs of other mammalian species.

In common with bovine, canine and porcine SCFs, fSCF has an extra amino-acid (Glu^{130}) when compared to human and rodent SCF sequences. Four cysteine residues (aa 4, 43, 89, 139), implicated as important in forming intramolecular disulphide bridges in rat SCF (Lu *et al.*, 1991), are conserved in fSCF. A proteolytic cleavage site has been identified in rat SCF between Ala^{164} and Ala^{165} and/or Ala^{165} and Ser^{166} (Martin *et al.*, 1990; Lu *et al.*, 1991). The amino-acid sequence in this region is well conserved in fSCF suggesting the existence of an analogous site in fSCF, and thus a similar soluble form of fSCF.

3.4.2.3 Transmembrane domain (aa 191 to 213)

The transmembrane domain of membrane associated proteins is characteristically hydrophobic. Such a region can be seen in the predicted feline SCF protein between

amino acids 190 and 211 (Figure 3.9 and Figure 3.11); this largely coincides with the transmembrane domain predicted by alignment with other species SCFs.

3.4.2.4 Intracellular domain (aa 214 to 249)

The predicted intracellular domain stretches from amino acids 214 to 249. This region is mainly hydrophilic in character and its likely function is to anchor the transmembrane domain within the lipid bilayer of the plasma membrane. It has also been proposed that the intracellular domain may have additional biological function(s) (Brannan *et al.*, 1992).

3.4.2.5 Post translational modifications

Proteins secreted via the endoplasmic reticulum are often modified by the addition of oligosaccharides, a process known as glycosylation. The addition of these moieties generally occurs via asparagine (*N*-linked glycosylation) or by serine, threonine or hydroxylysine residues (*O*-linked glycosylation). Potential *N*-glycosylation sites within the extracellular domain of fSCF are present at amino-acids 65, 72, 120 and 171; the site corresponding to Asn^{93} of human SCF is absent in fSCF. Studies on native rat stem cell factor, isolated from Buffalo rat liver conditioned medium have implicated the site at Asn^{120} as being glycosylated, with variable glycosylation of the residues at Asn^{109} (not present in feline SCF) and Asn^{65} (Lu *et al.*, 1991). In addition, native rat SCF has a high degree of O-linked glycosylation (Zsebo *et al.*, 1990b). Given the high level of interspecies conservation such modification is likely to be present in the feline homologue.

The isolation of feline SCF sequences provides pivotal information enabling new investigations into the of the role of SCF in feline haemopoiesis. This information also allows the expression of feline SCF *in vitro* by a number of potential techniques. The DNA sequence data appears in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number D50833 and is reported by Dunham and Onions (1996).

CHAPTER FOUR - EXPRESSION OF FELINE RECOMBINANT STEM CELL FACTOR IN *ESCHERICHIA COLI*

4.1 INTRODUCTION

Heterologous protein expression systems are frequently used to produce proteins for biological uses. They have the advantages of ease of manipulation and frequently enable the production of protein in higher levels than would be achieved by direct isolation of the native protein (Olins, 1996). Recombinant proteins have been successfully expressed in a wide variety of different systems which include bacterial, yeast, fungal, insect, plant or mammalian (including transgenic livestock) (Hodgson, 1993). This chapter gives an overview of the more frequently used expression systems and outlines the factors which affect the choice of system. The expression of feline stem cell factor as a fusion protein in *Escherichia coli* is then described.

There have been a number of reviews of the different expression systems available and factors governing the choice of system for a particular application (Goeddel, 1990; *in* Ausubel *et al.*, 1994). There are no predetermined rules for the expression of a foreign protein in a heterologous system. However, the choice of system used can be guided by an appreciation of the advantages and disadvantages of each, by a consideration of the properties of the protein to be expressed (e.g. size, degree of post-translational modification) and by the desired use of the recombinant protein (e.g. antigen, structural studies, investigation of biological activity).

4.1.1 SYSTEMS FOR HETEROLOGOUS GENE EXPRESSION

4.1.1.1 Expression in prokaryotic organisms

A number of different bacteria have been used for the expression of recombinant mammalian proteins including *Escherichia coli, Bacillus subtilis, Lactococcus lactis* and *Corynebacterium glutamicum* (Billman-Jacobe, 1996). Of these, *E.coli* is the most fully characterised and most widely used. *E.coli* expression systems have the advantages of ease of use and simplicity; standard recombinant DNA techniques may be used to generate an overexpressing strain in a relatively short time. The bacteria may be grown in inexpensive media making the system relatively economical. Expression systems using *E.coli* have been developed to allow the expression of

foreign proteins at high yields, whereby the recombinant protein may be expressed at levels of up to 30% of the total cellular protein production (Brent, 1994).

The expression of foreign proteins in *E.coli* is not without its disadvantages. Proteins which are normally produced as secreted proteins (including the cytokines) are synthesised in eukaryotes by ribosomes of the endoplasmic reticulum, whereas their synthesis in *E.coli* takes place in the cytoplasm. This may lead to incorrect folding of the recombinant protein and the precipitation of the expressed protein as insoluble aggregates called inclusion bodies. The isolation of soluble proteins in such circumstances requires solubilisation using denaturing agents (e.g. urea, guanidine hydrochloride) and subsequent refolding (Kohno *et al.*, 1990). Such manipulations may, however, result in a low yield of properly refolded protein or protein with low biological activity; in the case of larger proteins, correct refolding may not be possible. Secondly, eukaryotic proteins, produced in *E.coli*, are not post-translationally modified (by glycosylation, sialalylation etc.) as the native protein would be; this is an important consideration when contemplating the expression of cytokines, as they are generally undergo extensive post-translational processing.

Experience with many cytokines expressed in *E.coli* has shown that authentic post translational modifications are not essential for biological activity, indeed in a number of cases the protein expressed in *E.coli* is more active than its counterpart expressed in a eukaryotic system. This is seen with rhSCF, the *E.coli* expressed form inducing greater proliferation of a human megakaryocyte cell (UT-7) line *in vitro* than the glycosylated (COS cell derived) counterpart (Langley *et al.*, 1992). However, the absence of such modifications may lead to changes associated with distribution, biological half life *in vivo* or antigenicity of the recombinant cytokine. Human interferon-γ expressed in *E.coli* has a reduced circulatory half life compared to natural hIFN-γ (Bocci *et al.*, 1985). *In vitro* studies have shown that the presence of glycan residues on the recombinant protein protect it from degradation by proteases, including crude granulocyte proteases, elastase and plasmin (Sareneva *et al.*, 1995). A number of human recombinant cytokines used in clinical trials including hGM-CSF and hIFN-α have been shown to be antigenic, leading to the development of

neutralising antibodies in some patients. Neutralising antibodies to hIFN-α may be associated with relapse of disease (Steis *et al.*, 1988; Steis *et al.*, 1991), whilst antibodies to rGM-CSF may cross-react with the endogenous cytokine (Wadhwa *et al.*, 1996). Such cross-reactivity may potentially lead to a decrease in endogenous cytokines and subsequent cytopenia. This has been reported following the administration of canine G-CSF to rabbits, for the purpose of antibody production (Reagan *et al.*, 1995). In the case of IFN-α, the incidence of neutralising antibodies was higher, in one study, in patients treated with *E.coli* derived rIFN-α compared to those treated with natural IFN-α, isolated from lymphoblastoid cells (Antonelli *et al.*, 1991). Despite these concerns, recombinant human cytokines expressed in *E.coli* are being increasingly used within the clinical field.

A number of features are required to allow efficient expression of cloned genes in *E.coli* :

- a selectable marker to ensure maintenance of the vector in the host strain (e.g. ampicillin resistance).
- a strong inducible promoter (e.g. *lac*, *trp* or tac); following induction this will direct the synthesis of large amounts of mRNA.
- a ribosome binding site that is not blocked due to secondary structure and a start codon (ATG).
- polylinker sequences to allow insertion of the gene of interest into the vector.

The efficiency of expression is also influenced by the codon usage within the cloned gene, especially at the 5' end of the gene, notably the second codon (Stormo *et al.*, 1982); use of non-preferred codons can lead to premature termination of translation. This may be overcome by taking advantage of the degeneracy of the genetic code and modifying the coding sequence of the cloned gene to use preferred codons. An alternative solution is to express the protein as a fusion with a carrier protein. The expression vector is designed with the coding sequence of the carrier protein directly 5' to the site of insertion of the cloned gene; upon translation an N-terminal fusion protein is produced. The carrier protein can be from any gene that is highly expressed in *E.coli*; examples include *trp*E fusions, histidine-tagged proteins, maltose-binding

protein fusions and glutathione S-transferase (GST) fusions. The use of a fusion protein system may be associated with additional advantages. Expression of human growth hormone in a vector encoding the *E.coli* signal peptide *ompA*, results in secretion of the recombinant protein into the periplasm, cleavage of the signal peptide and facilitates proper disulphide bond formation and folding of the recombinant protein (Hsiung *et al.*, 1986). The presence of the fusion partner may enhance the solubility of the expressed fusion protein, an advantage associated with the GST gene fusion system. The N-terminal sequence may be used as an aid to purification of the expressed fusion protein, using antibody affinity purification (*trp*E or β-gal fusions), affinity to metals in the case of His-tagged proteins or specific affinities such as the binding of GST fusion proteins to glutathione sepharose. Antibodies directed against the fusion partner may be used to identify expressed proteins by Western blot analysis. The presence of the fusion partner may, however, confer disadvantages in addition to those discussed above. The biological activity of the recombinant protein may be modified or the fusion may interfere with immunological techniques, for example protein-A fusions will interact non-specifically with antibodies. Other fusion partners may affect the use of the protein as a specific immunogen to raise antibodies to the target protein. This problem may be overcome however, by cleavage of the desired recombinant protein from its fusion partner by chemical or proteolytic methods (Riggs, 1994).

4.1.1.2 Expression of proteins in yeasts

Yeasts have a number of advantages for the expression of foreign proteins. Like *E.coli*, yeasts are unicellular organisms which grow rapidly and are easy to manipulate. Additionally, as eukaryotic organisms, they possess much of the cellular machinery to perform accurate post-translational processing and modification of many mammalian proteins (Buckholz and Gleeson, 1991). Expressed proteins can be directed to the secretory pathway, simplifying harvesting and purification (Brake *et al.*, 1984). The majority of recombinant proteins produced in yeast have used *Saccharomyces cerevisiae* (bakers yeast) as a host, due to familiarity with its genetics and growth characteristics. However, *S. cerevisiae* based expression systems often produce only low yields of protein. Furthermore, the glycosylation patterns of proteins produced in *S. cerevisiae* differ from those of native mammalian proteins in

that the glycosyl groups are more extensive and composed largely of mannose residues. This can lead to a decreased circulating half life of the protein *in vivo*, changes in immunogenicity and function (Eckart and Bussineau, 1996). A number of alternative yeast host strains have been developed including *Pichia pastoris* and *Hansula polymorpha*, which seem less prone to producing such hyperglycosylated proteins and may therefore be more suitable for the expression of mammalian glycoproteins (Hodgson, 1993; Eckart and Bussineau, 1996).

4.1.1.3 Mammalian expression systems

The expression of proteins from higher eukaryotes in a mammalian system has certain inherent advantages. Proteins are usually expressed in the correct cellular compartment and appropriately modified. Authentic modifications reduce the likelihood of the expressed protein proving immunogenic or having altered biological activity or pharmacokinetics. However, mammalian expression systems tend to be more technically demanding and expensive to use (Brent, 1994). Transient expression systems are commonly used to produce small amounts of proteins to evaluate the functional activity of a novel cDNA. COS cells are often used for such short term expression. These cells are derived from African green monkey kidney cultures that have been transformed with SV40 virus carrying a defective origin of replication (Gluzman, 1981). The cells do not, therefore, produce whole virus, but do produce large quantities of the viral protein, SV40 large tumour (T) antigen. This protein directs the amplification of vectors containing the SV40 origin of replication to high levels (10,000 - 100,000 copies per cell) 48 - 72 hours following transfection. Plasmids containing a cDNA or genomic DNA insert can therefore direct the synthesis of large amounts of protein in a short time, under the control of an appropriate promoter. However, because the protein production machinery of the transformed cell is effectively 'hijacked', cells generally die after a number of days (Aruffo, 1994). Large scale production of proteins in mammalian systems requires the establishment of stably transfected cell lines and gene amplification. Chinese hamster ovary (CHO) cells have been widely used for the large scale expression of human proteins including erythropoietin, granulocyte colony-stimulating factor and growth hormone (Hodgson, 1993).

4.1.1.4 Baculovirus expression systems

The baculovirus expression system uses insect cells to propagate a virus into which the cloned gene has been introduced, in place of a highly expressed, yet non-essential protein. The most widely used baculovirus system utilises the virus *Autographa californica* multiply enveloped nuclear polyhedrosis virus (AcMNPV) belonging to the family *Baculoviridae,* a large dsDNA virus that infects arthropods (Luckow and Summers, 1989). Infection with baculovirus leads to production of two types of viral particles: extracellular (non-occluded) and polyhedra-derived (occluded). Extracellular virus is released from the cell by budding (> 10 hours p.i.) and leads to secondary infection of neighbouring cells and tissues. Polyhedra-derived virus, appearing as viral inclusions, is seen after approximately 18 hours p.i. and accumulate within the nucleus of infected cells until released by cell lysis (up to five days p.i.). Viral inclusions consist of virus particles imbedded in proteinaceous material, the main component of which is polyhedrin. In nature the polyhedrin serves to protect viral particles from proteolytic digestion as the host tissue decomposes. The occluded virus is released in the alkaline conditions of the gut following ingestion of contaminated food by a new host, thus resulting in propagation of the virus. Whilst the polyhedrin protein is therefore important in nature, the virus is able to survive and propagate in tissue culture without it. The baculovirus expression system takes advantage of this, whereby recombinant baculoviruses are generated by replacing the polyhedrin gene with a foreign gene via homologous recombination. Viruses lacking polyhedrin have a distinctly different appearance which provides a method for the selection of recombinant virus (reviewed by Miller, 1988, Luckow and Summers, 1988).

Baculovirus expression has the advantage of allowing high level expression of protein which generally remains soluble in insect cells. The system performs many of the post-translational modifications occurring in higher eukaryotes. Proteins are generally appropriately secreted or targeted to the nucleus, cytoplasm, cell membrane. Post-translational modifications such as signal peptide cleavage, removal of hormonal pro-sequences, glycosylation, myristolation, palmitylation and phosphorylation have all been documented. However, whilst *N*-linked glycosylation is often performed at the

correct sites, the extent of the glycosylation generally does not approach that of higher eukaryotes. Mammalian proteins expressed in baculovirus systems tend to be high in mannose, lacking sialic acid, galactose and fucose residues of the native protein (Luckow and Summers, 1988; van Die *et al.*, 1996). However, it has been shown that the use of different cell lines, such as *Estimene acrea*, to those commonly used for expression (*Spodoptera frugiperda*) can produce a glycosylation pattern more akin to the native mammalian protein (Wagner *et al.*, 1996)

4.1.2 EXPRESSION OF RECOMBINANT FELINE STEM CELL FACTOR

The choice of system for expression of recombinant fSCF was principally directed by ease of use and the ability to produce a sufficient quantity of protein of a relatively high purity, within a limited time scale, to enable characterisation of the protein's biological activity. These considerations led to the choice of the *E.coli* based glutathione S-transferase gene fusion system (Smith and Johnson, 1988). This utilises one of a series of pGEX plasmids which are designed for high level expression of genes as fusion proteins with *Schistosoma japonicum* glutathione S-transferase (GST). The plasmids contain a *tac* promoter that allows high level expression, inducible with the lactose analogue IPTG. The presence of an internal *lac* I^q gene enables the plasmid to be used in any *E.coli* host. Fusion proteins produced in this system are often soluble, increasing the likelihood of them possessing full biological function and removing the need for resolubilisation and refolding steps. Purification of the fusion protein relies on the affinity of the GST tag to glutathione bound to a matrix (Sepharose 4B). Throughout purification mild (physiological) conditions may be maintained, thus minimising as far as possible the effects of the procedure upon protein antigenicity and functional activity. The plasmids contain either thrombin or factor Xa protease recognition sites; this allows the desired protein to be cleaved from the fusion product following affinity purification. The presence of the GST tag allows identification of the fusion protein using specific anti-GST antibody in Western blotting protocols. It also enables estimation of yields of recombinant protein by means of the CDNB (1-chloro-2,4-dinitrobenzene) assay (Habig *et al.*, 1974). GST has a strong affinity for CDNB and catalyses the conjugation of CDNB to glutathione, resulting in a product which shows strong absorption at 340 nm; by measuring the

change in absorbance at this wavelength, an estimate of the amount of GST fusion protein in the sample may be obtained.

This chapter describes the expression of the soluble form of feline stem cell factor in *E.coli*. PCR was used to subclone the coding sequence for amino acids 1 - 165 of the mature fSCF protein into the pGEX vector (i.e. the sequence encoding the 5' signal peptide and 3' amino acids 166 - 249 were omitted). The protein was then expressed and purified by affinity chromatography. An overview of the experimental protocol is shown in Figure 4.1.

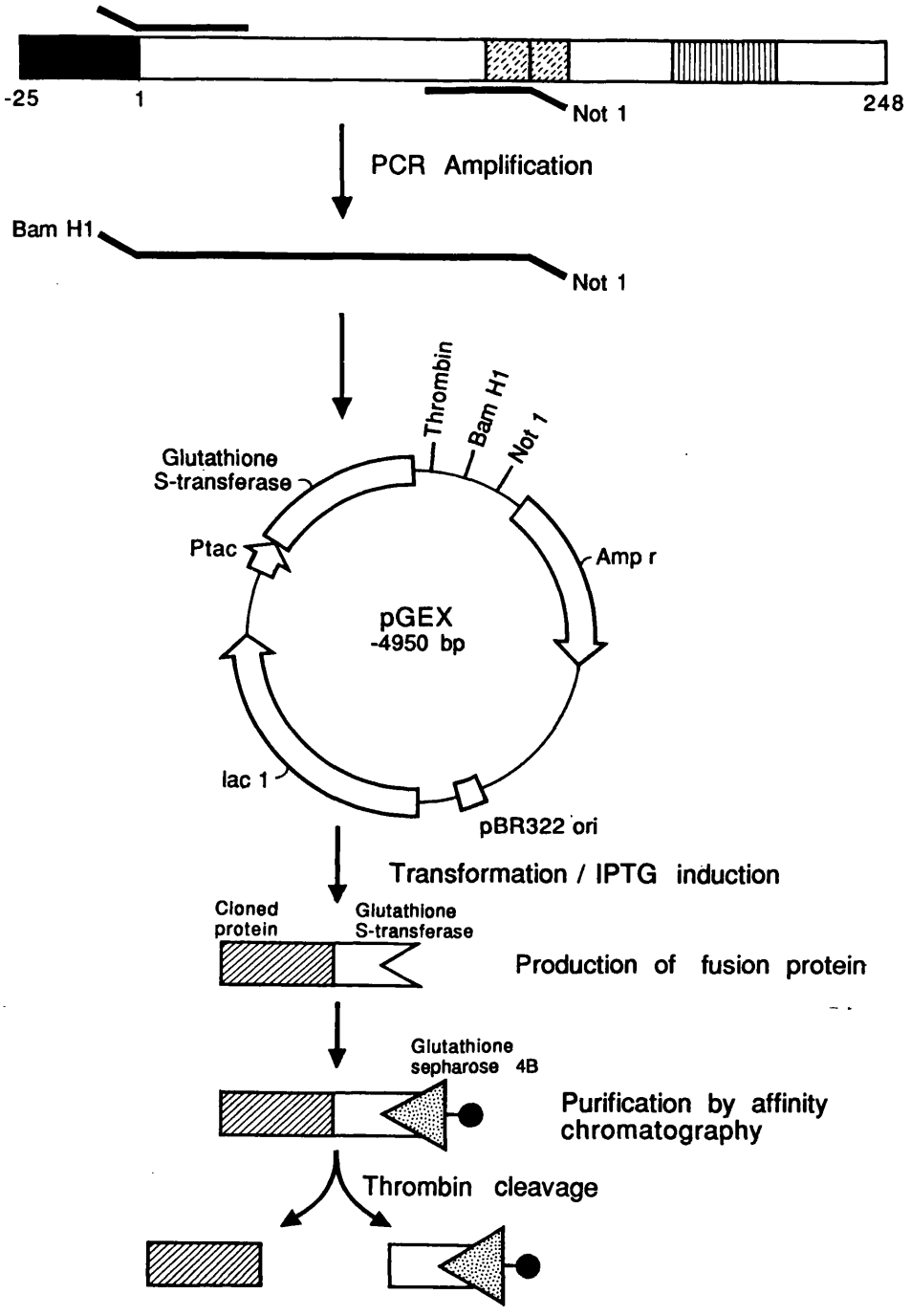


Figure 4.1: Expression of soluble rSCF protein using the GST gene fusion system. The coding sequence for amino acids 1 - 165 of the predicted mature fSCF protein was amplified using the polymerase chain reaction. This was then cloned into the pGEX vector 4-T1. The vector was introduced into *E.coli* and the protein expressed following induction with isopropyl-1-thio-β-D-galactoside. The recombinant protein was purified by affinity chromatography and cleaved using thrombin.

4.2 MATERIALS AND METHODS

4.2.1 PRODUCTION OF RECOMBINANT EXPRESSION VECTOR

The recombinant plasmid fSCF-pGEX (pGEX-4T-1 plasmid containing the coding sequence for predicted soluble feline stem cell factor) was produced as described below.

4.2.1.1 Preparation of cDNA encoding soluble fSCF

Primers were designed for PCR amplification of DNA encoding amino-acids 1-165 of the predicted mature fSCF protein, as follows:

5' Primer: 5'-GCG-CGG-ATC-CAA-AGG-GCT-CTG-CAG-GAA-CCG-3'

3' Primer: 5'-GCG-CGG-CCG-CAT-TAT-GCA-ACA-GGG-GGT-AAC-3'

The 5' primer contains a GCGC clamp and a BamHI restriction site at the 5' end whilst the 3' primer has a GC clamp and a NotI restriction site at its 5' end. These modifications allowed directional cloning of the amplified PCR product into the pGEX 4T-1 vector as detailed below. The 3' primer also encodes a stop codon (TAA(T)).

The primers were each used at 1.0 μM concentration in a 100 μl volume reaction mix. The template was provided by 40 ng of recombinant pCR™ II vector containing the full length feline stem cell factor insert (clone 52C). Cloned *Pfu* DNA polymerase (Stratagene) was used in preference to *Taq* polymerase. *Pfu* polymerase is isolated from the hyperthermophilic marine archaebacterium *Pyrococcus furiosus* and unlike *Taq* polymerase it possesses a 3' to 5' exonuclease activity or proof reading ability; this can lead to an increase in the fidelity of DNA synthesis of 12 fold over *Taq* DNA polymerase. This was used in order to minimise the chance of introducing base changes into fSCF during amplification. To the reaction mix was added 10 μl of 10 x cloned *Pfu* buffer (200 mM Tris-HCl (pH 8.75), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, 1 mg/ml BSA) and 2.5 units cloned *Pfu* DNA polymerase. Deoxynucleoside triphosphates were included at a final concentration of 100 μM.

The reaction mix was overlain with mineral oil and the tubes transferred to a thermal cycler. The following cycle was programmed: an initial denaturation step of 94°C for five minutes, followed by 30 cycles of: 94°C, for one minute; 58°C, for one minute; 72°C, for one minute; with a final extension step of 72°C for 10 minutes. The tubes were then brought to and held at 4°C until collected. Seven 100 µl reactions were set up and run concurrently. Upon completion of thermocycling, the reactions were removed from the overlying mineral oil and combined into a single tube. The DNA was precipitated overnight at -20°C following the addition of 0.1 volumes of 3M sodium acetate and 2.5 volumes of ethanol. The DNA was pelleted by centrifugation in a microcentrifuge at 13,000 rpm for 10 minutes, the supernatant removed and the DNA pellet dried in a vacuum desiccator. The pellet was then resuspended in 40 µl dH_2O. Ten microlitres of this solution was then loaded onto five lanes of a 1% TEA agarose minigel and electrophoresed for two hours. The DNA bands were visualised under UV illumination following staining with ethidium bromide, excised using a sterile scalpel and the DNA extracted from the gel slices using a GenecleanII Kit (as detailed in section 2.2.2.6); the DNA was eluted in 30 µl of dH_2O.

The purified insert DNA was digested with the restriction enzymes NotI and BamHI. Thirty microlitres of plasmid DNA was digested in 50 µl volume using 20 units of each restriction enzyme, five microlitres of ReactIII buffer and 5 mM spermidine; following incubation for four hours at 37°C the reaction was stopped by the addition of 5.5 µl DNA gel loading buffer. The insert DNA was then quantified by polyacrylamide gel electrophoresis (loading a 2 µl aliquot). Following staining with ethidium bromide and inspection under UV illumination the amount of DNA was compared to that of the molecular weight standard DNA (ϕX174 RF DNA/Hae III fragments).

4.2.1.2 Preparation of pGEX vector DNA

A working stock of pGEX-4T-1 vector DNA was prepared, briefly, as follows. JM105 *E.coli* were transformed with 10 ng of the vector as described in section 2.2.2.8.2. Following selection on LB agar plates containing 50 µg/ml ampicillin and

20 mM glucose (LBAG plates) a single colony was picked and used for large scale DNA preparation by the CsCl method as detailed in 2.2.2.2.1.

In order to prepare the vector for cloning with the fSCF insert, the vector was digested with the restriction enzymes NotI and BamHI. Five micrograms of plasmid DNA were digested in 50 µl volume using 20 units of each restriction enzyme, five microlitres of ReactIII buffer and 5 mM spermidine; following incubation for 2.5 hours at 37°C the reaction was stopped by the addition of 5.5 µl DNA gel loading buffer. The efficiency of restriction digest was checked by electrophoresis of five microlitres of DNA through a 1.0% TEA agarose gel for two hours followed by staining with ethidium bromide and inspection under UV illumination. The vector DNA was purified from the excised insert using a GenecleanII Kit. The procedure followed that detailed in 2.2.2.6, however separation of the DNA fragments by prior agarose gel electrophoresis was not carried out; this was not considered necessary as the excised restriction fragment was too small to bind efficiently to the glassmilk. The purification protocol was carried out twice in the same manner followed by collection of the purified DNA in 20 µl of dH_2O. Two microlitres of the DNA was used to estimate the DNA concentration by running the sample on a 1.0% TEA agarose gel. After staining with ethidium bromide the gel was examined under UV illumination and the quantity of DNA present estimated by comparison with a known quantity of molecular weight marker (λ DNA/HindIII fragments).

4.2.1.3 Ligation of vector to insert DNA

The insert DNA was ligated to the vector DNA at a 5:1 molar ratio; 60 ng insert DNA and 100 ng vector DNA were combined with one unit (one microlitre) T4 DNA ligase and four microlitres of 5x ligase buffer in a final volume of 21 µl. The reaction was incubated at 14°C overnight.

4.2.1.4 Transformation of bacteria

Competent JM105 *E.coli* were freshly prepared and transformed with an aliquot of the ligation reaction as detailed in section 2.2.2.8.2. Colonies were selected by growth overnight at 37°C on LBAG plates. Colonies were picked and small scale

DNA preparations made using the Wizard Miniprep method (section 2.2.2.2.3). The presence of an insert of the correct size (approximately 500 bp) was verified by subjecting a quantity of the DNA to restriction enzyme digestion with NotI and BamHI enzymes followed by electrophoresis through a 5.0% polyacrylamide gel.

4.2.1.5 Sequencing of recombinant plasmids

Two clones containing recombinant plasmids with the appropriate sized insert were sequenced using Sequitherm DNA polymerase and IRD41 labelled primers as detailed in section 2.2.5.2. The primers used were:

5' pGEX primer: 5'-GGG-CTG-GCA-AGC-CAC-GTT-TGG-TG-3'

3' pGEX primer: 5'-CCG-GGA-GCT-GCA-TGT-GTC-AGA-GG-3'

The sequencing reactions were run on a Licor model 4000 sequencer. The fSCF sequence in both clones was found to be identical to that previously reported. A stock of recombinant plasmid DNA (clone two) was prepared using the caesium chloride method (section 2.2.2.2.1).

4.2.2 EXPRESSION OF SOLUBLE fSCF FUSION PROTEIN

4.2.2.1 Transformation of BL21 E.coli

Competent BL21 *E.coli* were freshly prepared and transformed with the recombinant pGEX plasmid containing the fSCF coding sequence (section 2.2.2.8.2); the cells were also transformed with the parental pGEX plasmid for use as a control in expression studies. Single colonies were picked after overnight growth on LBAG agar plates and used for production of recombinant protein.

4.2.2.2 Small scale screening for recombinant protein expression

Prior to large scale production of recombinant protein, a small scale screening procedure was carried out. This followed the same protocols outlined in 4.2.2.3 and 4.2.2.4 below, however a final culture volume of 400 ml was used and quantities of reagents were reduced accordingly. The presence of fSCF fusion protein in the final eluate was verified by SDS-PAGE as detailed in 4.2.3 below.

4.2.2.3 Large scale production of bacterial sonicate

A single colony of BL21 *E.coli* containing the fSCF-pGEX plasmid was used to inoculate 150 ml of 2YTA medium (2YT medium containing 100 μg/ml ampicillin) and grown overnight with shaking at 37°C. A BL21 *E.coli* colony containing the plasmid pGEX-4T-1 was used in parallel as a control, bacterial expression and purification was carried out identically except that a 50 ml overnight culture was grown and subsequent quantities of reagents reduced proportionally. The overnight cultures were diluted 1:20 by the addition of fresh 2YTA medium, and divided into one litre aliquots. The cultures were grown with shaking at 30°C to an OD_{600} of approximately 1.0 (for two and a half to three hours). Production of the recombinant protein was then induced by the addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a final concentration of 0.1 mM; the cultures were incubated at 25°C with shaking for further 90 - 120 minutes.

The bacterial culture was transferred to 500 ml centrifuge flasks (Beckman) and pelleted by centrifugation at 8,000 rpm for 10 minutes in Beckman JA-10 rotor. The supernatant was discarded and the bacterial pellet resuspended in ice cold PBS containing 1 mM PMSF (a protease inhibitor). The cell suspension was sonicated for approximately 30 - 45 seconds to disrupt the bacteria. A Model XL 2020 sonicator (Heat Systems Inc., Farmingdale, NY.) equipped with a standard probe was used for sonication; the sonicator was tuned prior to use, following the manufacturers instructions. The power level was generally set to between five and six (where level 10 was maximal) which avoided frothing (which may denature fusion proteins) yet produced satisfactory cell lysis. The degree of sonication was judged by a decrease in viscosity and slight darkening in colour of the cell suspension. Triton X-100 (20% stock solution) was then added to final concentration of 1% and mixed for five minutes on ice to aid solubilisation of the fusion protein. Cell debris was pelleted by centrifugation in polypropylene centrifuge tubes for 10 minutes at 18,000 rpm in a JA-20 rotor (Beckman) The supernatant was then removed to a clean tube prior to purification of the fusion protein by affinity chromatography. If purification was not carried out immediately then the supernatant was stored at -20°C. After storage a

precipitate was generally seen which was removed by centrifugation as before; this did not appear to affect the yield of recombinant protein.

4.2.2.4 Affinity column purification of fusion protein

Glutathione sepharose 4B was supplied as a 75% slurry in 20% ethanol (Pharmacia Biotech). This was used in accordance with the manufacturers recommendations; one millilitre bed volume was taken as sufficient to purify the sonicate derived from two litres of bacterial culture (approximately five milligrams of fusion protein assuming average yields). Prior to use the matrix was resuspended by shaking and the required amount of slurry (1.33 ml of slurry per millilitre of required bed volume) removed to a 15 ml or 50 ml centrifuge tube. The matrix was sedimented by centrifugation at 500 x g for five minutes and the supernatant gently removed. The glutathione sepharose 4B was washed by the addition of 10 ml ice cold PBS per 1.33 ml of the original slurry and the matrix sedimented by centrifugation as before. The supernatant was discarded and a 50% slurry of glutathione sepharose 4B prepared by addition of one millilitre of PBS for each 1.33 ml of original slurry dispensed.

Three millilitres of 50% glutathione sepharose 4B slurry was added to the sonicate derived from three litres of bacterial culture and incubated at room temperature for 30 minutes with gentle agitation. The matrix was then sedimented by centrifugation at 500 x g for five minutes, the supernatant discarded and the matrix resuspended in 20 ml PBS. The slurry was loaded into a disposable PD10 column (Pharmacia) to facilitate washing. The matrix was washed with PBS until the OD_{280} of flow through reached zero, compared to blank of PBS. The fusion protein was then eluted using 1.5 ml of glutathione elution buffer (10 mM glutathione, 50 mM Tris-HCl (pH 8.0)); the glutathione sepharose 4B was incubated for 10 minutes at room temperature with the elution buffer prior to collecting the eluate. Elution and collection steps were repeated two further times and the eluates pooled and stored at -70°C.

4.2.2.5 Thrombin cleavage

The concentration of fusion protein in the pooled eluate was **estimated** by measuring the OD_{280}, compared to a blank of elution buffer, and taking one OD unit as equal to approximately 0.5 mg/ml (this estimate was derived from the manufacturer's

information that 0.5 mg/ml of the GST affinity tag protein has an OD_{280} of approximately one). Ten cleavage units of bovine thrombin (Pharmacia Biotech) were added per milligram of fusion protein; digestion was allowed to proceed overnight with gentle agitation.

4.2.2.6 Dialysis

In order to remove free glutathione from the eluate the digested fusion protein solution was dialysed against PBS. Three batches of fusion protein, prepared as described above, were pooled and then loaded into a 15 ml Slide-a-lyzer cassette with a 10 kDa molecular weight cut off (MWCO) dialysis membrane (Pierce Chemical Co., Rockford, IL, USA). Dialysis was carried out against five litres of PBS, for approximately 24 hours, at 4°C, with four to five changes of buffer.

4.2.2.7 Removal of cleaved GST tag

The GST affinity tag was removed from the fSCF protein by affinity chromatography using glutathione sepharose 4B. Fifty per cent slurry, prepared as described above, was added to the protein solution (using one millilitre of slurry per one and a half millilitres of original eluate volume) and incubated for 30 minutes. The sepharose beads were pelleted by centrifugation at 500 x g for five minutes and the supernatant containing the fSCF protein moiety removed to a fresh tube.

4.2.3 ALTERNATIVE PROCEDURE FOR CLEAVAGE OF FUSION PROTEIN

During the latter stages of this project an alternative method of releasing the fSCF moiety from the complete fusion protein was employed. The above protocol was modified as follows. The fusion protein was produced and purified as detailed in 4.2.2.4, however the protein was not eluted from the glutathione sepharose 4B column following washing. Instead the fusion protein was cleaved whilst still bound to the column. This was performed by adding thrombin solution (50 cleavage units of thrombin contained in one millilitre of PBS per millilitre of bed volume) to the glutathione sepharose matrix and incubating overnight, at room temperature, with gentle mixing. The eluate was then collected, removed to a clean tube and stored at -20°C prior to further purification, as detailed in chapter five.

4.2.4 IDENTIFICATION OF SCF RECOMBINANT PROTEINS

4.2.4.1 Identification on Coomassie stained gels

Recombinant proteins were identified by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with coomassie blue, as outlined in section 2.2.6.2. Typically between 2.0 - 10 µl of each sample was loaded into each well of a 8.0 x 7.3 cm gel, when using a 10 well, 0.75 mm thick, comb. This was used to estimate the molecular size, yield and purity of the recombinant protein.

4.2.4.2 Identification by Western Blot analysis

In order to confirm the identify of the protein band seen on coomassie stained gels as a GST-fusion protein, immunoblotting was performed as outlined in section 2.2.6.3. The primary antibody used was a mouse anti-GST IgG monoclonal antibody (kindly provided by N. Spibey and T. Dunsford, Department of Veterinary Pathology, University of Glasgow); this was used at a 1:50 dilution. The secondary antibody used was an HRP conjugated sheep anti-mouse IgG polyclonal antibody (Sigma), used at a dilution of 1:1000.

The identity of the recombinant fSCF protein released following thrombin cleavage was confirmed similarly, using a primary antibody of anti-human SCF goat polyclonal IgG (R & D Europe Ltd., Abingdon, Oxon, UK) at a dilution of two micrograms per millilitre. The secondary reagent used was HRP conjugated rabbit anti-goat IgG (whole molecule) IgG fraction of antiserum (Sigma Chemical Co., St Louis, MO, USA); used at a dilution of 1:2000.

4.2.5 ENDOTOXIN ASSAY

Samples of recombinant fSCF were submitted to Q1 Biotech Ltd. (Glasgow, U.K.) for assessment of the level of endotoxin contamination. Endotoxin levels were measured using a chromogenic Limulus amoebocyte lysate (LAL) assay. The LAL assay utilises a natural defence mechanism of the horseshoe crab *Limulus polyphemus*. The horseshoe crab is a marine animal, which is in fact more closely related to spiders

than crustaceans. In response to injury the animal's haemolymph clots and thus provides a barrier to infection. This clotting mechanism is initiated by endotoxin (the majority of marine bacteria are gram negative) (Dawson, 1985). The basis of this clotting mechanism is the activation of a pro-enzyme by endotoxin to form an active clotting enzyme which then catalyses the cleavage of a clotting protein; once cleaved the protein becomes insoluble and forms a clot. The simplest LAL endotoxin assay is the gel-clot test which (semi-quantitatively) measures the endotoxin level in a test sample by comparison of the ability of the sample to form a solid clot, when mixed with the LAL enzyme and substrate, compared to known standards. The chromogenic method replaces the clotting protein with a synthetic peptide that forms a substrate for the enzyme. The synthetic peptide is covalently attached to a chromophore, para-nitroanilide (p-NA); following pre-incubation with endotoxin, the activated enzyme cleaves p-NA to form a coloured product which can be quantified by spectrophotometry (at 405 nm). The colour change is proportional to the amount of active enzyme and hence the endotoxin level of the test substance (Novitsky, 1983).

4.3 RESULTS

Analysis of affinity purified lysates from bacteria expressing SCF-GST reveals major products with relative molecular weights of 44 kDa and 28 - 34 kDa (Figure 4.2). These major products are confirmed as GST fusion proteins by immunoblotting (Figure 4.3). The larger product is the approximate size predicted for the SCF-GST fusion protein. The smaller products may have arisen due to the premature termination of protein synthesis at 'pause sites' within the fSCF coding sequence or from proteolytic degradation of the fusion protein by *E.coli* proteases. Following thrombin cleavage of the fusion protein, a major product of approximately 18 kDa is seen (Figure 4.2). This is the expected molecular weight of the expressed frSCF protein and its identity is confirmed by immunoblotting (Figure 4.4).

Typically, the **total** yield of protein, following affinity chromatography, was 1.1 - 1.4 mg/L of bacterial culture (estimated by Bradford assay, using BSA as a standard). However, the purity of rSCF-GST, as estimated by SDS-PAGE (Figure 4.2), was generally only 35 - 50%. Following cleavage of the GST moiety, the yield of frSCF

was estimated as 0.35 - 0.5 mg/L bacterial culture, with a purity of 60 - 70%. However, the estimates of yield are based on the use of BSA as a standard for the Bradford assay; this results in an approximate two-fold greater absorbance (A_{595}) than seen with pure frSCF and therefore underestimates the quantity of frSCF by a similar factor (Appendix A3). The yield of frSCF may therefore be estimated as 0.7 - 1.0 mg/L bacterial culture.

Levels of endotoxin contamination in all samples of frSCF protein solution submitted for LAL endotoxin assay were above the limits of the assay (reported as 'fail').

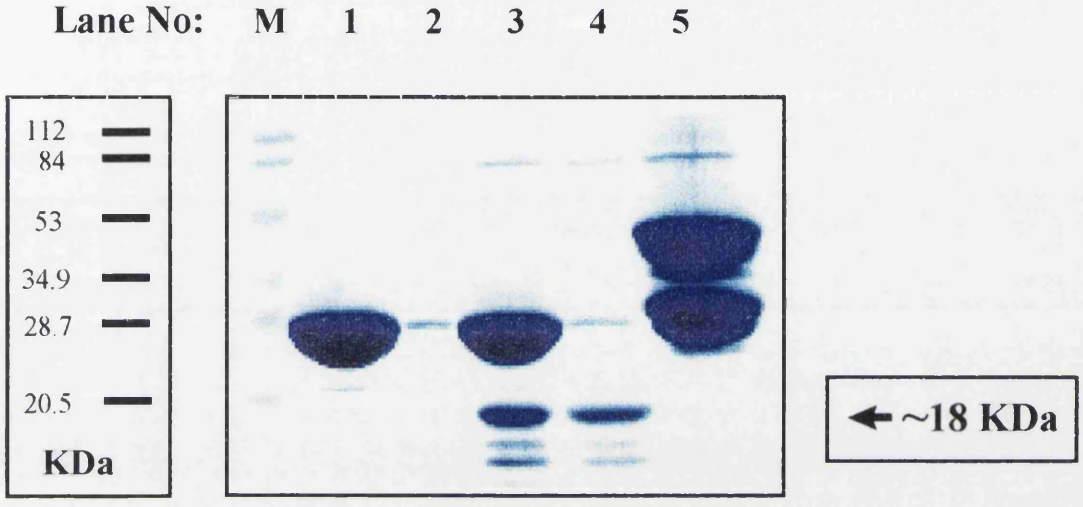


Figure 4.2: SDS-PAGE of recombinant feline stem cell factor proteins. Lane 1 - purified GST protein (2 µl); lane 2 - GST solution following affinity purification over glutathione sepharose 4B (10 µl); lane 3 - frSCF following thrombin cleavage **prior** to affinity purification (10 µl); lane 4 - frSCF following thrombin cleavage and affinity purification (10 µl); lane 5 - frSCF-GST fusion protein (10 µl); M - molecular weight markers.

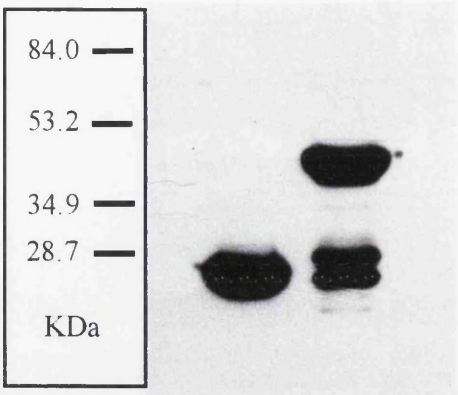


Figure 4.3: Identification of GST-fusion proteins expressed in *E.coli*. Proteins were detected using a murine anti-GST monoclonal antibody in conjunction with an HRP conjugated sheep anti-mouse IgG polyclonal antibody. The western blot was developed using ECL detection reagents. recombinant fSCF-GST fusion protein post affinity purification (10 µl); purified GST (two microlitres).

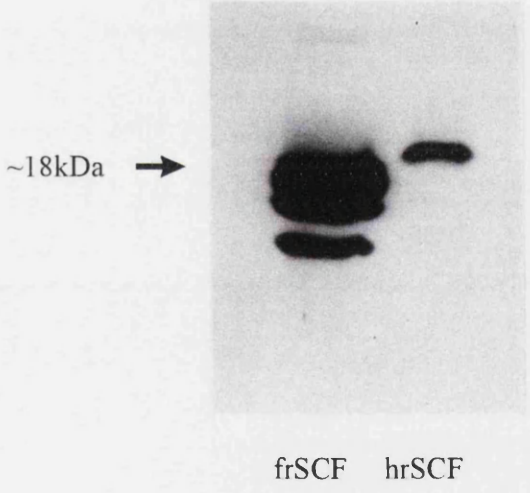


Figure 4.4: Identification of recombinant feline SCF. Proteins were detected using a primary antibody of anti-human SCF goat polyclonal IgG in conjunction with an HRP conjugated rabbit anti-goat IgG. The western blot was developed using ECL detection reagents. frSCF following thrombin cleavage - approximately two micrograms; hrSCF (*E.coli* expressed) - 25 ng.

4.4 DISCUSSION

The production of feline stem cell factor as a GST fusion protein was reasonably successful, with good yield of the protein achieved. Although there was minimal contamination of the purified product by *E.coli* proteins, there was significant contamination with truncated and/or degraded SCF-GST fusion protein. The reduction of these contaminants could greatly improve both the yield and purity of expressed frSCF. Techniques which can be used to reduce the proteolytic degradation of fusion proteins include the use of alternative host strains which are protease-deficient, addition of protease inhibitors to the lysis buffer and reducing the induction period following addition of IPTG. The *E.coli* host strain used in this project for protein expression was BL21, this strain lacks the outer membrane protease (*ompT*) which tends to prevent cleavage at exposed basic residues (Grodberg and Dunn, 1988). Alternative strains which could be considered in any future attempts to optimise expression of fSCF include *lon-* strains or *lon htp*R double mutants (Baker *et al.*, 1984). The serine protease inhibitor PMSF was routinely used in this project, although a reduction in protein degradation with its use was not consistently demonstrated. Other protease inhibitors which could be used include EDTA, aprotinine and benzamidine. Recommended induction periods, following addition of IPTG, vary from two to seven hours (GST Gene Fusion System Manual, 1994; Smith and Corcoran, 1994); the induction period used in this project was 90 - 120 minutes, this was chosen in an attempt to minimise proteolytic digestion of the fusion protein and also because it allowed sufficient time to purify the fusion protein by affinity chromatography on the same day.

In order to minimise the premature termination of protein synthesis, codons rarely used by *E.coli* should be avoided. One example of such a rare codon is AUA, encoding isoleucine. The feline soluble SCF coding sequence has four of these codons (I^{28}, I^{45}, I^{82} and I^{76} of the mature protein). Use of such rare codons can lead to termination of protein synthesis or errors in translation including amino acid substitutions or frame-shifts. These errors arise due to a corresponding rarity of the required tRNA molecules leading to a so-called 'hungry codon syndrome' (Kurland and Gallant, 1996).

The yield of fusion proteins expressed in *E.coli* can also be markedly affected by the protein's solubility. Production of insoluble products will lead to their loss as insoluble bacterial debris or require the use of resolubilisation techniques to harvest the protein. Cultures of frSCF expressing bacteria were grown at 30°C, decreased to 25°C following induction, as lower growth temperatures have been shown to increase the solubility of mammalian protein expressed in *E.coli* (Shein and Noteborn, 1988).

The protocol used gave sufficiently high yields and purity that further optimisation, which may have taken considerable time, was not considered appropriate. Should, however this expression system be used in the future to produce fSCF on a larger scale, optimisation would be warranted; the optimum conditions for large scale production may vary quite considerably from those used here. The endotoxin contamination of the protein solution was, however unacceptably high, such that further purification was deemed necessary prior to extensive evaluation of the biological activity of the protein. This is discussed further within the following chapter.

CHAPTER FIVE - PURIFICATION OF FELINE RECOMBINANT STEM CELL FACTOR

5.1 INTRODUCTION

The single-step purification of frSCF by affinity chromatography resulted in a product that was significantly contaminated both with protein and endotoxin, such that further purification was needed. The aims of purification were to remove endotoxin and contaminating proteins such as residual cleaved GST, truncated fSCF and native *E.coli* proteins, whilst minimising any reduction in activity of or loss of frSCF protein.

Endotoxins are derived from components of the cell wall of gram-negative bacteria and consist of lipopolysaccharide and variable amounts of protein and lipid. The biological effects of endotoxin are numerous and include activation of complement and coagulation pathways and modulation of the activity of platelets, neutrophils, monocyte/macrophages and endothelial cells (Morrison and Ulevitch, 1978). The importance of reducing the endotoxin load within recombinant cytokine preparations is clearly illustrated by reference to early preparations of erythropoietin (EPO). All widely available preparations of EPO were found to contain significant concentrations of endotoxin, ranging from 1.0 - 7,692 ng/U EPO. Consequently the *in vivo* effects of some EPO preparations included marked stimulation of marrow granulopoiesis but no increase (or even a decrease) in marrow erythropoiesis. Following endotoxin removal, EPO administration increased marrow erythropoiesis, with no stimulation of marrow granulopoiesis, in accord with the effects of stimulation of endogenous EPO production, by haemolysis or phlebotomy (Zuckerman *et al.*, 1979).

An overview of the purification procedure is shown in Figure 5.1. The diagram shows the quantity of frSCF solution at each stage of the purification procedure and indicates the number of chromatography runs performed.

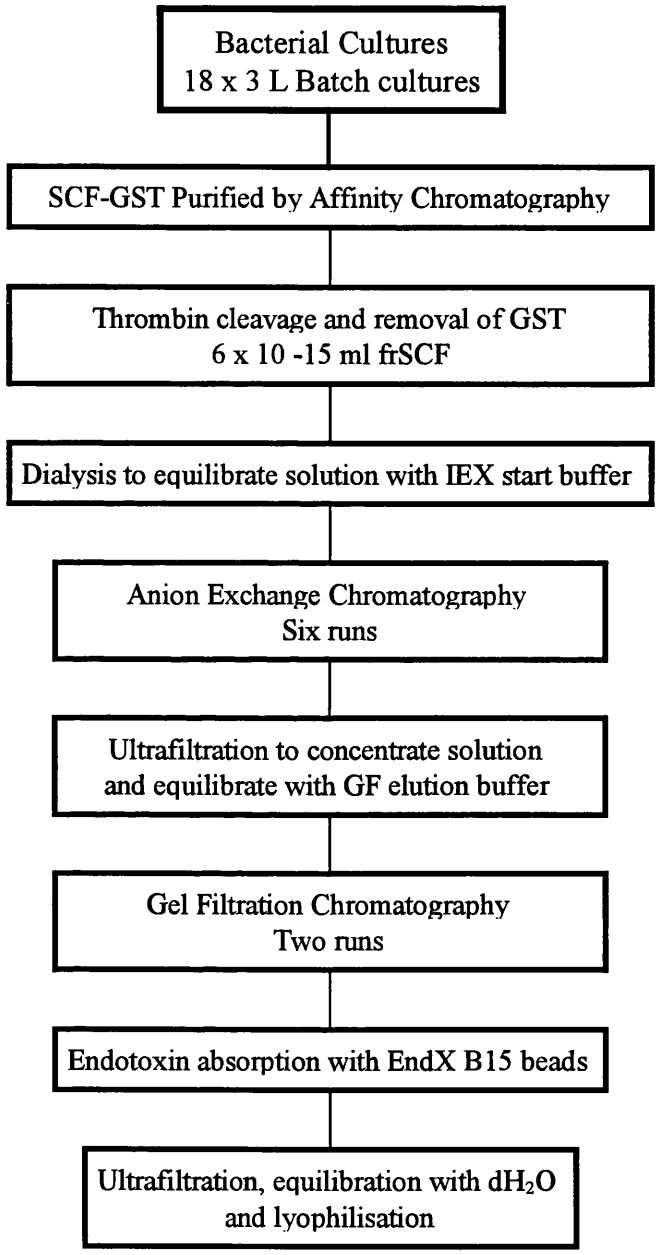


Figure 5.1: Flow diagram showing the steps used in the purification of feline recombinant stem cell factor. The number of times each major purification step was performed is also shown. IEX = ion exchange; GF = gel filtration.

5.2 MATERIALS AND METHODS

Feline recombinant SCF was purified by sequential anion exchange and gel filtration chromatography. This was carried out using a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia Biotech) which allowed automation of the majority of the procedure, thus maximising accuracy and consistency. All buffer solutions were filtered and degassed using a 0.22 μm bottle top filter (Sigma). Protein samples (and solvents) were filtered through a 0.22 μm syringe filter (Gelman Sciences) prior to loading onto a column.

5.2.1 ANION EXCHANGE CHROMATOGRAPHY

Ion exchange (IEX) chromatography is frequently used in the purification of charged biomolecules including proteins and nucleic acids, and is included in up to 75% of purification protocols (Bonnerjea *et al.*, 1986). Separation of charged substances depends upon their differential interaction, with immobilised ion exchange groups of opposite charge, due to differences in their charges and charge densities. These interactions can be modified by controlling conditions such as pH or ionic strength of the solute. Positively charged exchangers associate with negatively charged counter-ions and are thus termed anion exchangers. For the purification of frSCF a Mono Q HR 5/5 anion exchange column (Pharmacia Biotech) was used. This column consists of 10 μm polyether beads with substituted quaternary amine groups (hence 'Q') packed into one millilitre bed volume (Pharmacia Biotech manual, 1994). The conditions used for anion exchange chromatography were modified from those used for the purification of murine SCF from buffalo rat liver culture supernatant (Zsebo *et al.*, 1990b).

Prior to IEX chromatography the recombinant protein solution was equilibrated with start buffer (20 mM Tris-HCl pH 8.0) by dialysis. SCF solution was loaded in 15 ml aliquots into a 15 ml dialysis cassette (Slide-A-Lyzer - Pierce & Warriner UK Ltd., Chester, UK) and dialysed for 12 - 16 hours against 5 L start buffer, with stirring, at 4 °C (buffer was changed three times during this period).

The liquid chromatography controller LCC-500 (Pharmacia Biotech) was programmed as detailed in Appendix A2, providing automation of the purification procedure. The sample was loaded onto a Mono Q HR 5/5 column previously equilibrated with start buffer. Column loading of 7.5 - 10 ml of solution (containing less than 10 mg protein) was facilitated using a 50 ml Superloop (Pharmacia Biotech). The column was then washed with 30 ml start buffer. SCF was eluted in a linear gradient of 0 - 350 mM NaCl (20 mM Tris-HCl pH 8.0) at a flow rate of 1 ml/min; the gradient volume was 30 ml. The eluate was collected automatically using a FRAC-100 fraction collector (Pharmacia Biotech). This was programmed to discard the first 10 ml of eluate and thereafter collect the eluate in 0.5 ml fractions. The eluted protein was detected by measurement of its absorption at 280 nm. The elution profile was recorded automatically by a chart recorder (REC-482, Pharmacia Biotech), with sensitivity set at 2.0 absorbance units full scale deflection (AUFS).

Following each run the column was cleaned by equilibration with 1 M NaCl 20 mM TrisHCl (pH 8.0), until the baseline deflection shown by the chart recorder had stabilised. After every third run the column was cleaned as recommended by the manufacturers. This was carried out by reversing buffer flow through the column and applying start buffer at a flow rate of 0.25 ml/min. The column was washed by sequentially applying 0.5 ml of 2 M NaCl, 0.5 ml of 2 M NaOH and 0.5 ml 75% acetic acid. The column was thoroughly washed with start buffer after the application of each solvent until the baseline of the chart recorder had stabilised. Prior to use the column was washed with 10 ml 1 M NaCl 20 mM TrisHCl (pH 8.0) to change counter ions and then equilibrated with start buffer.

The identity and purity of frSCF in the peak fractions was checked by SDS-PAGE following each chromatography run. Fractions composed principally of frSCF were pooled and stored at - 70°C prior to confirmation of their bioactivity (see chapter six) and further purification.

5.2.2 GEL FILTRATION CHROMATOGRAPHY

Gel filtration (or size exclusion) chromatography separates molecules based on differences in their molecular size. The chromatography column is formed by spherical beads which contain pores of a specific size distribution. Smaller molecules enter the pores of the gel matrix and their flow is retarded, whilst larger molecules do not enter the pores and thus pass rapidly through the column (Porath and Flodin, 1959). To purify frSCF a HiLoad 16/60 column containing Superdex 75 (prep grade) was used (Pharmacia Biotech). Superdex is a composite gel which comprises cross-linked porous agarose beads to which dextran is covalently bonded. Superdex 75 has a fractionation range for globular proteins of 3 - 70 kDa.

The column was washed with one bed volume (120 ml) elution buffer (0.15 M NaCl, 12.3 mM KH_2PO_4, 37.7 mM Na_2HPO_4, pH 7.0) to remove storage solution (20% ethanol), at a flow rate of 0.75 ml/min. The column was then equilibrated with two bed volumes of elution buffer at a flow rate of one millilitre per minute. The operation of the column was checked by running one millilitre of a sample containing the proteins cytochrome c (1.0 mg/ml) and Bovine serum albumin (5.0 mg/ml) (both supplied by Sigma Chemical Co.) at a flow rate of one millilitre per minute. The eluted protein was detected by measurement of its absorption at 280 nm. The elution profile was recorded automatically by a chart recorder, with sensitivity set at 2.0 AUFS.

The resolution of gel filtration chromatography is affected by the sample volume applied. A maximal volume of 0.5 - 4.0% of bed volume (0.6 - 4.2 ml) is recommended for the HiLoad 16/60 Superdex 75 column. To minimise the number of chromatography runs required, the frSCF protein solution was concentrated by ultrafiltration using a Centriplus 10 centrifugal concentrator (Amicon Inc., MA) in accordance with the manufacturers instructions. This also enabled the solution to be equilibrated with the elution buffer. The purification of frSCF was carried out in a similar manner to the calibration procedure, with the liquid chromatography controller LCC-500 programmed as detailed in Appendix A2. The column was equilibrated with elution buffer prior to use. The recombinant protein (1.2 ml sample containing

less than 10 mg protein) was loaded using a two millilitre sample loop. The protein was then eluted at a flow rate of one millilitre per minute and the elution monitored as above. The eluate was collected automatically using a FRAC-100 fraction collector which was programmed to discard the first 40 ml of eluate and thereafter collect the eluate in 1.0 ml fractions. The identity and purity of frSCF in the peak fractions was checked by SDS-PAGE. Fractions composed principally of frSCF were pooled and stored at 4°C prior to confirmation of their bioactivity (see chapter six) and further purification.

5.2.3 ENDOTOXIN REMOVAL

The further reduction in the endotoxin load of recombinant protein solution was achieved by use of a commercial endotoxin removal device (End-X B15 - Associates of Cape Cod, Woods Hole, MA). This device consists of endotoxin neutralising protein (ENP) immobilised on glass microspheres, contained within a microcentrifuge tube. ENP is derived from *Limulus polyphemus* amoebocytes and specifically binds endotoxin thus allowing its removal from solution. The minimal capacity of a single 1.8 ml End-X B15 tube is stated as 50 ng endotoxin.

Each aliquot of frSCF solution, prepared as described above, was mixed in a 15 ml Falcon tube with the beads taken from two EndX B15 tubes. The tube was incubated overnight at 4°C with gentle rotation. The tube was then centrifuged at 3,300 x g for four minutes to pellet the resin. The supernatant was removed and stored at 4°C prior to final processing.

5.2.4 LYOPHILISATION

In order to accurately quantify the amount of frSCF produced the protein was freeze dried. Prior to lyophilisation the protein solution was equilibrated with tissue culture grade dH_2O (Gibco Life Technologies, Paisley, UK) using a Centriplus 10 centrifugal concentrator (Amicon Inc., MA) in accordance with the manufacturers instructions. The solution was then transferred in two aliquots to preweighed microcentrifuge tubes, frozen in dry ice and lyophilised overnight in an Edwards freeze dryer

Modulyo. The microcentrifuge tubes were then re-weighed to quantify the yield of fSCF.

5.3 RESULTS

5.3.1 ANION EXCHANGE CHROMATOGRAPHY

The elution profile from a typical chromatography run is shown in Figure 5.2. By comparison with SDS-PAGE of the collected fractions (Figure 5.3) it can be seen that frSCF is the first major peak to be eluted from the column. The protein was generally eluted between 160 - 250 mM NaCl, which varied between runs. Contaminating proteins, including GST, were eluted mainly in later fractions.

5.3.2 GEL FILTRATION CHROMATOGRAPHY

An example of the elution profile seen during calibration of the gel filtration column using albumin and cytochrome c is shown in Figure 5.4. The elution profile of fSCF is shown in Figure 5.5. This can be compared to fractions analysed by SDS-PAGE (Figure 5.6). It can be seen that fSCF has an elution volume (V_e) of approximately 59 ml. Although the molecular weight of frSCF cannot be accurately determined as the void volume of the column is not known, comparison with the elution volumes of albumin and cytochrome c suggests that fSCF elutes in a lower volume than would be expected for its molecular weight of approximately 18 kDa. This implies that, like SCF in other species, frSCF exists in solution as a dimer under non-denaturing conditions. This unfortunately limits the separation of fSCF by gel filtration chromatography, from the major contaminating protein, GST, as can be seen in Figure 5.6, due to their similar size under non-denaturing conditions.

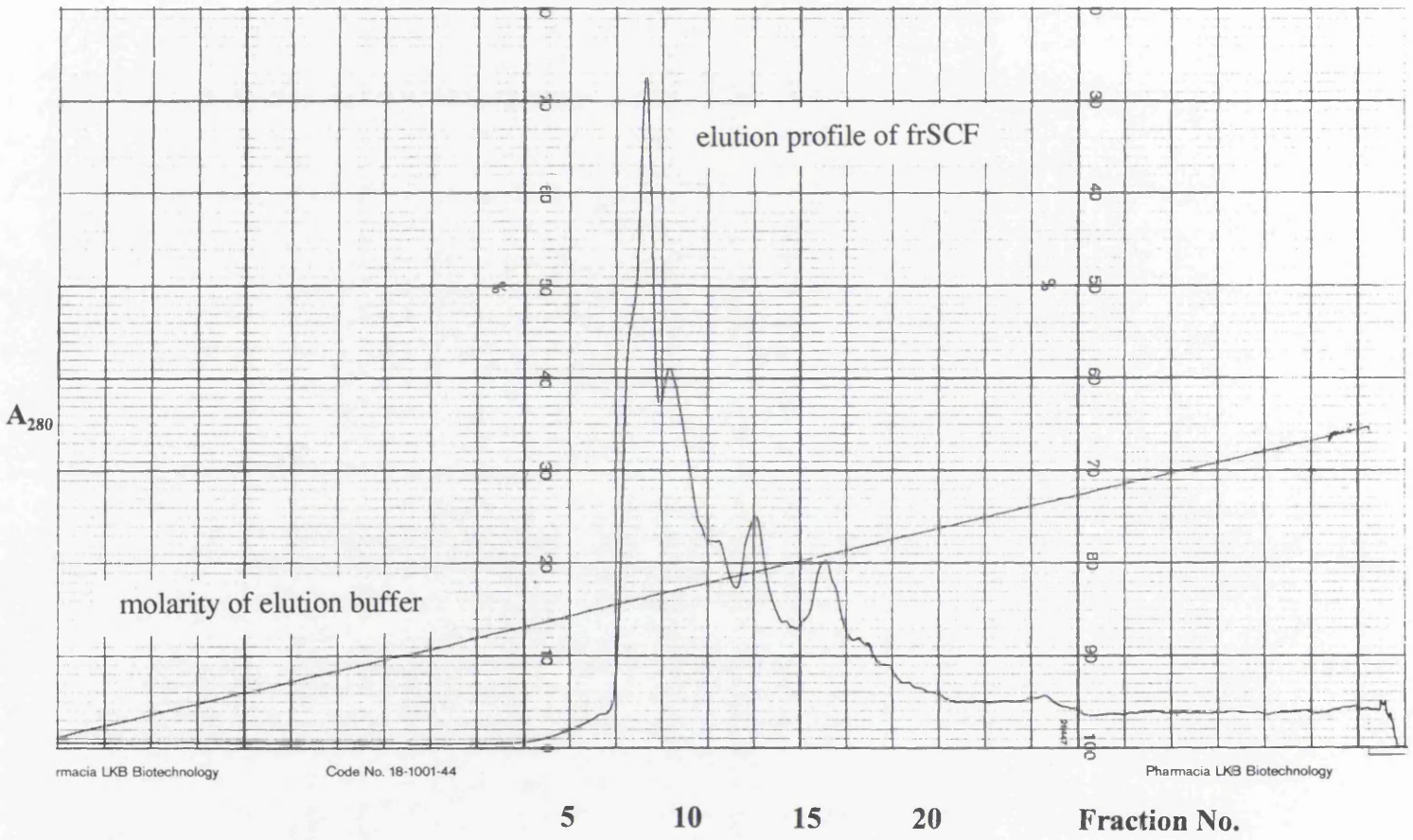


Figure 5.2: Elution profile of fSCF seen with anion exchange chromatography. The curved trace depicts the A_{280} of the sample (AUFS = 2.0) whilst the straight line shows the increasing molarity of NaCl in the elution buffer (10% = 100 mM). Feline SCF corresponds to the first major peak, eluted between 160 - 170 mM mM NaCl, in this example. Flow rate = 1.0 ml/min; chart speed = 1.0 cm/min.

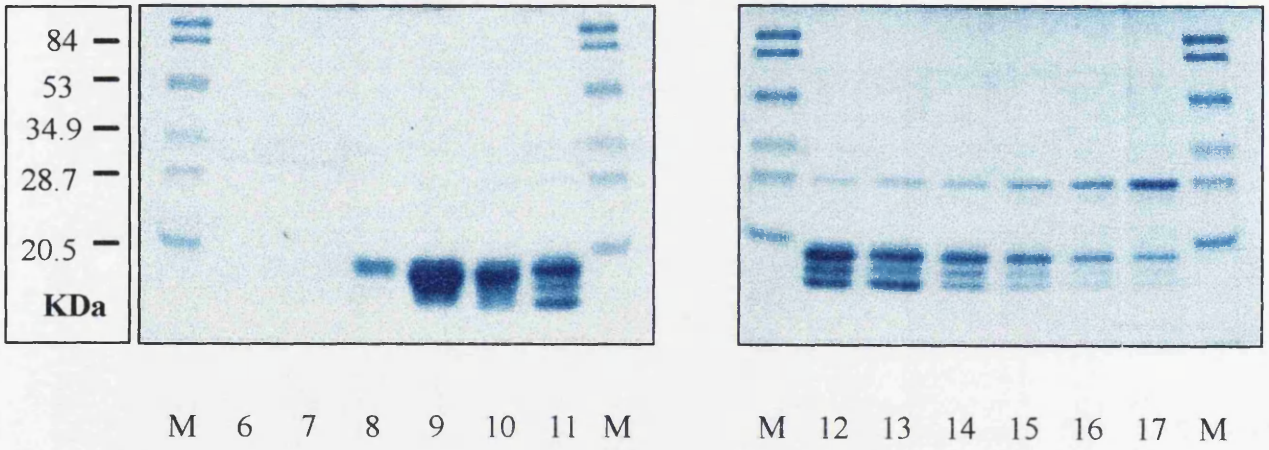
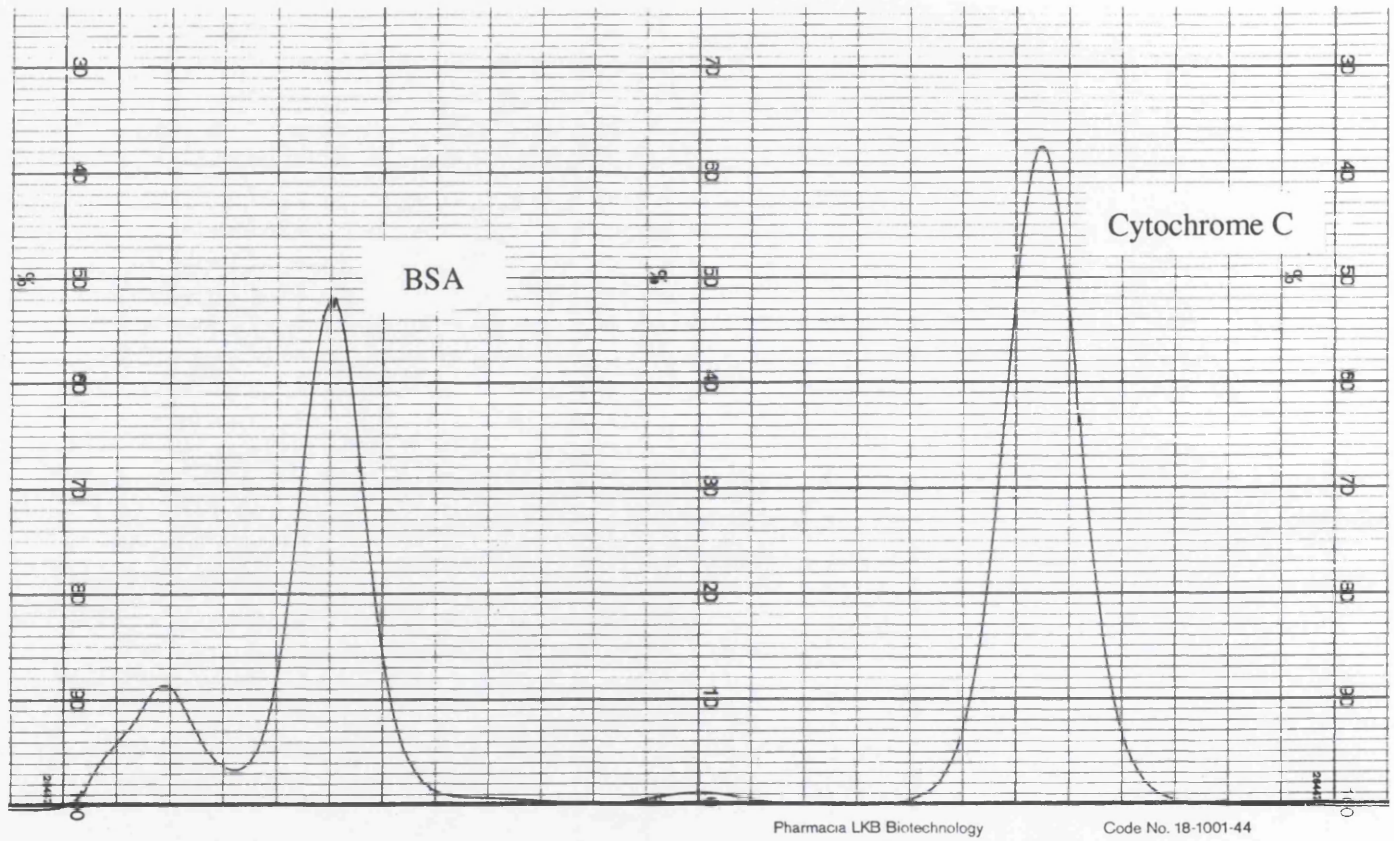


Figure 5.3: SDS-PAGE of eluted fractions obtained following anion exchange purification of feline recombinant stem cell factor. The fraction number is shown under the respective lane; five microlitres of solution were loaded into each lane. In this example, fraction numbers 8 - 10 were pooled and retained for further purification, whilst the remaining fractions were discarded. M = molecular weight markers.



A_{280}
(10% = 0.1 AU)

Figure 5.4: Calibration of HiLoad 16/60 gel filtration chromatography column with the proteins bovine serum albumin and cytochrome c. BSA (66 kDa) is eluted in a volume of 55 ml, whilst cytochrome c (12.4 kDa) is eluted in 82 ml. Flow rate = 1.0 ml/min; chart speed = 0.5 ml/min.

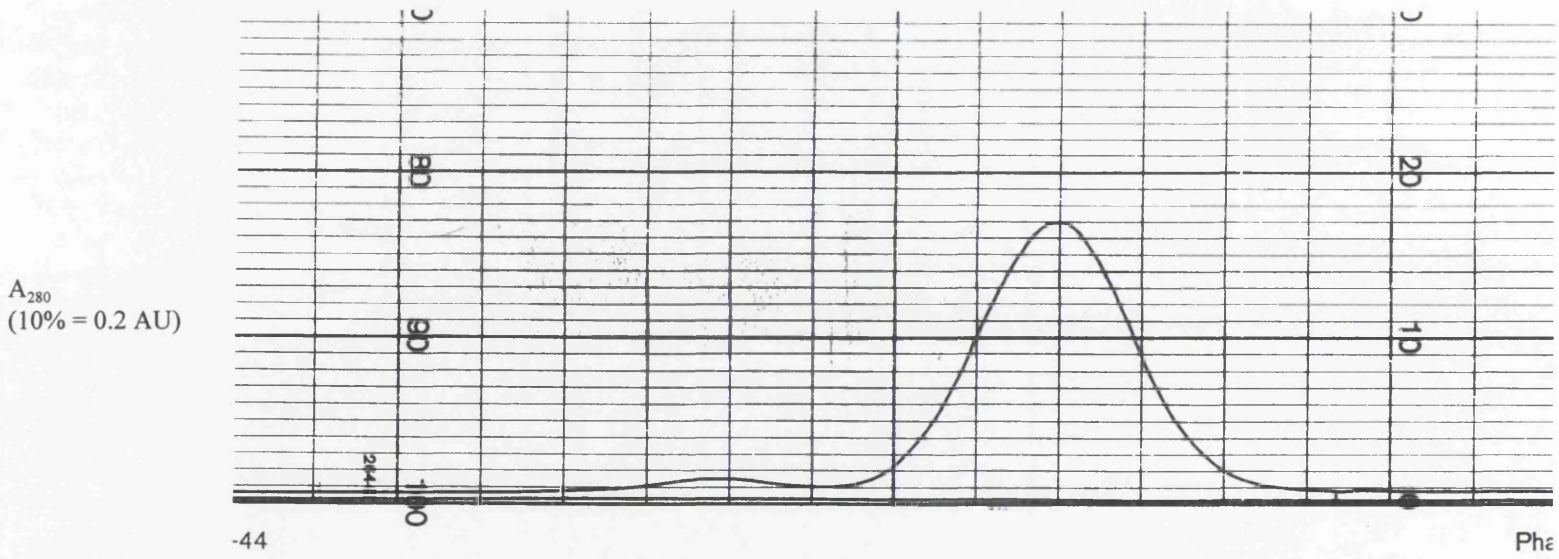


Figure 5.5: Elution profile of frSCF with gel filtration chromatography using a HiLoad 16/60 column. Peak elution occurs at a volume of 59 ml. Flow rate = 1.0 ml/min; chart speed = 0.5 cm/min.

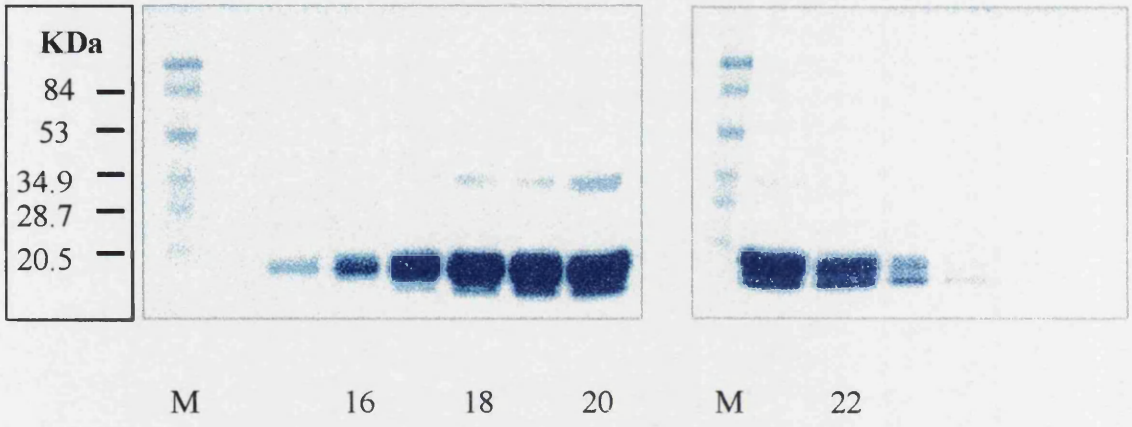


Figure 5.6: SDS-PAGE of eluted fractions obtained following gel filtration chromatography of feline recombinant stem cell factor. The fraction number is shown under the respective lane; two microlitres of solution were loaded into each lane. . In this example, fraction numbers 15 - 19 and 20 - 22 were retained and their bioactivity evaluated (see chapter six). M = molecular weight markers.

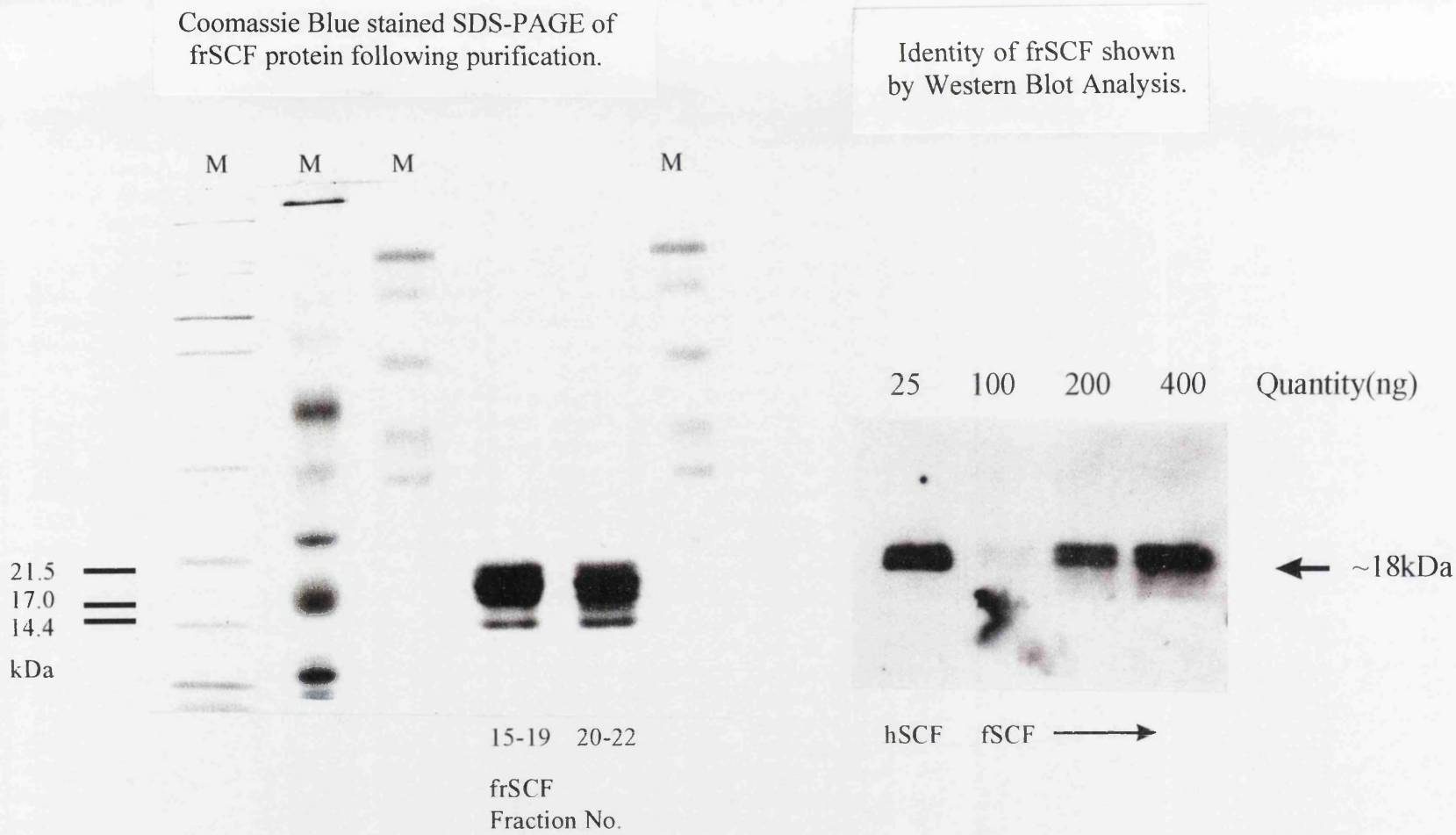


Figure 5.7: SDS-PAGE and immunoblot of frSCF following complete purification protocol as detailed in the text. Ten micrograms of protein was loaded in each lane on the SDS-PAGE gel; these fractions were subsequently pooled after confirmation of their bioactivity.. The immunoblot was performed as described in chapter four. Proteins were detected using a primary antibody of anti-human SCF goat polyclonal IgG in conjunction with an HRP conjugated rabbit anti-goat IgG. The western blot was developed using ECL detection reagents. Lane 1 - hrSCF (*E.coli* expressed) (25 ng); lanes 2 - 4, frSCF (100, 200, 400 ng respectively). M = molecular weight markers.

5.3.3 YIELD AND PURITY OF RECOMBINANT PROTEIN

Figure 5.7 shows the identity and purity of frSCF following the final stage of purification. The approximate yields and estimated purity of frSCF after each major purification step is shown in Table 5.1. The final yield of protein from an original volume of 56 L of bacterial culture was 13 mg, determined by direct measurement of dry weight.

Purification Step	*Yield Protein*	*Estimated Purity* [c]	*Endotoxin* (EU/mg frSCF)
Affinity Chromatography	40 - 56 mg [a]	60 - 70%	FAIL
IEX Chromatography	23 mg [b]	85 - 90%	2.0
GF Chromatography	ND	~90%	3.1
EndX B15 Beads	ND	~90%	2.0
Lyophilised protein	13 mg	~90%	ND

ND Not determined.
a Estimated by Bradford assay then corrected for frSCF protein by multiplying by a factor of two. This assay used BSA as a standard, which was found to produce approximately 2-fold greater colour change than seen with purified frSCF.
b Estimated by SDS-PAGE in comparison to known quantity of purified frSCF.
c Estimated by SDS-PAGE.

Table 5.1: Purification of frSCF. Approximate yield and purity following each purification step is given. Yields refer to the total amount of protein obtained at each stage; these were obtained from 54 L (18 x 3 L batches) of bacterial culture.

5.4 DISCUSSION

Although the yields at each stage were measured in different ways and are only approximate it can be seen that major losses of protein occurred during the purification procedure. The loss of protein following anion exchange chromatography occurred mainly due to the limitations in resolution obtained with the procedure, which is evident in Figure 5.3 (i.e. fractions containing frSCF were discarded because of protein contamination). A loss of approximately 40% of the yield was in the latter stages of purification; this may have been due to losses during ultrafiltration or GF chromatography. It is also apparent that the GF chromatography step did little to increase the purity or reduce the endotoxin concentration of the purified protein.

Anion exchange chromatography significant improved both parameters, although the efficacy of each procedure *per se* cannot be directly compared as the IEX step was performed first. As mentioned above, though, GF chromatography is unable to effectively separate frSCF from the major contaminating species (GST) due to their similar molecular weights under non-denaturing conditions. Future experiments would benefit from attempts to improve the separation efficiency of IEX chromatography, which might be achieved by the use of a different elution gradient. Additionally the use of a larger chromatography column would reduce the number of separate runs required. It is possible that the GF chromatography step may be dispensed with in future experiments. Although the final preparation of fSCF has not been purified to homogeneity, the major contaminants are likely to be truncated fSCF proteins which may possess biological activity. The analysis of variant soluble forms of hSCF expressed in *E.coli* showed that whilst loss of N-terminal amino acids 1 - 5 abrogated biological activity, loss of the C-terminal amino acids 142 - 165 did not (Langley *et al.*, 1994).

The endotoxin content of the recombinant protein solution was effectively reduced by the purification procedure. The minimum pyrogenic dose for man, given intravenously, is 4 - 8 EU per kilogram, for rabbits the figure is 10 - 15 EU/kg (Hochstein, 1987). The maximum dose of frSCF that was expected to be given to cats was 200 μg/kg (see chapter eight); cats receiving this dose would therefore be given 0.4 EU/kg which is well within the above limits. Additionally the endotoxin content of the purified frSCF is comparable to that reported for rSCFs given *in vivo* to primates (0.033 ng/mg; Andrews *et al.*, 1991), mice (2 ng/mg; Bodine *et al.*, 1993) and dogs (0.033 ng/mg; Schuening *et al.*, 1993) (1 ng endotoxin is approximately 10 EU, but this varies depending on the source of endotoxin). Unfortunately the final endotoxin removal step effected little reduction in endotoxin content of the protein solution although the theoretical endotoxin binding capacity of the matrix was not exceeded. In future experiments it may be worth using a new batch of the same product or trying an alternative product to investigate whether this aspect of purification can be improved. Additionally, future experiments would benefit from screening of buffer solutions for endotoxin content prior to use. This was not

routinely performed during this experiment and thus endotoxin contamination of buffer solutions cannot be ruled out.

CHAPTER SIX - *IN VITRO* BIOLOGICAL ACTIVITY OF FELINE RECOMBINANT STEM CELL FACTOR

6.1 INTRODUCTION

6.1.1 *IN VITRO* ASSAYS FOR STEM CELL FACTOR

The ultimate aim of this project was to produce feline SCF as a recombinant protein that could be used both in the study of feline haemopoiesis *in vitro* and as a novel therapeutic agent in this species. As previously discussed in chapter four, proteins expressed in *E. coli* may have a low biological activity due to aberrant folding or absence of authentic post translational modifications. Thus it was necessary to confirm the bioactivity of the expressed protein prior to further evaluating its effects on haemopoiesis *in vitro* and its potential use as a therapeutic agent *in vivo*.

A number of types of assay have been used to measure cytokine levels in biological fluids and laboratory samples. These may be broadly classified as immunoassays, bioassays or receptor binding assays (Wadhwa *et al.*, 1995). Immunoassays are generally easier and faster to perform. An ELISA for human stem cell factor is available commercially (R & D Systems, Abingdon, Oxon) and is particularly sensitive (3-4 pg/ml). However, immunoassays give no indication of the integrity of biological activity of a recombinant protein as they may detect inactive denatured or fragmented molecules. Due to the specificity of the antibody used in such assays they are generally restricted to use within a single species. A radioreceptor assay has been used to detect murine and human SCF. The assay measures the ability of SCF to competitively bind to the SCF receptor in purified plasma membrane preparations of the human erythroleukaemia cell line, OCIM1. The assay can detect 0.3 - 0.5 ng/ml of SCF, but is unsuitable for the measurement of SCF levels in serum due to the presence of inhibitory substances (Smith and Zsebo, 1993). Whilst highly specific, the radioreceptor assay may also identify SCF molecules that are unable to effect signal transduction.

Bioassays can be performed using primary cell cultures or, more conveniently, continuously growing, cytokine dependent or independent cell lines. Assays may measure the ability of a cytokine to induce cell proliferation, death (cytotoxic assays), chemotaxis, protection from viral lysis or other parameters such as the upregulation of

cell surface proteins. A number of continuously growing cell lines can be used to detect SCF *in vitro*, based on its ability to induce cell proliferation. These include the human megakaryoblastic leukaemia cell line, UT-7 (Miura *et al.*, 1990) and erythroleukaemia cell line, TF-1 (Kitamura *et al.*, 1989) and the murine mast cell line MC/9 (Nabel *et al.*, 1981; Galli *et al.*, 1982). SCF shows some cross reactivity between species, so the use of these cell lines for bioassays is not species restricted. Rat, murine and human SCF are active on human cells *in vitro*, however, human SCF is relatively inactive on mouse cells (Martin *et al.*, 1990; Broxmeyer *et al.*, 1991a). The sensitivity of such bioassays to a given cytokine is expressed in terms of its EC_{50} (effective concentration required for 50% maximal stimulation). Human TF-1 cells show an EC_{50} to hrSCF of 1 - 2 ng/ml and mrSCF of 5 - 10 ng/ml. Murine MC/9 cells show an EC_{50} to mrSCF of 2 - 4 ng/ml but are relatively insensitive to hrSCF, with an EC_{50} of 2 - 3 μg/ml (Martin *et al.*, 1990). Cell line based bioassays are often not cytokine specific, for instance TF-1 cells proliferate in response to IL-3, IL-4, IL-5, EPO and GM-CSF in addition to SCF. However, specificity can be demonstrated by the ability of a specific neutralising antibody to block cytokine-induced cell proliferation (Wadhwa *et al.*, 1995).

Murine MC/9 cells were initially used to assess the bioactivity of frSCF, however TF-1 cells were later used and became the preferred bioassay, as discussed below.

6.1.2 EFFECTS OF STEM CELL FACTOR ON HAEMOPOIESIS *IN VITRO*

The earliest bioassays devised to study colony stimulating factors used their ability to generate morphologically recognisable colonies of haemopoietic cells in semi-solid media (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966). The colonies generated in such assays consist of clones of cells derived from single progenitor cells (colony forming cells) (Bradley and Metcalf, 1966). Such assays are poorly suited to the accurate determination of cytokine levels but provide a powerful tool to study the effects of growth factors on their normal target cells. Furthermore, by using purified populations of progenitor cells in serum-free cultures, clonal assays may be used to investigate the direct and indirect actions of cytokines in haemopoiesis (Testa *et al.*, 1995).

The effects of SCF on haemopoiesis *in vitro* have been discussed in chapter one. As a single agent, SCF shows limited stimulation of haemopoiesis *in vitro,* but in synergy with other cytokines supports the growth of progenitor cells of most lineages. There have been only limited studies on feline haemopoiesis *in vitro*, the majority of these have centred around the effects of feline immunodeficiency virus or feline leukaemia virus infection on haemopoiesis. These studies have used a variety of experimental protocols including long term marrow cultures and erythroid (BFU-E) and granulocyte macrophage (CFU-GM) colony forming cell assays (Testa *et al.,* 1983; Rojko *et al.,* 1986; Linenberger and Abkowitz, 1992; Linenberger *et al.,* 1995). The use of clonal assays in the cat is somewhat limited by the lack of recombinant colony stimulating factors (CSFs) with demonstrable activity on feline haemopoietic cells. Conditioned medium provides a useful source of such CSFs, but is uncharacterised and can suffer from batch to batch variability. Its use therefore introduces further inconsistency into an assay which is already prone to inter-assay variability (Grant, 1995). The choice of assay and protocol for the investigation of the activity of frSCF on the development of feline bone marrow cells *in vitro* was based on the studies of Dr. S. Grant, performed within the Department of Veterinary Pathology, University of Glasgow. The GM-CFC assay described herein therefore used an established protocol and previously batch tested reagents. Human granulocyte colony stimulating factor (hGCSF) and a single batch of feline phytohaemagglutinin lymphocyte conditioned medium (fPHA-CM) were used as a sources of colony stimulating activity. PHA-CM provides a cocktail of growth factors which likely includes IL-1, G-CSF, and GM-CSF (Coutinho *et al.,* 1993).

6.2 MATERIALS AND METHODS

6.2.1 MC/9 CELL PROLIFERATION ASSAY

The murine mast cell line MC/9 was maintained as described in section 2.2.1.3. Prior to performing an assay, the cells were deprived of conditioned medium for 24 hours in order to reduce background levels of cell proliferation. This was achieved by washing the cells three times with sterile PBS, followed by centrifugation at 250 x g for 10 minutes. Viable cells were counted as detailed in section 2.2.1.1.2 using trypan blue

exclusion and the cells resuspended to 4 x 10^5 cells/ml in complete DMEM-10 (DMEM with 10% FBS, 2 mM L-glutamine, 50 μM 2-ME, 1% NEAA, 100 iu/ml penicillin, 100 μg/ml streptomycin, and additional 32 mg/L L-arginine, 36 mg/L L-asparagine and 2 mg/L folic acid).

The cell suspension was prepared for an assay as follows. Cells were pelleted by centrifugation at 250 x g for 10 minutes. Viable cells were counted using trypan blue exclusion and the cells resuspended to 4 x 10^4 cells/ml in RPMI-1640 medium containing 4% FBS, 2 mM L-glutamine, 100 iu/ml penicillin and 100 μg/ml streptomycin.

Assays were prepared in 96 well tissue culture plates (Costar, Cambridge, MA). All samples were assayed in triplicate. Human stem cell factor was used as a standard with serial two fold dilutions prepared starting from 200 ng/ml. Feline stem cell factor was diluted similarly; preliminary assays used a starting concentration of approximately 10 μg/ml. A solution prepared from lysates of GST expressing cells, and purified in an identical manner to frSCF, was used as a negative control. Dilutions were performed directly within the 96 well plate with each dilution contained within 100 μl volume. One hundred microlitres of medium was added to three wells (to determine background levels of cell proliferation). One hundred microlitres of washed cells was then aliquoted to each well. Two hundred microlitres of medium was added to each of three wells as a negative control. The plates were incubated for 48 hours, at 37°C, in humidified CO_2 incubator.

A solution of tritiated thymidine was prepared in a sterile universal by adding 50 μl [^{3}H]-thymidine to 2.5 ml of culture medium. Fifty microlitres of this solution (containing 0.5 μCi [^{3}H]-thymidine) was then aliquoted to each well and the plates incubated for a further four hours. The contents of each well were harvested onto microplate filters (Unifilter-96 - Packard Instrument Co. Inc., Meriden, CT) using a cell harvester (Filtermate 196 - Packard Instrument Co. Inc.). The filters were then dried in an oven at 60°C for 30 - 60 minutes. The underside of the filters were sealed with adhesive film and 25 μl of scintillant (Microscint O - Packard Instrument Co.

Inc.) added to each well. The top of the plate was sealed using adhesive film (Top Seal A - Packard Instrument Co. Inc.) and liquid scintillation counting performed using a microplate scintillation counter (Parkard Top Counter).

6.2.2 TF-1 CELL PROLIFERATION ASSAY

TF-1 cells were maintained as described in section 2.2.1.4. Cells were taken two to three days after passaging and washed three times in RPMI-1640. The washed cells were counted, cell viability assessed by trypan blue exclusion and the cells resuspended to a final concentration of 1 x 10^5 cells/ml in RPMI-5 (RPMI-1640 containing 5% FBS, 2 mM glutamine, 100 iu/ml penicillin and 100 µg/ml streptomycin).

Recombinant feline stem cell factor was serially diluted in 100 µl volume of RPMI-5. This was performed in triplicate, in 96 well microtitre plates as detailed for the MC/9 assay. Recombinant murine stem cell factor was used as a positive control, with a starting concentration of 100 ng/ml. Demonstration of the specificity of feline stem cell factor activity was achieved by pre-incubating the cytokine with anti-human SCF goat polyclonal IgG neutralising antibody (R & D Europe Ltd., Abingdon, Oxon, UK) at a concentration of 40 µg/ml for one hour. To each well of the plate was added 100 µl of washed cells. Controls were included as detailed for the MC/9 assay. The plates were then incubated for 48 hours at 37°C in humidified CO_2 incubator. Tritiated thymidine was added and incorporated radioactivity determined after four hours incubation, exactly as described for the MC/9 assay.

6.2.3 GRANULOCYTE-MACROPHAGE COLONY FORMING CELL ASSAY

Feline bone marrow mononuclear cells (BMMC) and fPHA-CM were prepared as detailed in sections 2.2.1.7 and 2.2.1.8 respectively. Feline BMMC were resuspended at a concentration of 5 x 10^4 cells/ml in a mixture containing 0.66 ml batch-tested fetal bovine serum (FBS Advanced Protein Products Ltd., Brockmoor, W. Midlands UK), 0.033 ml batch-tested bovine serum albumin (BSA, Sigma Fraction V, Sigma Chemical Co, St Louis, USA) and IMDM (containing 100 iu/ml penicillin, 100 µg/ml streptomycin and 4 mM L-glutamine) to a total volume of 2.97 ml. Growth factors

were included (as single factors or combination) at the following concentrations: hG-CSF, 20 ng/ml; frSCF, 100 ng/ml; fPHA-CM, 5%. Control cultures were set up omitting all growth factors. An aliquot of 3.3% agar, was then brought to boiling point in a water bath and 0.33 ml added to the cell suspension. The resultant mix was then transferred in one millilitre aliquots to three 35 x 10 mm petri dishes, allowed to set and incubated in humidified conditions at 37°C, 5% CO_2 for 8 days. Unstained colonies comprising over 50 cells were then counted using a Leitz Labovert FS (Leitz, Wetzlar, Germany) inverted microscope at four times magnification.

6.2.4 STATISTICAL ANALYSES

6.2.4.1 TF-1 cell proliferation assay

Data were analysed by two-way analysis of variance with treatment and concentration as fixed effects. The dependent variable was $\log_e(\text{count}+1)$.

6.2.4.2 GM-CFC Assay

Comparison of CFC assay results was performed by a one way analysis of variance using a $\log_e(x+1)$ transformation of the count data. A Newman-Keuls multiple range test was carried out to assess pairwise differences between the treatment group means.

6.3 RESULTS

6.3.1 CELL PROLIFERATION ASSAYS

The response of MC/9 and TF-1 cells to frSCF is shown in Figures 6.1 and 6.2, respectively. Background levels of cell proliferation seen upon growth factor deprivation were low. TF-1 cells showed background levels of thymidine incorporation of 3,173 +/- 155 cpm and MC/9 cells 4,315 +/- 456 cpm (mean +/- standard deviation). Control wells containing no cells showed thymidine incorporation of 100 - 250 cpm for each assay. Each cell line shows a sigmoid dose response curve to frSCF. Below a threshold level of approximately 2 ng/ml for TF-1 cells or 5 ng/ml for MC/9 cells only background levels of cell proliferation are seen.

A linear dose response is seen between approximately 4 to 30 ng/ml for TF-1 cells and 10 to 80 ng/ml for MC/9 cells. Above this limit the dose response curves plateau as cell proliferation reaches maximal levels.

MC/9 cells showed an almost ten-fold greater proliferative response to thrombin cleaved frSCF compared with the SCF-GST fusion protein. This is greater than the two to three fold greater response that would be expected per nanogram, given that the molecular weight of frSCF is approximately 18 kDa and that of SCF-GST is 44 kDa. The difference may be explained in part due to the use of the Bradford assay to measure the protein concentration of the solutions used for the MC/9 assay (SCF concentrations used in the TF-1 assay were assessed by weight). The assay may have underestimated the concentration of frSCF as this protein produces a relatively small change in A_{595} of the Bradford dye compared other proteins (e.g. BSA). However, the presence of the GST tag in the fusion protein may have reduced this tendency to underestimate the protein concentration of the solution. Another possible explanation which may contribute to the observed discrepancy is that the presence of the GST tag reduced the biological activity of the SCF fusion partner.

The use of a negative control in the MC/9 assay was important because the protein solutions used in that assay contained relatively high concentrations of endotoxin as they had not been purified by FPLC. The 'GST' control had been subjected to exactly the same purification steps as the frSCF used in the assay and thus could be expected to contain a similar level of endotoxin contamination. The MC/9 cells show no response to this preparation, thus effectively excluding the possibility that endotoxin contamination affected the proliferation of these cells in response to frSCF.

TF-1 cells respond to frSCF with a similar sensitivity to mrSCF showing an EC_{50} of approximately 10 ng/ml. Murine MC/9 cells are a less sensitive bioassay for frSCF, having an EC_{50} of approximately 40 ng/ml. TF-1 cells show a parallel dose response to frSCF and mrSCF suggesting that the cells are responding in an analogous manner and that other components within the frSCF solution are not responsible for the proliferation seen. The specificity of action of frSCF on TF-1 cells was also shown by

the ability an anti-hSCF neutralising antibody to inhibit the stimulatory effect of frSCF (Figure 6.2). This effect is overcome by increasing concentrations of frSCF due to saturation of the neutralising capacity of the antibody.

6.3.2 GM-CFC ASSAY

The effect of frSCF, alone and in combination with two sources of colony stimulating activity (CSA) on the growth of CFU-GM in agar is shown in Figure 6.3. Growth of colonies in the absence of any growth factors is minimal (5 +/- 3 colonies/10^5 cells). Alone, frSCF simulates the growth of CFU-GM in agar (23 +/- 8 colonies/10^5 cells). Human G-CSF showed little stimulation of colony numbers in the assay shown (9 +/- 6 colonies/10^5 cells), although this was statistically more than seen in the absence of growth factors. Additionally, colonies grown in the presence of hG-CSF tended to be larger than those seen in control plates. Feline PHA-CM was a more effective stimulant of colony formation than hrG-CSF but promoted fewer numbers of colonies than frSCF, at the concentrations used, although these colonies tended to be larger.

The combination of fPHA-CM and SCF resulted in the growth of larger numbers of colonies than with either factor alone. The addition of frSCF to hrG-CSF led to a marginal increase in colony numbers, but this was not statistically significant. When frSCF was combined with either source of CSA the growth of a number of larger colonies was promoted; an effect not seen with either growth factor alone (Figure 6.4). The majority of colonies grown in these assays had morphological characteristics typical of granulocyte, macrophage or granulocyte-macrophage colonies but a number of smaller colonies consisted of a densely packed 'sheet' of larger cells which may have been less differentiated cells.

frSCF increases ^{3}H-thymidine incorporation by murine MC/9 cells

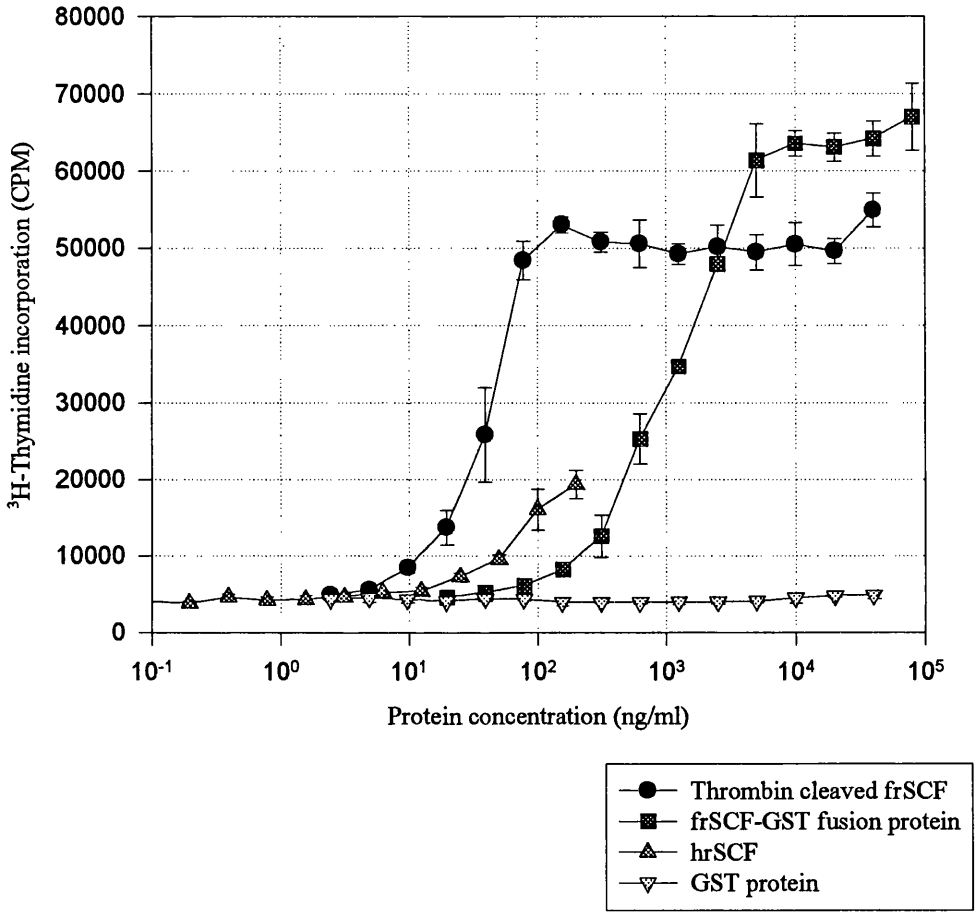


Figure 6.1: Incorporation of ^{3}H-thymidine by murine MC/9 cells in response to both frSCF and fSCF-GST proteins. The concentration of frSCF and fSCF-GST is as shown on the x axis. Data points for the GST protein solution represent the concentration that would have been present assuming the solution had been one of fSCF-GST i.e. equivalent volumes were used. The EC_{50} in response to thrombin cleaved frSCF is approximately 40 ng/ml.

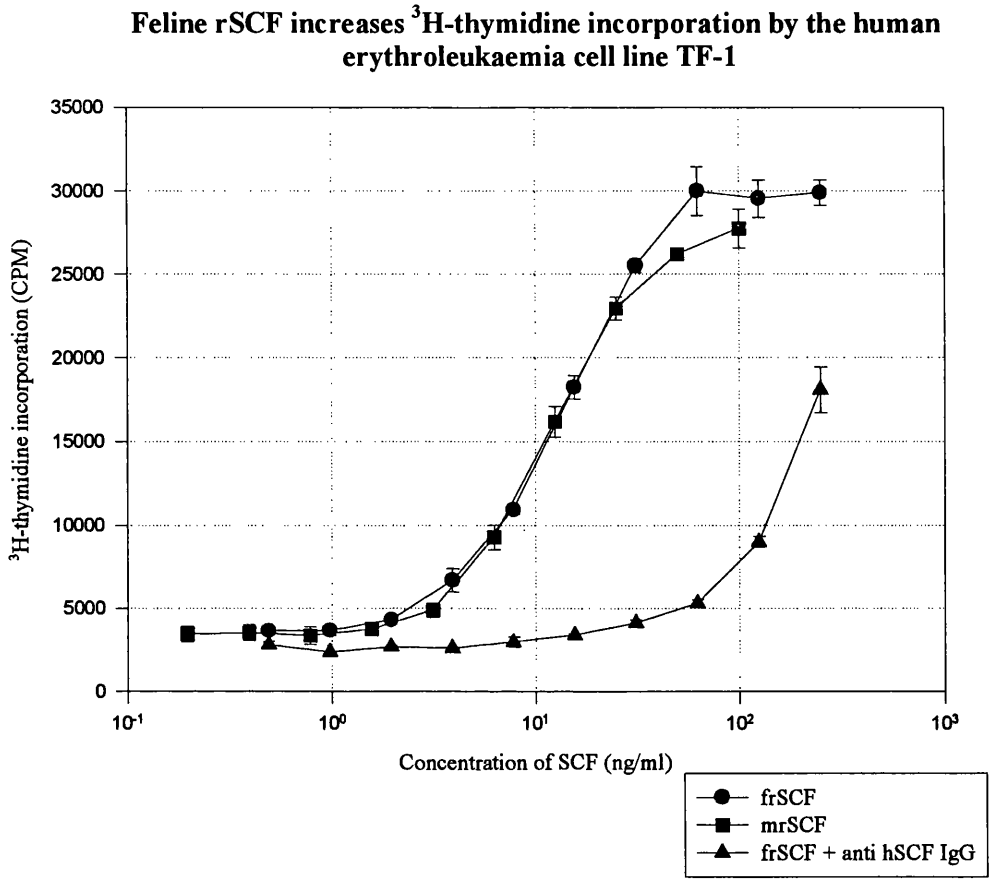


Figure 6.2: Incorporation of ^{3}H-thymidine by the human erythroleukaemia cell line TF-1 is increased by stimulation with frSCF. TF-1 cells respond to frSCF with a similar sensitivity to mrSCF showing an EC_{50} of approximately 10 ng/ml. The specificity of frSCF induced proliferation is demonstrated by preincubation with a neutralising antibody (although the neutralising effect of this antibody is overcome with higher concentrations of frSCF). There is a statistically significant treatment x concentration interaction ($p<0.000$) indicating that the response profiles of the fSCF and fSCF + anti hSCF IgG groups were different.

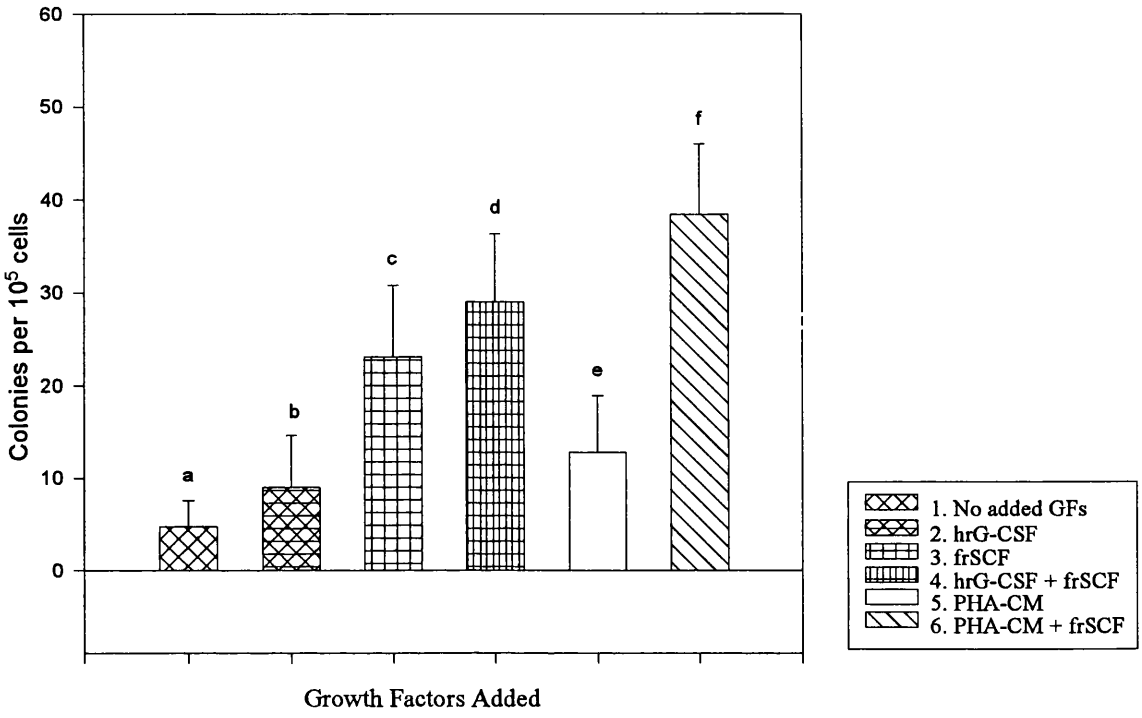


Figure 6.3: Effect of frSCF, alone and in combination with hrG-CSF or feline phytohaemagglutinin lymphocyte conditioned medium on the CFU-GM growth in agar cultures. Each column shows the mean number of colonies per 10^5 cells (n = 9) with the standard deviation indicated by the vertical line. Letters above the bars show where statistically significant differences exist between different treatment groups ($p<0.05$ - Newman-Keuls multiple range test). **a.** cf. all other treatment groups **b.** cf. groups 1,3,4,6 **c.** cf. groups 1,2,5,6 **d.** cf. groups 1,2,5 **e.** cf. groups 1,3,4,6 **f.** cf. groups 1,2,3,5.

Figure 6.4: Colonies seen in the GM-CFC assay. **A.** Smaller colonies (10 x objective magnification) with characteristics of mixed granulocyte - macrophage colonies. **B.** Larger colony (4 x objective magnification) consisting of cells with typical characteristics of cultured granulocytes.

6.4 DISCUSSION

6.4.1 USE OF CELL LINES AS BIOASSAYS FOR FELINE SCF

The results presented in this chapter clearly demonstrate the ability of frSCF to effect the proliferation of both the TF-1 and MC/9 cell lines *in vitro*. The limited ability of hrSCF to stimulate proliferation of MC/9 cells is in agreement with previous reports (Martin *et al.*, 1990). It is therefore not possible to compare the relationship between the dose response of these cells to frSCF and a standard preparation of SCF. Human rSCF was not used at higher concentrations in the assay due to limited availability of the cytokine. Attempts to repeat the assay at a later stage using mrSCF as a standard were unsuccessful despite the consistent use of early passage cells. This may have been due to a change in the batches of cell culture media used or the use of a new batch of conditioned media to maintain the cell line.

Due to these problems with the MC/9 assay, TF-1 cells were used as an alternative bioassay. These cells are not only more sensitive to frSCF, but are also easier to maintain as they do not require animal derived conditioned medium for growth. Additionally, TF-1 cells are more stable in culture than MC/9 cells and show less tendency to become factor independent (A.R. Mire-Sluis, personal communication). TF-1 cell proliferation therefore became the preferred bioassay and was used to monitor the activity of frSCF throughout purification. The assay was relatively easy to perform and showed an acceptably low degree of inter-assay variation. It is therefore suitable for routine measurement of frSCF in laboratory samples. Its use in for measurement of fSCF in biological fluids, such as serum, may also be considered. However, normal serum levels of SCF in man are only 2 - 4 ng/ml (Langley *et al.*, 1993), which is at the limit of sensitivity for the TF-1 assay.

The poor stimulation of the growth of murine MC/9 cells by human in comparison to murine SCF likely reflects differences in receptor binding and subsequent induction of receptor dimerisation and signal transduction. The use of interspecies and homologous SCF mutant proteins have implicated the predicted first, third and fourth SCF alpha-helices as important regions required for full biological function (Matous *et*

al., 1996). Of these the fourth helix is largely conserved between species and probably contributes little to the differences in specificity of the murine and human homologues. In contrast, the first and particularly the third helices have a number of amino acid differences that could contribute to the observed specificity. Feline SCF shows a high overall degree of homology to the murine and human homologues. In the MC/9 assay it is a more potent stimulant of cell growth than hSCF, but less so than mSCF. It is possible that any of the observed amino acid changes within the feline molecule are responsible for these differences. Interestingly, though fSCF has a number of amino acids within the predicted third helix that are conserved in comparison to mSCF but not hSCF (K^{81}, E^{91}, and N^{97}); it is possible that these residues are required for full biological activity of murine SCF; their alteration in human SCF may explain its comparative lack of activity on MC/9 cells.

6.4.2 ROLE OF SCF IN COLONY FORMING ASSAYS

The effects of frSCF on the growth of feline CFU-GM in cultures are similar to those reported for the human and murine growth factors. Feline SCF promotes the development of increased numbers of CFU-GM in combination with PHA-CM compared with either growth factor alone. Additionally, frSCF promotes significant colony formation as a single agent, although colonies formed were generally smaller than seen with a combination of growth factors. This effect is in contrast to the lack of stimulation of CFU-GM colony formation by canine (Shull *et al.*, 1992) or human SCFs (Martin *et al.*, 1990) but is comparable to the modest stimulation of such colonies by murine rSCF (Broxmeyer *et al.*, 1991b; Metcalf and Nicola, 1991). The limited stimulation of feline CFU-GM growth by hG-CSF is surprising, in view of its documented efficacy, *in vivo*, in this species (Fulton *et al.*, 1991). However, previous studies have shown that hG-CSF is inconsistent in the stimulation of feline CFU-GM growth (Grant, 1995). Whilst the reasons for this remain unclear, it emphasises the need for feline specific cytokines to enable further studies of haemopoiesis in this species.

SCF has also been shown to stimulate the growth of erythroid colonies *in vitro* in combination with erythropoietin, increasing both the size and the number of colonies generated (Anderson *et al.*, 1990; Nocka *et al.*, 1990a; Broxmeyer *et al.*, 1991c;

McNiece *et al.*, 1991). Indeed, purified human erythroid progenitors show an obligate requirement for rhSCF for proliferation and differentiation in serum-free medium (Dai *et al.*, 1991). It would be interesting to evaluate the effect of feline SCF on cells of the erythroid series; given the degree of conservation of the protein, it is likely to be similar to that described for other species. Unfortunately, attempts to evaluate this aspect of SCF function, using a methylcellulose based assay, previously used in this laboratory by Grant (1995), were not successful due to a number of factors including fungal overgrowth of cultures and excessive drying of plates. Further experience with the assay technique would likely eliminate these problems.

The concentrations of growth factors used for the GM-CFC assay were chosen because they were considered likely to elicit maximal stimulation of progenitor cells. Whilst this maximised the likelihood of demonstrating colony formation by frSCF it may have obscured the potential for growth factor combinations to act in an additive manner. The use of lower concentrations of stimulatory cytokines in the CFU-GM assay may provide a greater insight into the ability of frSCF and other growth factors to act in this way. Indeed, such results are likely to be more relevant to haemopoiesis *in vivo*.

The mechanism for enhancement of colony formation by combinations of growth factors has been discussed by Metcalf (1993b). The generation of greater numbers of daughter cells (i.e. larger colonies) from single progenitor cells is referred to as 'synergy'. The ability to increase the number of colonies formed can be termed 'recruitment'. The combination of two or more growth factors may increase colony numbers either because they are acting on two distinct populations of progenitor cells which respond exclusively to one factor or because some progenitors require the simultaneous stimulation by multiple growth factors in order to proliferate. Feline SCF enhanced colony formation in combination with fPHA-CM, by both recruitment and synergy, increasing colony size and numbers. In combination with hG-CSF some increase in colony size was seen, but no significant increase in colony numbers. This could be due to hG-CSF stimulating a sub-population of the SCF responsive progenitors. However, the results are in contrast to those reported in other species (Martin *et al.*, 1990; McNiece *et al.*, 1991). It seems likely that the limited efficacy of

hG-CSF in stimulating progenitor cell growth resulted in the failure to demonstrate increased colony numbers in combination with frSCF.

SCF and G-CSF both stimulate the development of similar numbers of granulocytic colonies of small size from murine BM cells. SCF additionally supports the growth of undifferentiated blast cell colonies. When the growth factors are combined colony numbers are increased in a less than additive manner. However, the number of cells in immature granulocytic colonies and blast cell colonies is greatly increased. The increased size of these colonies is thought to be due to the enhanced amplification of cell numbers at the level of the committed progenitor, each progenitor cell producing a similar number of progeny. Many of the blast cell colonies generated were unable to mature further unless stimulated by additional growth factors, a clear example of recruitment. This halt in differentiation in response to G-CSF and SCF may be due to a downmodulation of cell surface receptors for these cytokines or an acquired unresponsiveness (Metcalf, 1993a).

No attempt was made to confirm the cell type of developing colonies or to quantify colony sizes in the assays described herein. Future assays could adopt a more objective approach to evaluating colony size, although this is difficult. Replating experiments would enable assessment of the number of clonogenic cells supported by each growth factor and combination. In order to further investigate the cell types supported by frSCF, colonies could by picked from agar plates for cytological examination.

6.4.3 HAEMOPOIETIC CELL SURVIVAL AND DIFFERENTIATION

Cytokines may act on haemopoietic cells to promote cell survival, differentiation and proliferation. It has been shown that a number of cytokines prevent the death of haemopoietic cells by apoptosis (Williams *et al.*, 1990). Many studies do not discriminate between the ability of cytokines to affect cell survival and their induction of cell expansion or differentiation. However, low concentrations of macrophage-colony stimulating factor promote macrophage survival whilst higher concentrations permit both survival and proliferation (Tushinski *et al.*, 1982). SCF and its receptor belong to the same family as M-CSF which suggests that whilst the target cells of

SCF differ, an analogous effect may exist for SCF. The potential would therefore exist for low levels of SCF to facilitate cell survival whilst higher levels enable cell proliferation. To the authors knowledge this aspect of SCF function has not been studied. However, it has been shown that the survival of primitive progenitors and potentially stem cells themselves is promoted by SCF in the absence of cell division (Keller *et al.*, 1995). Furthermore, the respective abilities of soluble and membrane associated SCF to promote survival or proliferation may differ.

The majority of stem cells within the bone marrow are dormant during steady-state haemopoiesis, in G_0 of the cell cycle. Several models have been proposed for the mechanism of transition of stem cells from G_0 to active cell cycling and their subsequent self-renewal or differentiation (discussed by Ogawa *et al.*, 1983, Fairbairn *et al.*, 1993). The inductive model (Curry and Trentin, 1967) proposes that the differentiation of haemopoietic cells is determined by stimuli to which the cells are exposed; such stimuli include cytokines, cell adhesion molecules and components of the extracellular matrix. The stochastic model (Till *et al.*, 1964) suggests that the 'decision' of a stem cell to self-renew or differentiate is determined by chance and that changes in the stem cell compartment occur by changes in the probability of self renewal (p). The role of growth factors in the stochastic model is a permissive one, whereby they allow the development and proliferation of 'genetically programmed' progenitors. The stem cell competition model proposed by Van Zant and Goldwasser (1977) suggests that different growth factors such as EPO and the CSFs compete to determine the developmental pathway of stem cells. Such alternate theories are not mutually exclusive.

The ability of cytokines to determine the lineage of developing progenitor cells, in agreement with an inductive model, has been shown by a number of studies e.g. (Metcalf, 1991b). However, the demonstration that paired murine progenitor cells can produce colonies of different lineages under identical culture conditions, lends support to the stochastic model of differentiation (Suda *et al.*, 1984). The ability of haemopoietic cells to survive and differentiate in the absence of cytokines was shown by the transfection of FDCP-Mix cells with the human *bcl-2* gene (Fairbairn *et al.*, 1993). This prompted the suggestion that cytokines provide a survival signal to

haemopoietic cells and that their subsequent differentiation is intrinsically determined (although the ability of cytokines to influence differentiation was not excluded). The balance of experimental evidence tends to support a stochastic model, with the probability of a stem cell following a certain pathway influenced by factors such as metabolic state of the cell, cell receptor expression, exposure to growth factors and other environmental components (Dexter, 1989; Ogawa, 1993).

Broadly speaking, haemopoietic cytokines may be divided into three categories: late-acting lineage specific factors, intermediate-acting non-lineage restricted factors and factors affecting the kinetics of dormant stem cells (Dexter, 1989). Late acting, lineage specific factors include erythropoietin acting on erythroid progenitors and IL-5 affecting eosinophil development. Cytokines which affect the development of a variety of progenitor cells include GM-CSF, IL-4 and IL-3 (multi-CSF). Cytokines affecting the progression of stem cells from G_0 to active cell cycling include IL-1, IL-3, IL-6, IL-11, IL-12, IL-4 and SCF; indeed a combination of these cytokines seems to be required to recruit stem cells into the differentiation pathway (Metcalf, 1993b).

Cytokines may also act upon haemopoietic cells in an inhibitory manner; such cytokines include TGF-β, TNF-α, MIP-1α and the interferons. Whilst the effects of the interferons and TNF-α are not lineage restricted, TGF-β and MIP-1α appear to act preferentially to inhibit the more primitive precursors and possibly stem cells (Ogawa, 1993). TGF-β antagonises the actions of a number of early acting cytokines including SCF, IL-3 and IL-1 (Lardon *et al.*, 1994; Jacobsen *et al.*, 1995) and inhibits the proliferation of primitive haemopoietic cells (Sitnicka *et al.*, 1996). Such negative regulators may facilitate the survival of stem cells (Verfaillie *et al.*, 1994). TGF-β and possibly other negative regulators may also safeguard against neoplastic transformation of haemopoietic cells due to the activation of intracellular tumour suppressor proteins (Serra and Moses, 1996).

CHAPTER SEVEN - IMMUNOLOGICAL DETECTION OF THE CD34 ANTIGEN

7.1 INTRODUCTION

CD34 is a transmembrane glycoprotein expressed predominantly on primitive haemopoietic cells, vascular endothelial cells and embryo fibroblasts. Cell surface expression of CD34 on haemopoietic cells is highest on the most immature progenitors and decreases progressively with cell maturation. Whilst only 1.5% of bone marrow mononuclear cells express CD34, nearly all haemopoietic colony forming cells are found within this population. The function of CD34 remains elusive; it may be an adhesion factor, mediating the anchorage of stem and progenitor cells to BM stroma and/or may be important in preventing the terminal differentiation of immature progenitor cells and thus maintaining the BM progenitor cell population. Despite this, the use of anti-CD34 monoclonal antibodies has found widespread clinical applications. CD34 expression has been used as a marker for diagnosis of leukaemia and sub-classification of the disease. The expression of CD34 in childhood acute lymphoblastic leukaemia is associated with a good prognosis, whilst its expression in acute myeloid leukaemia correlates strongly with expression of the multidrug resistance protein. CD34 is finding increasing utility in the management of BM transplantation, allowing quantification of cells with repopulation potential and furthermore permitting the purification of such cells from a heterogeneous population of cells, an approach which may allow the purging of contaminating tumour cells from allogenic grafts or T-cell depletion to prevent graft versus host disease (reviewed by Krause *et al.*, 1996).

The aim of this part of the project was to raise antibodies that could be used to identify feline haemopoietic cells expressing CD34. The availability of such antibodies would aid studies into the *in vivo* effects of feline stem cell factor. The cDNAs for human (Simmons *et al.*, 1992), murine (Brown *et al.*, 1991), and canine (McSweeney *et al.*, 1996) CD34 have been cloned and antibodies to the proteins have been raised, enabling the study of CD34 expression in these species. The degree of inter-species homology is, however, relatively low (the human CD34 protein shows 66% identity overall to the murine homologue, with lower homology within the extracellular domain) thus the use of such antibodies is species restricted. Since feline CD34 has yet to be cloned and time considerations prevented such an approach it was decided

to attempt to raise antibodies, to conserved intracellular epitopes of the CD34 molecule, using immunogenic peptides. Unfortunately, the antisera raised, whilst recognising the synthetic peptides against which they had been raised, showed poor affinity for the native protein.

The use of synthetic peptides to raise antibodies against previously uncharacterised proteins was first described in 1980 (Sutcliffe *et al.*, 1980; Walter *et al.*, 1980). Due to their small size, linear peptides may not be immunogenic on their own. Conventionally, a linear peptide is coupled to a carrier protein such as bovine serum albumin or keyhole limpet hemocyanin (KLH). The use of a carrier protein ensures that class II T-cell receptor sites are present on the immunogen; the peptide then serves as a hapten. The choice of peptide is largely empirical, but certain features may be used to predict the likelihood of a given peptide being immunogenic (reviewed by Harlow and Lane, 1988). Hydrophilic residues and those with high flexibility are more likely to be exposed on the surface of the native protein and form epitopes. Similarly amino and carboxy terminal regions are often exposed and thus make good choices for synthetic peptides. Peptides are generally chosen to be 10-15 amino acids in length, although longer peptides may be used.

The use of peptide-carrier protein conjugates as immunogens has certain inherent disadvantages. The peptide comprises only a small portion of the immunogen, thus antibodies to the peptide may represent only a fraction of the total antibody response. The conjugation procedure may alter the conformation of the peptide so that the antibodies raised are less likely to recognise the native protein. The carrier protein may also be toxic or suppress the immune response to the epitope of interest (Tam and Shao, 1993). To overcome these limitations, the multiple antigen peptide (MAP) system was developed (Tam, 1988). MAPs consist of an inner branched matrix of lysine residues to which may be attached up to 16 copies of the peptide antigen. The inner core is small and non-immunogenic. It is the multiple copies of the peptide that form the bulk (93% of the mass) of the immunogen. The branching nature of the MAP allows mobility of the peptide chains and therefore enhances their immunogenicity (Tam and Shao, 1993).

7.2 MATERIALS AND METHODS

7.2.1 PRODUCTION OF CD34 PEPTIDES

7.2.1.1 Design and synthesis of synthetic peptides

The cDNA sequences of murine (Brown *et al.*, 1991) and human (Simmons *et al.*, 1992) CD34 were obtained from the EMBL database. GCG software (Version 7 for UNIX) was used to derive the predicted proteins using the 'Translate' program. Alignment of human and murine CD34 protein sequences was performed using the 'BestFit' program and is shown in Figure 7.1. The antigenicity of the human CD34 molecule was predicted using the 'PeptideStructure' program. This utilises the method of Jameson and Wolf (Wolf *et al.*, 1987) to predict an antigenic index, calculated by summing a number of measures of secondary structure; the output from this program is shown in Appendix A4. Predicted antigenic regions of the human CD34 protein are shown graphically in Figure 7.2. Two areas with a high antigenic index, conserved between human and murine CD34, situated within the intracellular region were found by manual inspection. These directed the choice of two peptides for use as immunogens (the amino acid position relative to human CD34 is given in parenthesis):

Peptide 55	NGTGQATSRNGHS	(aa 350-362)
Peptide 56	SWSPTGERLGEDPYY	(aa 304-318)

Peptides were synthesised within the Department of Veterinary Pathology on a 432A Protein Synthesiser (Applied Biosystems). Peptide 55 was synthesised as a linear peptide and subsequently conjugated to a carrier protein as detailed below. Peptide 56 was produced as a multiple antigen peptide. Peptides were weighed and then dissolved in dH_2O. The peptides were then aliquoted into eppendorfs, freeze dried, and stored in aliquots at -70°C.

```
humCD34    1 MPRGWTALCLLSLLPSGFMSLDNNGTATPELPTQGTFSNVSTNVSYQETT 50
murCD34   13 LPWRWVALCLMSLLH.....LNNLTSATTETSTQGISPSVPTNESVEENI 57
humCD34   51 TPSTLGSTSLHPVSQHGNEATTNITETTVKFTSTSVITSVYGNTNSSVQS 100
murCD34   58 TSSIPGSTSHYLIYQDSSKTTPAISETMVNFTVTSGIPSGSGTPHTFSQP 107
humCD34  101 QTSVISTVFTTPANVSTPETTLKPSLSPGNVSDLSTTSTSLA.TSPTKPY 149
murCD34  108 QTSPTGILPTTSDSISTSEMTWKSSLPSINVSDYSPNNSSFEMTSPTEPY 157
humCD34  150 T.SSSPILSDIKAEIKCSGIREVKLTQGICLEQNKTSSCAEFKKDRGEGL 198
murCD34  158 AYTSSSAPSAIKGEIKCSGIREVRLAQGICLELSEASSCEEFKKEKGEDL 207
humCD34  199 ARVLCGEEQADADAGAQVCSLLLAQSEVRPQCLLLVLANRTEISSKLQLM 248
murCD34  208 IQILCEKEEAEADAGASVCSLLLAQSEVRPECLLMVLANSTELPSKLQLM 257
humCD34  249 KKHQSDLKKLGILDFTEQDVASHQSYSQKTLIALVTSGALLAVLGITGYF 298
murCD34  258 EKHQSDLRKLGIQSFNKQDIGSHQSYSRKTLIALVTSGVLLAILGTTGYF 307
humCD34  299 LMNRRSWSPTGERLGEDPYYTENGGGQGYSSGPGTSPEAQGKASVNRGAQ 348
murCD34  308 LMNRRSWSPTGERLGEDPYYTENGGGQGYSSGPGASPETQGKANVTRGAQ 357
humCD34  349 KNGTGQATSRNGHSARQHVVADTEL 373
murCD34  358 ENGTGQATSRNGHSARQHVVADTEL 382
```

Figure 7.1: Alignment of the predicted polypeptide sequences of human and murine CD34, performed using the BestFit program using the algorithm of Smith and Waterman (1981) (GCG Software - Version 7 for UNIX). The predicted transmembrane region is depicted in bold type. The amino acid sequences of the peptides chosen for use as immunogens are shown in red.

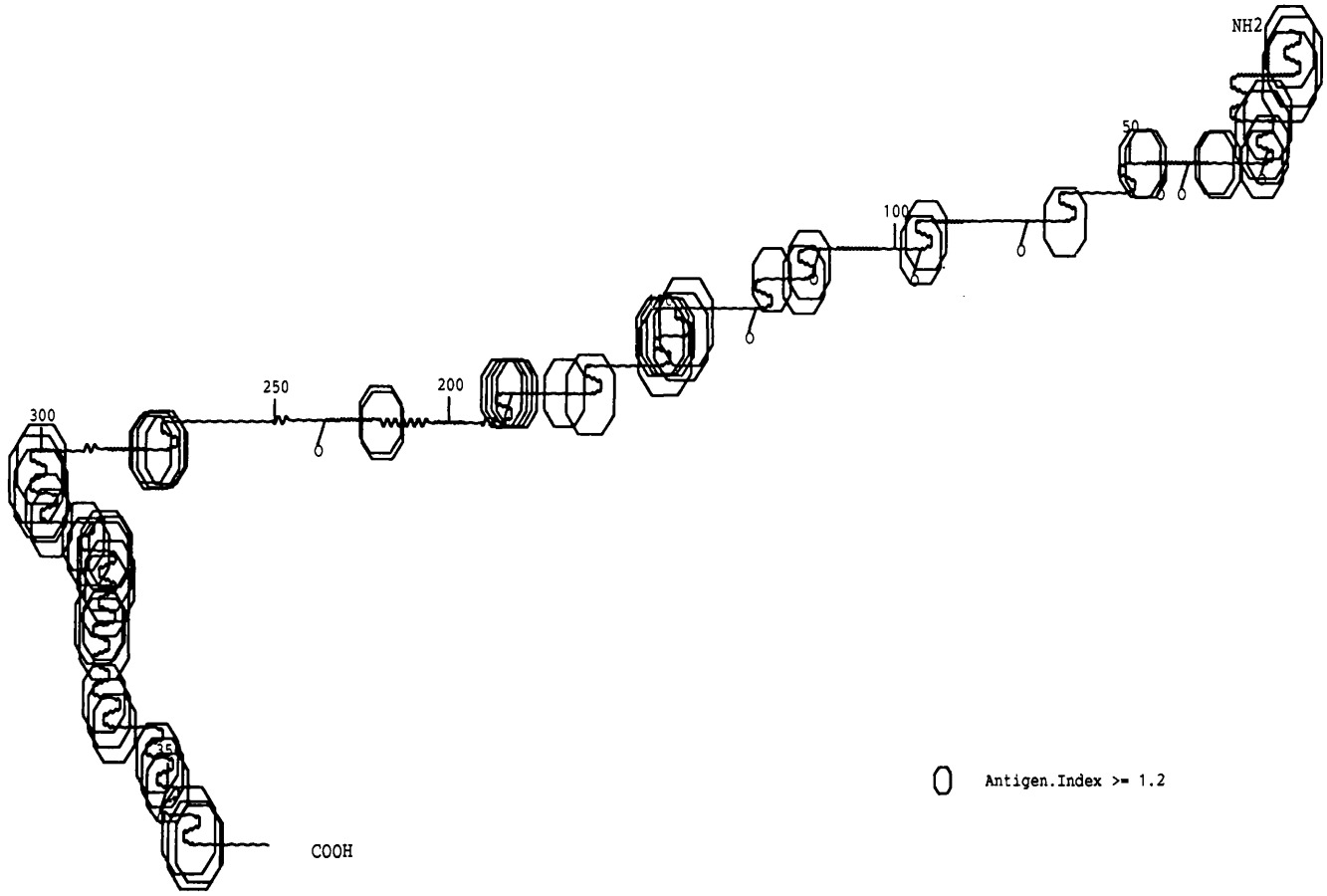


Figure 7.2: Predicted antigenicity of the human CD34 antigen. The diagram was created using the GCG program 'PlotStructure' using the output generated by the 'PeptideStructure' program. Predictions of secondary structure according to Chou and Fasman (1978) are overlain by motifs indicating regions of the protein with high antigenicity scores.

7.2.2 CONJUGATION OF PEPTIDE 55 TO CARRIER PROTEIN

Peptide 55 was coupled to the carrier protein KLH using a one-step glutaraldehyde method, described by Maloy *et al.* (1993). Ten milligrams of KLH was dissolved, with stirring, in two millilitres of 0.1 M borate buffer (pH 10) in a 20 ml glass universal. Fourteen milligrams (10 μmol) of peptide 55 was added to the above solution and allowed to dissolve. One millilitre of 0.3% glutaraldehyde was added slowly and the coupling reaction was allowed to proceed for two hours, with continuous stirring. Unreacted glutaraldehyde was then neutralised by the addition of 0.25 ml of 1 M glycine, which was allowed to react for 30 minutes. The solution was dialysed overnight against three litres of borate buffer (pH 8.5) at 4°C, using a three millilitre Slide-a-Lyzer dialysis cassette (Pierce & Warriner UK Ltd.) The following morning the buffer was changed and dialysis continued for a further four hours. The peptide-protein conjugate was then freeze dried and weighed before it was dissolved in dH_2O, aliquoted into eppendorfs, freeze dried again and stored at -70°C.

7.2.3 PRODUCTION OF ANTI-PEPTIDE ANTISERA

7.2.3.1 Preparation of Immunogen

In order to maximise the immune response to the injected immunogen, peptides were combined with an adjuvant prior to injection. Adjuvants typically have two major properties: the ability to maintain high local levels of antigen by preventing its rapid catabolism or dispersal and the non-specific stimulation of the immune response. Freund's complete adjuvant (CFA) was used for primary immunisations, whilst subsequent immunisations used incomplete Freund's adjuvant (IFA). Freund's adjuvant (Freund *et al.*, 1937) consists of a water in oil emulsion in which the oils are non-metabolisable. CFA is prepared by the addition of *Mycobacterium tuberculosis*, which may be killed or attenuated (e.g. Bacillus Calmette-Guerin (BCG) vaccine); IFA lacks this bacterial component. FA is a particularly effective adjuvant and produces a strong, persistent stimulation of the immune response. Its use can, however, be associated with persistent granulomas and ulceration. The majority of such side effects are avoided by using incomplete FA for all booster immunisations (reviewed by Harlow and Lane, 1988).

Peptides were prepared for immunisation immediately prior to use with CFA or IFA as appropriate. CFA was prepared by the addition of 0.1 ml of intradermal BCG vaccine (Evans Medical Ltd., Leatherhead, UK) to 0.9 ml of aqueous peptide immunogen. The aqueous phase was then mixed with two volumes of non-ulcerative Freund's adjuvant (NUFA - Guildhay Ltd., Guildford, UK) to produce a stable water in oil emulsion, as recommended by the manufacturers. IFA was prepared similarly, without the addition of BCG vaccine.

7.2.3.2 Immunisation of Rabbits

Four adult New Zealand white rabbits were used to raise polyclonal antisera; two rabbits received peptide 55 and two peptide 56. Prior to immunisation, 20 ml of blood was collected from each rabbit to provide suitable control antibody for later experiments. Each rabbit was given a primary immunisation of 100 μg of antigen in CFA. Booster immunisations were given four weeks later; one rabbit in each pair was immunised with 100 μg antigen in IFA (designated A) whilst the other rabbit in each pair was given 25 μg antigen in IFA (designated B). The rabbits were subsequently given booster immunisations using the same preparations at six week intervals. All immunisations were given subcutaneously, in total volume of one millilitre, split between four sites.

Blood was collected 10 - 14 days after booster immunisations. Ten to fifteen millilitres of blood was collected from the marginal ear vein into sterile glass universals. The blood was allowed to stand for several hours at room temperature, then allowed to clot overnight at 4°C. The serum was then transferred to a clean universal and any residual blood cells pelleted by centrifugation at 5,000 x g for 10 minutes. The serum was stored in one millilitre aliquots at -20°C.

7.2.3.3 Anti-peptide ELISA

In order to verify that the rabbits had developed an antibody response to the injected peptides and to measure the level of response, an indirect antibody enzyme linked immunosorbent assay (ELISA) was performed using an ELISA starter kit (Pierce & Warriner, UK). ELISAs were initially performed for each polyclonal antiserum using

a two-dimensional serial dilution protocol, as recommended by the manufacturers (Figure 7.3). A solution of each antigen, peptide 55 (not coupled to carrier) or peptide 56 containing 5 μg/ml peptide in BupH™ carbonate-bicarbonate buffer was prepared. Ninety-six well microtitre plates (Pierce & Warriner, UK) were coated with a single antigen by adding 100 μl of antigen solution to each well and incubating for one hour at room temperature. The plates were then washed three times with 100 μl of wash buffer (modified Dulbecco's phosphate buffered saline containing Tween 20 and bovine serum albumin). Plates were then incubated for 30 minutes with 100 μl of 1% BSA solution to block unreacted binding sites in the wells. The plates were then emptied and 100 μl of wash buffer added to each well. To each well in row A was aliquoted 100 μl of antiserum at a 1/2 dilution. The antibody was serially diluted (1/4, 1/8 etc.) from row A to G; row H was used as a negative control. After incubation for one hour each well was washed three times with 100 μl wash buffer.

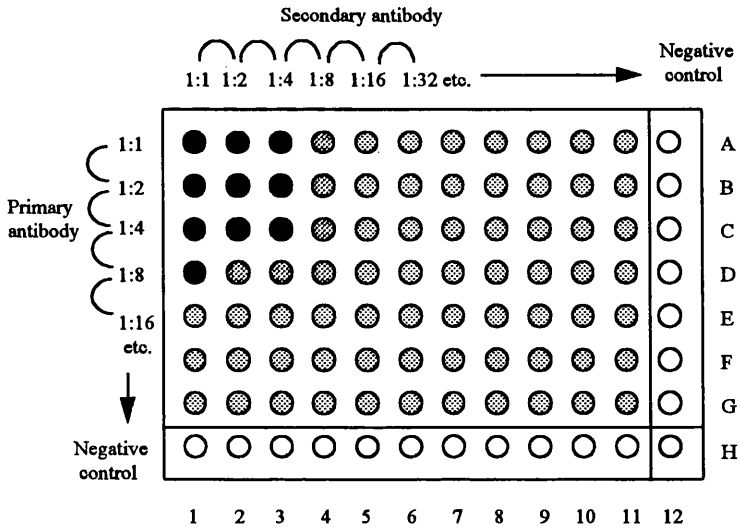


Figure 7.3: Two-dimensional serial dilution ELISA used to measure the level of antibody response in rabbits injected with immunogenic peptides.

The secondary antibody used was a goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (Pierce & Warriner, UK). One hundred microlitres of a 1/1000 dilution were aliquoted to each well in row 1; the antibody was then serially diluted, two-fold from row 1 to 11, with row 12 used as a negative control. After incubation for 30 minutes at room temperature the wells were washed three times with 100 μl

wash buffer, followed by a incubation with 100 µl wash buffer for five minutes. The final wash was discarded and 100 µl of enzyme substrate [2,2'-azino*bis*(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt - ABTS] added to each well. After incubation for 30 minutes, the absorbance of the reagents at 405 nm was measured using an ELISA plate reader (EL 312 - Bio-tek Instruments Inc., Winooski, VT).

In order to determine the degree of specific antipeptide antibody response, in comparison to non-specific antibody, a further ELISA was performed. Each antiserum was evaluated against the specific peptide used to raise the antiserum and another unrelated peptide (peptide 56 for antiserum 55 and *vice versa*). Additionally, pre-bleed serum was used as a negative control. ELISAs were conducted in a similar manner to that described above except that a single concentration of secondary antibody was used (1/5000 dilution).

7.2.4 PURIFICATION OF ANTISERUM

The immunoglobulin G fraction of antisera 55A and 56B and non-immune sera from these rabbits were purified using protein A affinity chromatography. Protein A is a 42 kDa polypeptide produced by most strains of *Staphylococcus aureus*. The protein has a high affinity for the Fc region of most IgG molecules. However, the protein A - immunoglobulin bond may be broken by lowering the pH. The purification of IgG was simplified by the use of a commercially available chromatography column consisting of protein A coupled to agarose beads (HiTrap protein A column - Pharmacia Biotech).

Prior to purification approximately three millilitres of each serum was equilibrated with start buffer (phosphate buffered saline, pH 7.4) using a centrifugal concentrator with a molecular weight cut off (MWCO) of 50 kDa (Centriplus - Amicon, Beverly, MA), as recommended by the manufacturers. The antiserum was then filtered using a 0.4 µm syringe filter (Gelman Sciences). A five millilitre HiTrap protein A column was equilibrated with five column volumes of start buffer, using a five millilitre syringe. The serum was loaded onto the column using a syringe and the column

washed with three column volumes of start buffer. The column was then eluted using 0.1 M citric acid, pH 4.5. Fractions of 0.5 ml were collected into eppendorf tubes into which 50 µl of 1 M Tris-HCl, pH 9.0 had previously been aliquoted (to minimise acid denaturation of the eluted protein). The immunoglobulin containing fractions were identified by measuring the absorbance at 280 nm of the eluted fractions (1 OD = approximately 0.8 mg/ml of IgG); fractions containing over ~ 0.5 mg/ml protein were pooled. The pooled fractions were concentrated by ultrafiltration using a 50 kDa MWCO centrifugal concentrator (Centriplus), as recommended by the manufacturers, to a final concentration of 5 mg/ml (assessed by measurement of the A_{280}). The purity of each antibody solution was confirmed by SDS-PAGE. After use the protein A column was cleaned by sequentially washing with three column volumes of 2 M urea, 1 M LiCl and 100 mM glycine (pH 2.5).

7.2.5 DETECTION OF CELLULAR CD34

The ability of the purified antipeptide antibodies to identify cellular CD34 was evaluated by immunohistochemistry against a cell line known to express CD34 (human erythroleukaemia cell line TF-1) and against feline bone marrow mononuclear cells (BMMC). Antibodies purified from pre-bleed serum were used as negative controls and controls omitting primary antibody were also included. An indirect detection method was used, with biotinylated swine anti-rabbit immunoglobulin as the secondary antibody. An avidin biotin complex (ABC) staining procedure was used. This method uses a biotinylated enzyme which is preincubated with avidin to form large complexes. On subsequent incubation with labelled cells a greater enzyme concentration is bound to any immobilised secondary antibody thus enhancing the sensitivity of the detection method.

TF-1 cells were harvested two days after passaging and feline BMMC prepared as described in section 2.2.1.7. Cytospins were prepared within the Department of Veterinary Haematology, University of Glasgow. The cells were then fixed and permeabilised by immersion in methanol/acetone (1:1) at -20°C for 20 minutes and stored at -20°C until use. The slides were thawed slowly prior to use then immersed in Tris buffered saline (TBS pH 7.6 - NaCl 0.15 M, Tris-HCl 0.05 M, Tris base 0.05

M) for five minutes. Cells were then incubated in 100 µl normal swine serum (20% dilution in TBS - DAKO A/S, Denmark) for 20 minutes, to block non-specific background. The slides were gently tapped to remove excess serum and then incubated with 100 µl of rabbit polyclonal antibody (5-10 µg/ml) for one hour. Slides were washed twice by immersion in TBS for three minutes. Cells were incubated with 50 µl secondary antibody (1:300 dilution of biotinylated swine anti-rabbit immunoglobulin - DAKO A/S) for 30 minutes and then washed as above. Cells were then incubated with 100 µl of ABcomplex/AP, prepared as directed by the manufacturers (DAKO A/S). The cells were then washed and incubated for 20 minutes with 200 µl of alkaline phosphatase substrate (Naphthol AS BI phosphate 40 mg, N,N-dimethylformamide 0.4 ml, 1M Tris-HCl pH 8.2 19.6 ml, 1M levamisole 200 µl, to 200 ml with dH_2O) containing 1 mg/ml Fast red TR. Slides were rinsed in dH_2O, counterstained with Mayer's haematoxylin for 20 seconds, washed in Scott's tap water substitute ($Na(CO_3)_2$ 3,5 g, $MgSO_4$ 20 g, dH_2O to 1 L) and mounted in aqueous mountant (Aquamount - BDH).

7.3 RESULTS

The result of a typical two-dimensional serial dilution ELISA are shown in Table 7.1. At a secondary antibody concentration of 1/16,000 an endpoint of 1/128 (half maximal A_{405}) is seen; at higher secondary antibody concentrations an endpoint is not reached. The specificity of each antipeptide response is shown in Table 7.2. All prebleed sera show no significant antipeptide antibody titre. The antisera raised by peptide 55 both generated strong positive results, with endpoints of 1/5120 and 1/320 for 55A and 55B respectively. However, antiserum 55A also shows a relatively strong non-specific response (to peptide 56). Antiserum 56B shows a specific response to peptide 56, with an endpoint of 1/160, whilst antiserum 56A shows no significant antipeptide antibody titre.

The purity of each antibody preparation following purification of the IgG fraction was assessed, by SDS-PAGE, to be over 95% (Figure 7.4).

TF-1 cells incubated with antibody 55A (raised against peptide-KLH conjugate) stained weakly (Figure 7.5). Those incubated with antibody 56B showed no staining. Staining of feline bone marrow cells was unsuccessful due to marked non-specific background staining, which was seen with both prebleed and immune antibodies but not with primary antibody negative controls (results not shown).

	1	2	3	4	5	6	7	8	9	10	11	12
A	>3.0	>3.0	>3.0	1.803	0.652	0.446	0.227	0.180	0.132	0.114	0.115	0.083
B	>3.0	>3.0	>3.0	>3.0	2.170	1.386	0.627	0.413	0.257	0.163	0.126	0.086
C	>3.0	>3.0	>3.0	>3.0	2.810	1.682	0.898	0.513	0.090	0.197	0.146	0.085
D	>3.0	>3.0	>3.0	>3.0	2.481	1.392	0.772	0.411	0.232	0.180	0.136	0.087
E	>3.0	>3.0	>3.0	>3.0	2.439	1.430	0.863	0.451	0.276	0.182	0.144	0.093
F	>3.0	>3.0	>3.0	2.915	2.032	1.254	0.817	0.457	0.268	0.177	0.144	0.088
G	>3.0	>3.0	>3.0	2.216	1.459	0.917	0.617	0.328	0.207	0.152	0.119	0.086
H	0.144	0.137	0.114	0.104	0.098	0.089	0.097	0.104	0.089	0.085	0.096	0.085

Table 7.1: Indirect antibody ELISA using antiserum to peptide 56. Primary antiserum is diluted down the plate (A to G); dilutions start at 1/2 and continue 1/4, 1/8 etc. Row H is a negative control. Secondary antibody is diluted across plate with serial two-fold dilutions from row 1 - 11, starting at 1/1000 dilution; row 12 is a negative control. Figures refer to the absorbance of the reagents at 405 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
55A-PRE	0.442	0.391	0.332	0.214	0.228	0.142	0.132	0.137	0.118	0.119	0.146	0.205
55A-S	2.665	2.680	2.739	2.723	2.680	2.626	2.527	2.345	1.881	1.601	1.106	0.126
55A-NS	2.508	2.590	2.569	2.262	2.022	1.327	0.844	0.590	0.359	0.213	0.165	0.099
55B-PRE	0.931	0.402	0.492	0.800	0.265	0.171	0.133	0.134	0.120	0.115	0.117	0.114
55B-S	2.693	2.757	2.579	2.278	1.946	1.546	1.003	0.689	0.435	0.290	0.203	0.120
55B-NS	0.744	0.563	0.431	0.304	0.246	0.185	0.162	0.136	0.117	0.102	0.103	0.102
56A-PRE	0.551	0.439	0.314	0.250	0.189	0.136	0.139	0.144	0.111	0.107	0.110	0.102
56A-S	0.676	0.560	0.469	0.291	0.216	0.137	0.120	0.122	0.106	0.107	0.114	0.104
56A-NS	0.692	0.389	0.314	0.265	0.296	0.532	0.488	0.284	0.150	0.143	0.153	0.122
56B-PRE	0.404	0.324	0.342	0.212	0.171	0.123	0.115	0.118	0.104	0.107	0.119	0.108
56B-S	2.480	2.370	2.176	1.925	1.483	0.950	0.579	0.391	0.250	0.173	0.143	0.104
56B-NS	0.692	0.389	0.314	0.265	0.296	0.532	0.488	0.284	0.150	0.143	0.153	0.122

Table 7.2: Results of indirect ELISA to measure antibody titres against peptides 55 and 56. Primary antibody (antiserum) is diluted across the plate (row 1 - 12); dilutions start at 1/10 and continue 1/20, 1/40 etc. Secondary antibody was used at a dilution of 1/5000. Pre = prebleed antiserum; S = specific antipeptide ELISA; NS = non-specific ELISA (antiserum 55A/B vs. peptide 56 and *vice versa*). Figures refer to the absorbance of the reagents at 405 nm.

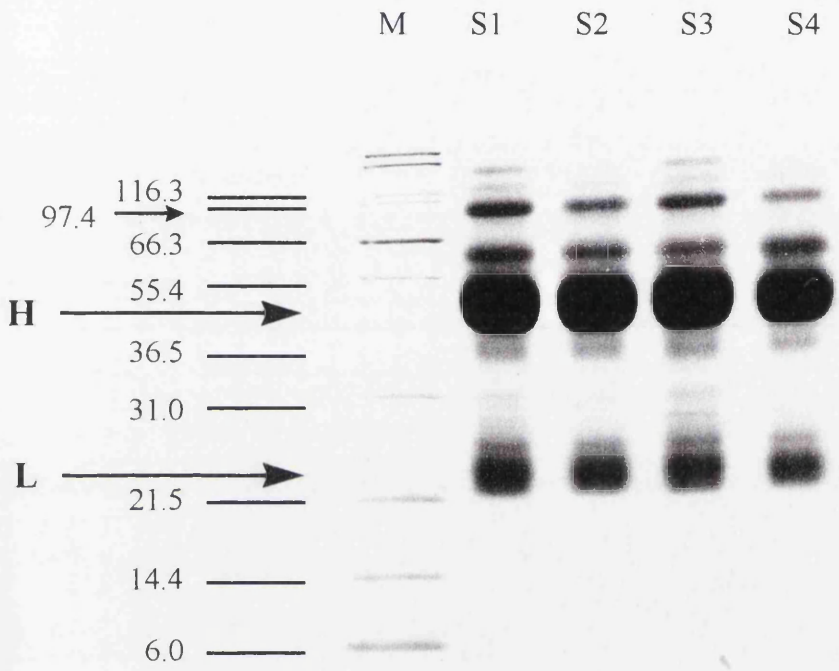
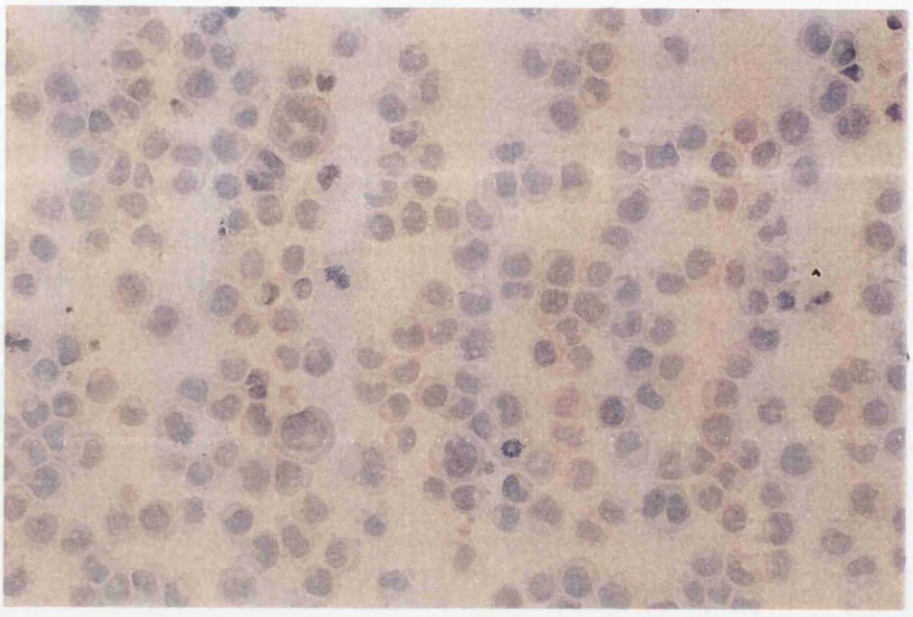


Figure 7.4: Purification of rabbit IgG fraction from polyclonal antiserum. Five micrograms of each antibody was resolved by SDS-PAGE using a 12.5% gel. The gel was subsequently stained with Coomassie blue. M = molecular weight markers; S1 - S4 are four different antisera following purification using Protein A chromatography. The letters H and L refer to the IgG heavy and light chains, respectively.

A.



B.

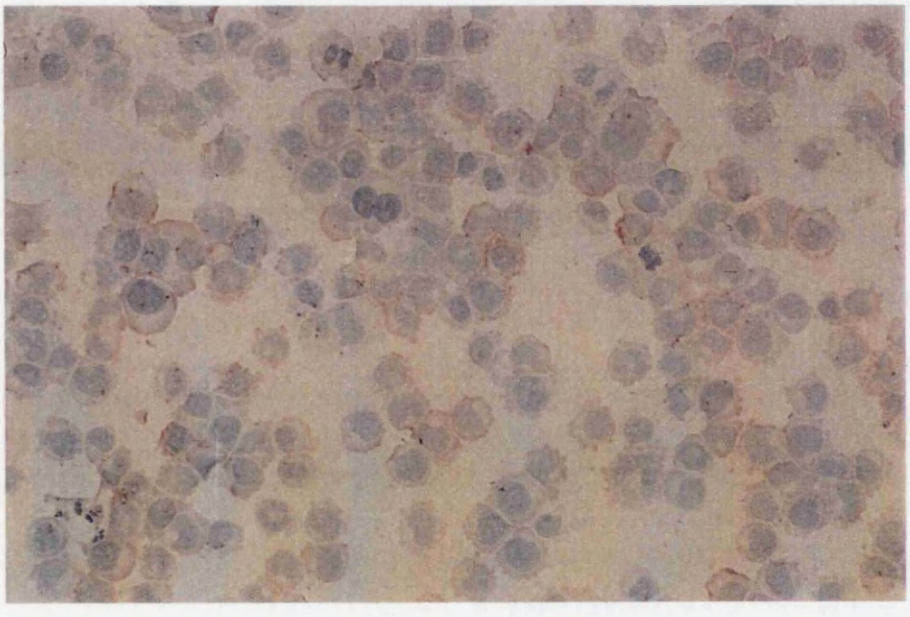


Figure 7.5: Human TF-1 cell line stained with rabbit antipeptide antibody 55A. **A.** Negative control - prebleed serum. **B.** Cells show weak staining with anti CD34 peptide antibody.

7.4 DISCUSSION

The antibodies generated by three of the four rabbits recognised the peptide against which they had been generated, with higher titres seen in sera raised using a KLH-peptide conjugate. However, the antibodies showed no, or poor affinity for the native protein. There are several possible reasons for this discrepancy. The peptide immunogens may have not accurately represented epitopes of the native protein either due to their site (e.g. in buried regions of the protein) or due to non-representative folding of the peptide. The epitopes may have been inaccessible due to insufficient permeabilisation of the cells or their conformation may have been altered by the method of fixation; alternative methods (e.g. use of paraformaldehyde) may produce better results. Furthermore, it was not proven whether the weak staining of TF-1 cells by antibody 55A was specific for CD34. Time limitations unfortunately prevented any further optimisation of the staining technique.

There have, however, been a number reports of the successful use of this experimental approach to raise antibodies to the cytoplasmic domain of human leucocyte markers, including CD3 and CD8 (Mason *et al.*, 1989; Mason *et al.*, 1992). Peptides were originally used to raise polyclonal antisera which could recognise these antigens in fixed tissues, in contrast to monoclonal antibodies available at that time.

Ultimately, the cloning of feline CD34 would be desirable. In particular this would allow the production of antibodies against the extracellular domain of CD34. Such antibodies could be use to identify CD34 positive cells using flow cytometry, without recourse to cell fixation and permeabilisation. This would enable the enumeration and sorting of live cells which would facilitate studies on purified progenitor cells *in vitro* and also the development of clinical protocols for marrow and peripheral blood stem cell transplantation.

CHAPTER EIGHT - EFFECTS OF FELINE STEM CELL FACTOR *IN VIVO*

8.1 INTRODUCTION

8.1.1 EFFECTS OF RECOMBINANT SCF ON HAEMOPOIESIS *IN VIVO*

The effects of recombinant SCF on haemopoiesis *in vivo* have been studied in a number of species including mice, dogs, baboons and humans. Administered as a single agent, rSCF has more dramatic effects than would be predicted by its *in vitro* effects.

Mice (Fleming *et al.*, 1993), baboons (Andrews *et al.*, 1992b) or dogs (De Revel *et al.*, 1994) given rSCF show increases in circulating haemopoietic progenitor cells, including those with the ability to repopulate the bone marrow of an irradiated recipient. rrSCF given to mice (100 μg/kg/day subcutaneously) produces a six fold increase in peripheral blood CFU-S after five days (Molineux *et al.*, 1991). rhSCF given to baboons produces a dose dependent increase in circulating progenitors (GM-CFC, BFU-E, CFU-Mix and HPP-CFC) at doses of 50 - 200 μg/kg/day given by intravenous infusion (Andrews *et al.*, 1992a). Similar effects have been reported in dogs (De Revel *et al.*, 1994). SCF also causes an acute dose dependent neutrophilia and lymphocytosis in rats, which peaks four to six hours and subsides between 12 and 24 hours, after a single intravenous injection (Ulich *et al.*, 1991). Longer term administration of rSCF to mice (100 μg/kg/day) produces a two to three fold increase in circulating leukocytes, predominantly neutrophils, after administration for 5 to 14 days (Molineux *et al.*, 1991; Bodine *et al.*, 1993); levels rapidly return to normal after cessation of growth factor administration. Baboons treated with rhSCF show increases in PB neutrophils, erythrocytes, lymphocytes, monocytes, eosinophils, and basophils (Andrews *et al.*, 1991).

The reported effects of SCF on the bone marrow are variable depending on species, dose and duration of treatment. One study showed an increase in BM mast cells, accompanied by an overall decrease in marrow cellularity after administration of rrSCF to rats for two weeks (Ulich *et al.*, 1991). Molineux *et al.* (1991) reported no significant change in BM cellularity after administration of a similar dose of rmSCF to

mice for two weeks, although an increase in the number of progenitor cells within the marrow was seen. Bodine *et al.* (1993) investigated the effect of rmSCF on the absolute number of pluripotent haemopoietic stem cells (PHSC) in mice, as measured by a competitive repopulation assay. The number of PHSC (per mouse) increased three-fold after rmSCF treatment for seven days; more specifically there was an increase in spleen and peripheral blood (PB) PHSC of 10 fold or more and a decrease in marrow PHSC of three fold. Dogs given 200 μg/kg/day rcSCF for 20 to 28 days show an increased BM cellularity associated with a seven-fold increase in granulocyte/macrophage progenitors (CFU-GM) (Schuening *et al.*, 1993). Baboons given 200 μg/kg/day rhSCF show an increase in marrow cellularity (150 - 200% c.f. control animals) and absolute number of granulocyte/monocyte (CFU-GM) and erythroid (BFU-E) progenitors in the marrow (Andrews *et al.*, 1991).

Administration of rSCF to mice, rats, non-human primates and humans produces increases in mast cells at the site of injection. Increased mast cell numbers are also seen at other sites, although the effect is dose dependent and species differences exist. SCF seems to be a more potent inducer of mast cell hyperplasia in rats than in other species, inducing increases in the number of mast cells in multiple organs including bone marrow, spleen, liver and lung, after intravenous administration of 100 μg/kg/day for two weeks (Tsai *et al.*, 1991a; Ulich *et al.*, 1991).

8.1.2 OBJECTIVES

The aims of this part of the project were to assess the safety and haemopoietic activity of feline recombinant SCF given to cats by the subcutaneous route. To evaluate safety, clinical parameters (e.g. demeanour, rectal temperature) and serum biochemistry profiles were monitored. The haemopoietic effects of SCF were evaluated by measuring peripheral blood haematological parameters and assaying granulocyte macrophage colony forming cells in both peripheral blood and bone marrow. Additionally, tissues were collected *post mortem*, enabling histopathology of haemopoietic organs and examination of tissues for mast cell infiltration.

8.2 MATERIALS AND METHODS

8.2.1 TRIAL DESIGN

The amount of feline recombinant SCF available limited the scale of the experiment in terms of number of cats that could be used, dose of SCF and duration of treatment. Seven specific pathogen free cats, aged between 16 and 24 months, were used. Cats were assigned randomly to one of three treatment groups or to the control group. The three treatment groups received 200 μg/kg, 100 μg/kg or 25 μg/kg frSCF and the control group 100 μg/kg bovine serum albumin. Each group contained two cats, except the 'group' receiving 25 μg/kg which contained one cat. Administration of injections, collection and assessment of all samples was carried out without knowledge of each animal's specific treatment. Figure 8.1 shows an overview of the experimental protocol. Bone marrow aspirates and blood for haematology and serum biochemistry were obtained prior to any treatments. Haematology was monitored at four, 12 and 24 hours and three and five days after starting treatment. Cats were euthanased on day eight, at which time BM aspirates, haematology and serum biochemistry were repeated and tissues collected for histopathology.

8.2.2 PREPARATION AND ADMINISTRATION OF 'TREATMENTS'

Feline recombinant SCF was prepared and purified as described previously. Bovine serum albumin solution (100 μg/ml) was prepared by dissolving BSA (Fraction V - Sigma cell culture, Sigma Chemical Co.) in phosphate buffered saline (pH 7.4). All treatments were given in a total volume of one millilitre; the desired dose for each cat was obtained by diluting either SCF or BSA solution with an appropriate volume of PBS. All injections were prepared prior to the start of the experiment and stored in labelled one millilitre syringes at 4°C. Treatments were given by subcutaneous injection, once daily, for seven days. Endotoxin content of the frSCF solution was approximately 2.0 EU/mg SCF and of the BSA solution 22 EU/mg protein.

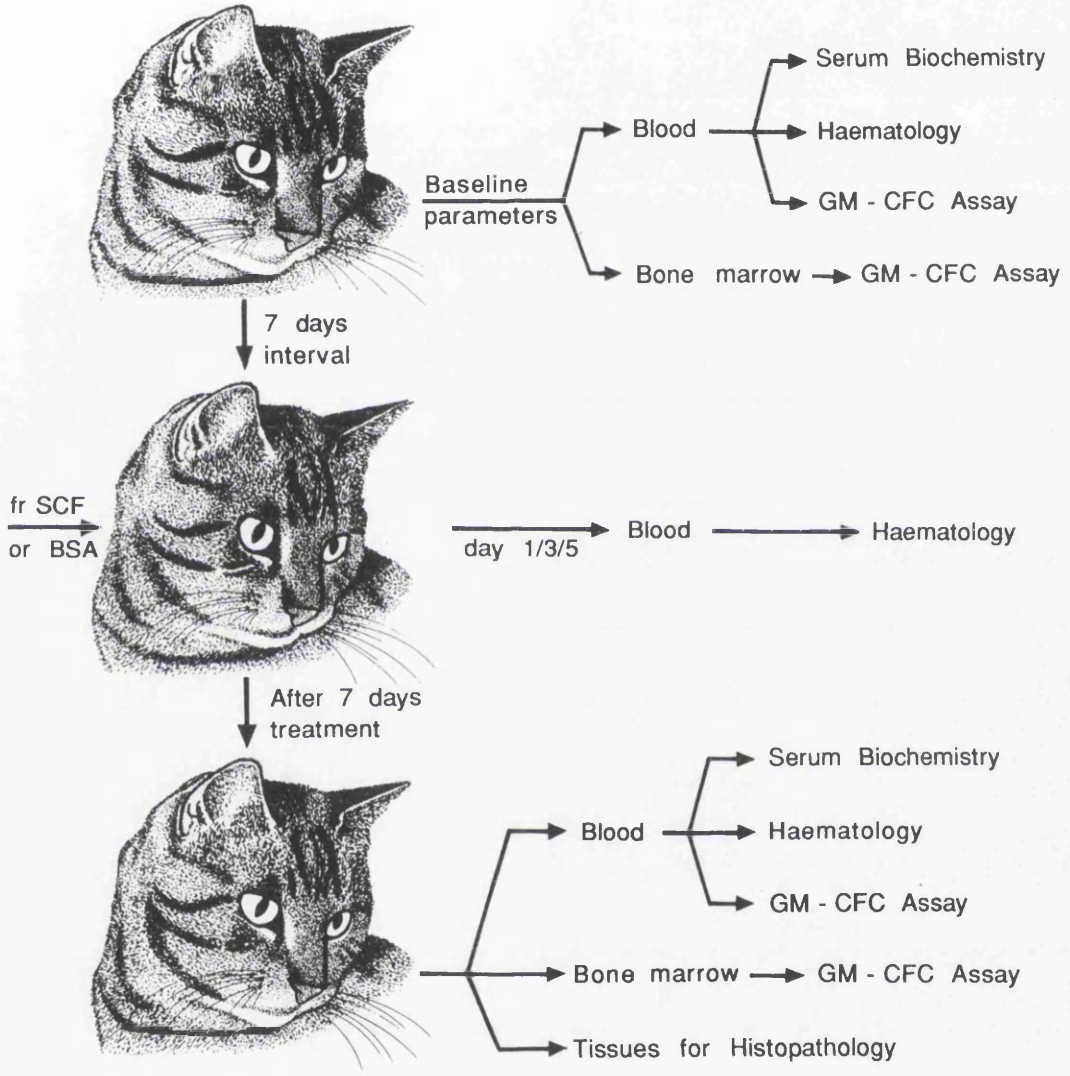


Figure 8.1: Overview of experimental procedure.

8.2.3 MONITORING OF ANIMALS

All cats were examined prior to injections and at intervals throughout the proceeding day. The animals demeanour and appetite were assessed and rectal temperature measured. In the event of any apparent departure from normal, a full clinical examination was carried out.

8.2.4 COLLECTION AND ANALYSIS OF SAMPLES

8.2.4.1 Blood

Animals were restrained manually and blood collected from the cephalic or jugular vein. Pre-euthanasia samples (day eight) were collected by intracardiac puncture whilst the animals were under general anaesthesia; a mixture of ketamine (Ketaset - Willows Francis Veterinary, Crawley, UK) and xylazine (Rompun - Bayer plc, Bury St Edmunds, UK), given by intramuscular injection, was used to anaesthetise the cats. Blood for haematology was collected into one millilitre tubes containing potassium-EDTA (Bibby Sterilin Ltd., Stone, UK) and for biochemistry into plain two millilitre tubes. All haematological analyses were performed in the Department of Veterinary Haematology, University of Glasgow by Mr R. Barron and Mr K. Williamson. Cell counts were obtained using an automated cell counter (ABX Minos Vet - Roche Products Limited) and differential counts performed manually using May-Grunwald-Giemsa stained smears. Serum biochemistry analysis was performed by the Department of Veterinary Biochemistry, University of Glasgow.

8.2.4.2 Bone Marrow

Pre-treatment bone marrow samples were collected under general anaesthesia and post-treatment samples were collected immediately following euthanasia, as detailed in section 2.2.1.7.

8.2.4.3 Tissues

Tissues for histopathology were collected immediately following euthanasia into formal-saline. Samples were processed and stained with haematoxylin and eosin and mounted by Mr I. Macmillan and the staff of the Histopathology Laboratory,

University of Glasgow. Bone marrow, spleen and liver sections were evaluated by Dr S. Toth, Department of Veterinary Haematology, University of Glasgow. Bone marrow, spleen, skin at the site of injection (between the scapulae), skin at a distant site (ventral abdomen), stomach, duodenum, colon and lung sections were stained with astra blue. Cells staining positive for astra blue with a morphology consistent with mast cells were counted using a Unilux-12 microscope (Kyowa, Japan) at 400 x magnification. Five replicate counts were made from each section with the field of view chosen at random.

8.2.5 GM-CFC ASSAYS

Bone marrow mononuclear cells were isolated over Ficoll as detailed in section 2.2.1.7. Peripheral blood was prepared for the GM-CFC assay by lysis of the red blood cells with ACK lysis buffer. Ten millilitres of ACK buffer was added to one to three millilitres of blood and incubated for approximately five minutes at room temperature. White blood cells (WBC) were pelleted by centrifugation at 250 x g for ten minutes. The WBC were then resuspended in tissue culture medium and kept on ice prior to setting up the GM-CFC assay.

The GM-CFC assays were performed as described in section 6.2.3. BMMC were plated at a concentration of 5 x 10^4 cells/ml and peripheral blood WBC at 1 x 10^5 cells/ml. Growth factors were included at the following concentrations: frSCF, 100 ng/ml and hrG-CSF, 20 ng/ml; control plates were also set up in which growth factors were omitted.

8.2.6 STATISTICAL ANALYSIS

Data from the peripheral blood GM-CFC assays were analysed as follows. Data for the single cat receiving 25 μg/kg frSCF was excluded because it would lead any analysis of data to be on an unbalanced design and also because data derived from a single animal could be non-representative. Pre- and post- treatment values were analysed separately in order to ascertain that there were no significant differences between the groups prior to administration of treatments. In one group missing a single data point, an estimate was inserted based on the mean of the five data points in that group. A $\log_e(x+1)$ transformation was performed on the data prior to analysis

and a nested ANOVA adopted with treatment as a fixed effect and cat as a random effect. When the pre- and post- treatment data were considered together, a nested two way analysis of variance with repeated measures and cat as random effect was used.

The number of mast cells in the skin of cats treated with frSCF was compared to the number of mast cells in the skin of control animals at the injection site and a distant skin site. Data was analysed using a three factor analysis of variance with the cat as the random factor.

8.3 RESULTS

Administration of fSCF to cats produced few undesirable side effects. Cats receiving 200 μg/kg frSCF showed mild oedema of the skin of the forelimbs distal to the elbow. This occurred five to seven hours after the first injection of frSCF; the degree of oedema subsided almost completely overnight and did not recur following further administrations of frSCF. No swelling or pain was seen at the site of injection. Cats receiving 200 μg/kg frSCF inconsistently showed mild malaise, manifested as a decrease in activity, which was most evident four to eight hour after administration of the growth factor. Rectal temperatures, monitored at the time of frSCF (or control protein) administration and 4.5 to 6 hours later are shown in Table 8.1. Although, individual cats show some increases over normal these are not consistent, nor are the changes more evident in cats receiving the higher doses of frSCF. This suggests that the relatively mild increases seen may be due to the physiological stress of handling and injections rather than a specific effect of the injected preparations. The experimental cats used for this trial were poorly accustomed to being handled, consequently stress induced pyrexia is not an unexpected finding. Although the control injections of BSA contained a higher level of endotoxin than frSCF preparations, there is no evidence that this had any effect in recipient cats.

The results of serum biochemistry parameters before and after treatments are shown in Table 8.2. Although some of the parameters lie outwith the laboratory reference ranges, there is no indication of any significant decrease in renal function (indicated by

urea and creatinine) or hepatocellular damage (indicated by SAP and ALT) due to administration of frSCF. The decrease of serum globulin to zero in one cat (H31) post-treatment is likely to be a laboratory artefact. Mild elevations in serum creatine kinase are seen in two cats post-treatment, one of which received 100 μg/kg frSCF (H31) the other of which received BSA (H45). Creatine kinase is released from muscle cells when damaged, such increases are non-specific and mild increases can be induced by poor venepuncture technique, injections or minimal exercise (Anderson *et al.*, 1976).

Haematology results are shown in Table 8.3. Results at day eight (pre-euthanasia) were obtained from blood collected from anaesthetised cats by cardiocentesis and are therefore not directly comparable to those obtained at other times. Red blood cell parameters show no significant changes in cats which received frSCF or in control cats except for the appearance of normoblasts (immature nucleated erythrocytes) in cats receiving frSCF. The appearance of normoblasts in the peripheral blood is suggestive of increased erythropoietic activity in medullary or extra-medullary sites. There was no significant change in platelet numbers in cats receiving frSCF (platelet clumping is an artefact commonly seen in feline blood samples). There is a dose dependent, acute increase in neutrophil counts in the cats receiving either 200 μg/kg or 100 μg/kg frSCF which occurs several hours after injection (Figure 8.2). This increase may be due to a mobilisation of the marrow granulocyte reserve or a shift of neutrophils from the marginating to the circulating compartments. The latter phenomenon is commonly seen in cats associated with physiological stress (Jain, 1993a). However, such a cause seems unlikely as minimal elevations are seen in cats receiving either 25 μg/kg frSCF or control cats; thus a specific effect of frSCF is probable. Subsequent neutrophil counts, in blood collected immediately prior to treatments, are within the normal range.

Feline SCF caused a dose dependent increase in circulating granulocyte-macrophage colony forming cells (GM-CFC) (Table 8.4). Few circulating GM-CFC were seen in cats prior to treatment or in cats following administration of BSA or 25 μg/kg frSCF; the colonies derived from these GM-CFC were generally small. Cats receiving 100 to

200 μg/kg frSCF for seven days showed increases in circulating GM-CFC of 10 to 20 fold (Table 8.4); additionally these GM-CFC gave larger colonies on culture (Figure 8.3). Statistical analysis of the data showed that there were no differences in the numbers of circulating CFC in the cats pre-treatment ($p>0.05$). Following treatment with either 100 or 200 μg/kg/day frSCF or control preparation, significant differences in circulating CFC were seen between each of the treatment groups ($p<0.05$). Additionally, the increases in circulating CFC following administration of 100 or 200 μg/kg/day frSCF were statistically significant ($p<0.000$).

GM-CFC assays showed no significant increases in the relative number of CFC in the bone marrow of cats following treatment with frSCF (Table 8.4). Histopathological examination of bone marrow sections revealed no evidence of altered cellularity in any of the cats.

The administration of frSCF produced a dose dependent stimulation of extramedullary haemopoiesis (EMH) within the spleens of treated cats. The spleens of cats receiving 200 μg/kg/day frSCF contained erythroid precursors with high mitotic activity at all stages of differentiation, numerous blast cells, small foci of granulopoiesis and immature megakaryocytes (Figure 8.4). Cats given lower doses of SCF had smaller foci of EMH, mainly erythroid, with some megakaryocytes, whilst these changes were absent in control animals. Occasional foci of erythroid precursors were seen in the liver of cats given 100 or 200 μg/kg/day frSCF but not in control animals.

The number of mast cells was increased approximately five fold in the skin of cats at the site of injection following treatment with 100 or 200 μg/kg/day frSCF for seven days (Table 8.5 and Figure 8.5). No significant increase in the numbers of tissue mast cells was seen in the skin at a distant site in these cats. Similarly, mast cell numbers were not increased in other tissues (Table 8.5).

		Treatment and cat ID No.						
Day	Time	200 μg/kg H32	200 μg/kg H36	100 μg/kg H33	100 μg/kg H31	25 μg/kg H35	control H34	control H45
1	am	ND	ND	ND	ND	ND	ND	ND
	pm	ND	102.3	ND	103.0	101.7	ND	100.6
2	am	101.0	99.4	100.4	100.9	101.2	99.2	100.0
	pm	101.5	102.3	100.4	102.6	101.3	100.0	100.0
3	am	ND	101.2	ND	100.2	101.7	ND	102.0
	pm	100.2	102.0	101.3	102.2	101.8	101.3	100.7
4	am	101.1	101.0	100.5	102.3	102.2	99.6	102.1
	pm	101.7	101.4	100.2	102.0	101.3	100.0	101.4
5	am	101.1	101.6	100.7	102.7	101.7	100.2	100.8
	pm	102.0	101.5	100.7	101.5	100.6	101.1	100.8
6	am	101.6	100.6	101.3	101.4	101.9	100.3	101.6
	pm	102.6	100.9	100.6	102.2	101.0	100.7	101.1
7	am	101.7	99.2	101.4	101.5	101.6	100.7	102.2
	pm	102.2	100.8	101.1	102.1	101.5	102.3	101.7

Table 8.1: Rectal temperature of cats during course of the experiment (°F). Temperatures were measured between 9am to 10am (AM), just prior to the administration of frSCF/control treatments, and between 2.30am to 3.30pm (PM). Normal feline rectal temperature is approximately 101.5°F. ND = not determined.

	200 µg/kg H36		200 µg /kg H32		100 µg /kg H33		100 µg /kg H31	
	PRE	POST	PRE	POST	PRE	POST	PRE	POST
Urea	**10.9**	**10.9**	**10.9**	**11.2**	**10.1**	**9.6**	11.2	**8.7**
Creat.	127	115	149	121	107	**88**	141	115
SAP	55	35	30	31	51	49	36	26
ALT	**61**	**48**	**47**	**48**	**57**	**58**	32	18
TP	61	**51**	68	**59**	64	**50**	64	**21**
ALB	28	26	28	28	28	27	27	**21**
GLOB	33	25	40	31	36	23	37	0
A/G	0.85	1.04	0.7	0.9	0.78	1.17	0.73	
CK	**161**	**679**	**238**	126	**277**	**821**	**156**	**1292**

	25 µg/kg H35		control H34		control H45	
	PRE	POST	PRE	POST	PRE	POST
Urea	9.7	8.4	8.9	9.4	10.4	8.4
Creat.	105	116	127	105	114	118
SAP	47	72	37	34	**102**	**108**
ALT	35	34	**69**	**57**	**41**	**36**
TP	63	**51**	**53**	**43**	61	**53**
ALB	27	28	27	26	30	31
GLOB	36	23	26	17	31	22
A/G	0.75	1.22	1.04	1.53	0.97	1.41
CK	**662**	**447**	131	**442**	**596**	**2030**

Laboratory reference ranges	
Urea	2.7 - 9.2 mmol/l
Creatinine (Creat.)	91 - 180 µmol/l
Serum alkaline phosphatase (SAP)	< 100 iu/l
Alanine aminotransferase (ALT)	< 35 iu/l
Total protein (TP)	60 - 85 g/l
Albumin (ALB)	26 - 36 g/l
Globulin (GLOB)	27 - 45 g/l
Creatine kinase (CK)	<150 iu/l

Table 8.2: Serum biochemistry parameters before and after treatment with frSCF. Cats received the stated dose of frSCF once daily by subcutaneous injection; control cats received 100 µg/kg BSA once daily. Figures in bold type lie outwith the laboratory reference range, the significance of which is discussed within the text.

Table 8.3: Full haematology results (continued overleaf).

H32	Time					
200 µg	0hr	4hr	12hr	3d	5d	8d
RBC	8.05	9.04	7.33	6.64	6.64	5.18
Hb	12.70	14.50	11.80	10.60	10.60	3.10
HT	36.80	42.20	34.60	29.90	30.60	23.60
MCV	46.00	47.00	47.00	45.00	46.00	46.00
MCH	15.70	16.00	16.00	15.90	15.90	45.60
MCHC	34.50	34.30	34.10	35.40	34.60	34.30
Retic	1.90	1.60	1.40	0.70	1.20	0.00
Norm				0.28		0.53
WBC	25.40	45.70	34.40	27.70	27.50	26.40
Neu	16.00	40.67	27.52	18.28	13.48	13.99
BNeu	0.25	1.37	0.00	0.00	0.00	2.11
Lym	6.60	2.74	5.16	6.09	10.18	6.86
Mon	0.25	0.91	0.69	1.11	2.75	1.06
Eos	2.03	0.00	1.03	1.94	1.10	1.58
Bas	0.25	0.00	0.00	0.00	0.00	0.26
PLT	Clump	Clump	100.00	202.00	Clump	Clump

H36	Time					
200 µg	0hr	4hr	12hr	3d	5d	8d
RBC	7.65	10.06	7.27	7.65	6.36	5.36
Hb	12.50	17.30	12.40	12.90	10.70	8.50
HT	34.30	46.30	34.30	35.30	29.90	24.80
MCV	45.00	46.00	47.00	46.00	47.00	46.00
MCH	16.30	17.10	17.00	16.80	16.80	15.80
MCHC	36.40	37.30	36.10	36.50	35.70	34.20
Retic	0.10	1.00	0.50	0.80	0.10	
Norm				0.16	0.91	
WBC	11.60	27.40	19.60	16.00	18.10	15.90
Neu	6.03	23.84	12.94	9.60	7.78	8.90
BNeu	0.00	0.00	0.00	0.00	0.18	0.00
Lym	3.83	1.92	6.08	3.52	7.06	6.04
Mon	0.23	1.10	0.20	0.80	0.36	0.16
Eos	1.51	0.55	0.39	1.28	1.63	0.64
Bas	0.00	0.00	0.00	0.64	0.18	0.16
PLT	236.00	165.00	Clump	115.00	Clump	50.00

H31	Time					
100 µg	0hr	4hr	12hr	3d	5d	8d
RBC	7.40	7.75	6.89	6.23	6.86	4.96
Hb	12.40	13.10	11.90	10.40	11.90	8.00
HT	34.30	36.80	33.30	30.00	33.70	23.60
MCV	46.00	47.00	48.00	48.00	49.00	48.00
MCH	16.70	16.90	17.20	16.60	17.30	16.10
MCHC	36.10	35.50	35.70	34.60	35.30	33.80
Retic	0.30	0.60	0.30	1.20	0.90	
Norm				0.20	0.49	0.18
WBC	16.70	34.20	23.50	20.20	24.70	18.30
Neu	9.35	30.10	15.28	13.13	11.61	13.73
BNeu	0.00	0.00	0.00	0.00	2.47	0.73
Lym	6.18	3.08	7.05	5.05	7.16	2.75
Mon	0.00	0.68	0.24	0.61	1.24	0.37
Eos	1.17	0.34	0.94	1.21	1.73	0.55
Bas	0.00	0.00	0.00	0.00	0.00	0.00
PLT	371.00	375.00	200.00	201.00	186.00	136.00

H33	Time					
100 µg	0hr	4hr	12hr	3d	5d	8d
RBC	Clotted	8.84	7.49	7.68	7.43	5.27
Hb		14.30	12.30	12.20	12.10	8.00
HT		40.30	34.90	34.30	33.10	23.30
MCV		46.00	47.00	45.00	45.00	44.00
MCH		16.10	16.40	15.80	16.20	15.10
MCHC		35.40	35.20	35.50	36.50	34.30
Retic		1.00	0.70	0.70	0.80	0.00
Norm				0.15		
WBC		22.30	20.70	14.90	14.00	11.10
Neu		18.96	18.21	9.69	8.26	9.10
BNeu		0.22	0.00	0.00	0.00	0.22
Lym		2.68	1.66	4.77	5.18	1.67
Mon		0.45	0.62	0.30	0.56	0.11
Eos		0.00	0.00	0.00	0.00	0.00
Bas		0.00	0.21	0.00	0.00	0.00
PLT	Clump	Clump	101.00	237.00	102.00	148.00

Table 8.3: Full haematology results (continued).

H35	Time					
25 µg	0hr	4hr	12hr	3d	5d	8d
RBC	6.83	7.17	7.05	6.86	6.86	5.09
Hb	11.70	12.60	12.30	11.90	12.20	8.30
HT	33.00	36.20	35.90	34.70	34.00	24.80
MCV	48.00	50.00	51.00	51.00	50.00	49.00
MCH	17.10	17.50	17.40	17.30	17.70	16.30
MCHC	35.40	34.80	34.20	34.20	35.80	33.40
Retic	0.30	0.60	0.50	0.60		
Norm			0.21	0.22		0.13
WBC	13.70	19.50	21.20	21.70	14.70	13.20
Neu	6.99	9.36	7.84	12.59	7.06	6.86
BNeu	0.00	0.00	0.00	0.21	0.58	0.26
Lym	4.25	5.85	7.63	6.29	4.41	3.56
Mon	0.00	0.20	0.42	1.09	0.74	0.66
Eos	2.33	3.71	4.88	1.30	1.62	1.32
Bas	0.14	0.39	0.21	0.00	0.15	0.40
PLT	Clump	Clump	20.00	Clump	Clump	21.00

H34	Time					
control	0hr	4hr	12hr	3d	5d	8d
RBC	7.78	7.46	7.19	7.40	7.05	5.12
Hb	12.50	12.00	11.70	11.80	11.50	7.60
HT	35.90	35.50	33.90	33.70	33.40	22.00
MCV	46.00	48.00	47.00	46.00	47.00	43.00
MCH	16.00	16.00	16.20	15.90	16.30	14.8
MCHC	34.80	33.80	34.50	35.00	34.40	34.50
Retic	1.40	0.70	1.00	0.70	0.70	0.00
Norm						
WBC	18.50	25.40	20.70	15.80	21.50	4.40
Neu	9.25	19.56	15.11	9.01	14.62	2.99
BNeu	0.00	0.25	0.00	0.00	0.00	0.00
Lym	8.70	4.06	4.97	6.32	6.45	1.32
Mon	0.19	1.27	0.41	0.47	0.22	0.09
Eos	0.00	0.00	0.00	0.00	0.00	0.00
Bas	0.19	0.25	0.21	0.00	0.22	0.00
PLT	Clump	Clump	89.00	116.00	Clump	204.00

H45	Time					
control	0hr	4hr	12hr	3d	5d	8d
RBC	8.35	8.29	7.84	6.52	7.81	5.52
Hb	14.40	14.30	13.60	11.40	13.90	9.20
HT	39.60	40.00	38.40	32.50	38.40	25.50
MCV	47.00	48.00	49.00	50.00	49.00	46.00
MCH	17.20	17.20	17.30	17.40	17.70	16.60
MCHC	36.30	35.70	35.40	35.00	36.10	36.00
Norm						
Retic	0.60	0.80	0.90	0.80	0.10	
WBC	14.50	20.90	22.90	21.10	20.60	7.40
Neu	8.84	11.50	9.62	10.97	10.71	4.59
BNeu	0.00	0.00	0.00	0.00	0.20	0.00
Lym	4.50	7.73	11.22	8.23	7.62	2.44
Mon	0.00	0.63	0.23	0.63	0.82	0.15
Eos	1.16	0.84	1.83	1.06	1.03	0.22
Bas	0.00	0.21	0.00	0.21	0.21	0.00
PLT	Clump	Clump	Clump	Clump	Clump	54.00

Parameter	Normal Range
RBC	7.77 +/- 1.32
Hb	12.16 +/- 2.01
HT	33.27 +/- 4.90
MCV	43.08 +/- 3.00
MCH	15.65 +/- 1.38
MCHC	36.42 +/- 1.62
Retic	
WBC	19.05 +/- 8.30
Neu	10.38 +/- 6.49
BNeu	
Lym	7.08 +/- 3.33
Mon	0.57 +/- 0.49
Eos	0.96 +/- 0.60
Bas	0.089 +/- 0.12
PLT	

RBC - red blood cell (x 10^{12}/L); **Hb** - haemoglobin (g/dl); **HT** - haematocrit (L/L); **MCV** - mean cell volume (fl); **MCH** - mean cell haemoglobin (pg); **MCHC** - mean cell haemoglobin concentration (g/dl); **Retic** - reticulocyte; **WBC** - white blood cell (x 10^{9}/L); **Neu** - neutrophil; **BNeu** - basophillic neutrophil; **Lym** - lymphocyte; **Mon** - monocyte; **Eos** - eosinophil; **Bas** - basophil; **PLT** - platelet (x 10^{9}/L).

Table 8.3: Full haematology results. Cat ID numbers and treatment are indicated (dose of frSCF in µg/kg/day or control). Normal values are derived from research cats housed in the same facility (Grant, 1995).

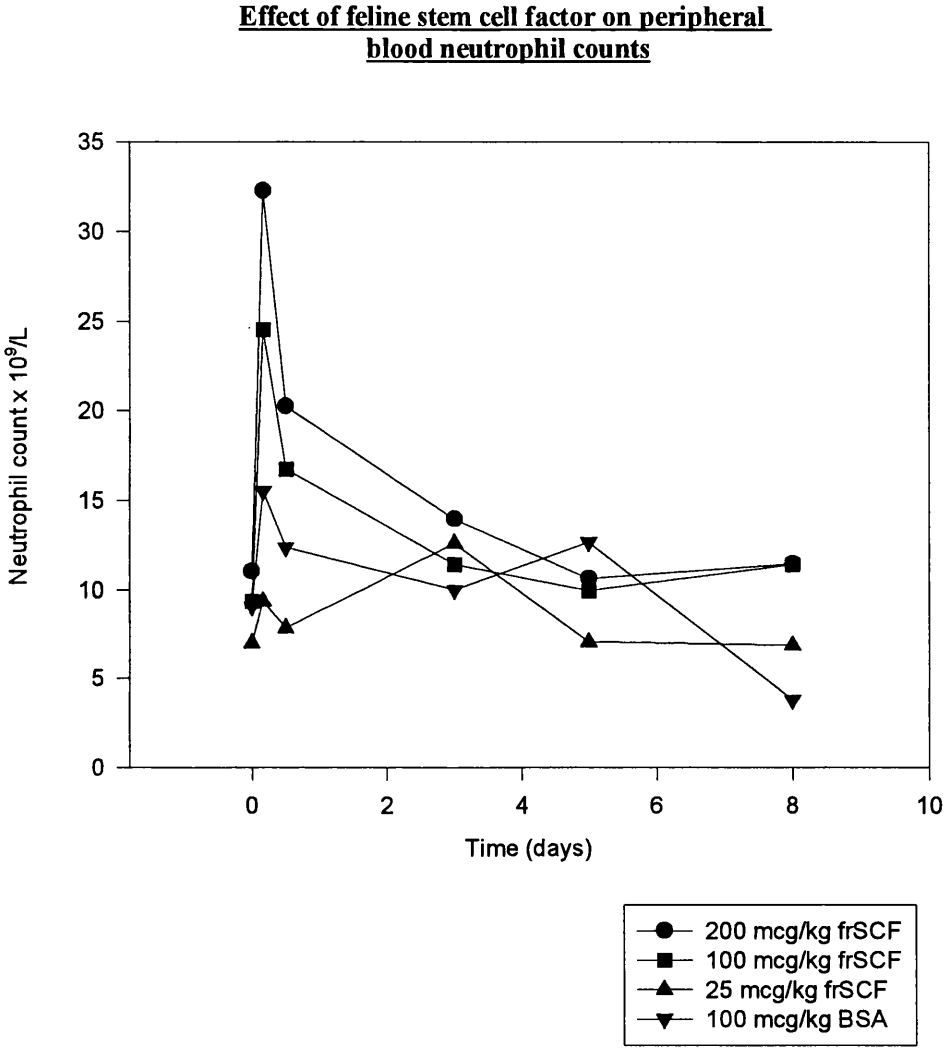


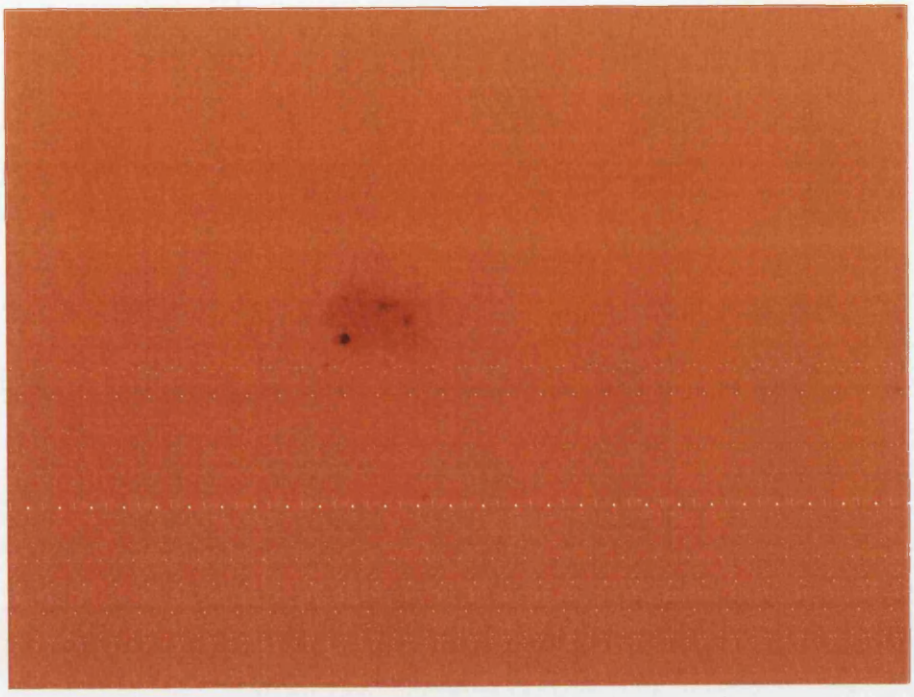
Figure 8.2: Effect of frSCF upon the peripheral neutrophil counts of cats. There is a dose dependent, acute increase in neutrophil counts in the cats receiving either 200 μg/kg or 100 μg/kg frSCF which occurs between 0 - 12 hours after injection.

Marrow	H32	200	H36	200	H31	100	H33	100	H35	25	H34	C	H45	C
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
A	4	NR	2	26	2	18	2	2	0	10	14	4	0	14
	8	2	2	12	2	6	4	0	0	4	12	4	0	4
	4	8	10	8	8	14	6	0	2	4	4	0	0	2
Mean	**5.3**	**5.0**	**4.6**	**15.3**	**4.0**	**12.7**	**4.0**	**0.7**	**0.7**	**6.0**	**10.0**	**2.7**	**0.0**	**6.7**
SD	2.3	4.2	4.6	9.4	3.4	6.1	2.0	1.2	1.2	3.5	5.3	2.3	0.0	6.4
D	60	NR	68	150	84	52	100	56	42	NR	70	30	NR	44
	64	36	62	98	48	38	70	36	48	58	70	78	NR	64
	40	36	54	108	42	52	80	8	50	28	28	62	NR	46
Mean	**54.7**	**36.0**	**61.3**	**119**	**58.0**	**47.3**	**83.3**	**33.3**	**46.7**	**43.0**	**56.0**	**56.7**		**51.3**
SD	12.9	0.0	7.0	27.6	22.7	8.0	15.3	24.1	4.2	21.2	24.3	24.4		11.0

Blood	H32	200	H36	200	H31	100	H33	100	H35	25	H34	C	H45	C
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
A	2	0	0	1	0	1	0	1	0	0	1	0	0	0
	0	0	0	2	0	1	1	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Mean	**1.0**	**0.0**	**0.0**	**1.0**	**0.0**	**0.7**	**0.3**	**0.4**	**0.0**	**0.0**	**0.7**	**0.0**	**0.0**	**0.0**
SD	1.0	0.0	0.0	1.0	0.0	0.6	0.6	0.6	0.0	0.0	0.6	0.0	0.0	0.0
D	0	16	0	16	0	6	0	3	0	NR	0	2	NR	1
	2	7	0	14	0	4	0	3	0	1	1	0	NR	0
	2	NR	0	6	1	1	0	3	0	0	0	0	NR	0
Mean	**1.4**	**11.5**	**0.0**	**12.0**	**0.4**	**3.7**	**0.0**	**3.0**	**0.0**	**1.0**	**0.3**	**0.7**		**0.4**
SD	6.4	6.4	0.0	5.3	0.6	2.5	0.0	0.0	0.0	1.0	0.6	1.2		0.6

Table 8.4: GM-CFC counts obtained before and following treatments indicated above (C = control; numbers refer to dose of frSCF in μg/kg/day). A = cells plated in the absence of growth factors; D = cells plated with frSCF (100 ng/ml) and hGCSF (20 ng/ml). Colony counts are given per 10^5 cells. NR = no results obtained.

A.



B.

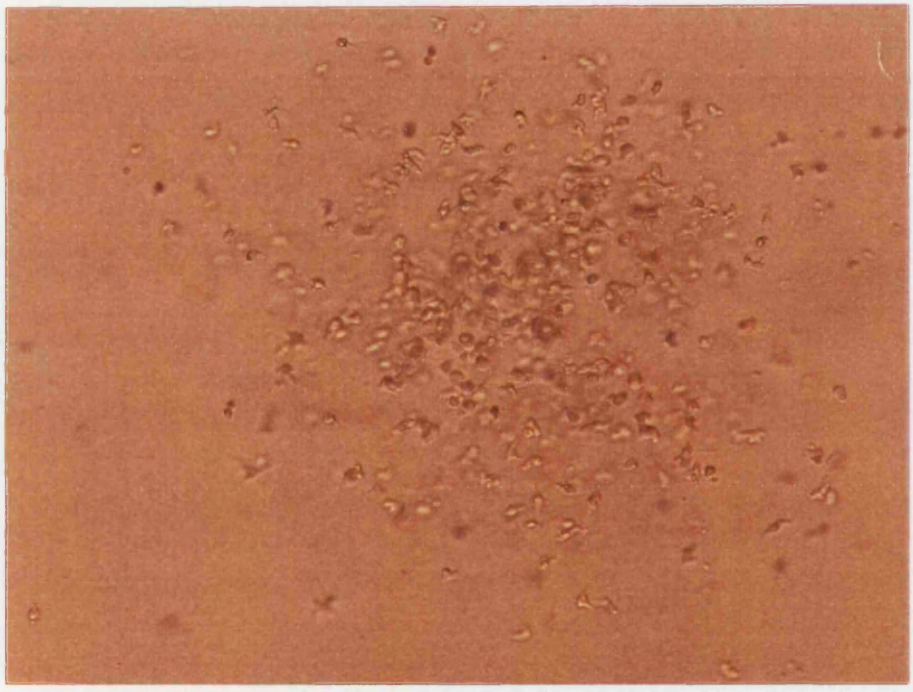
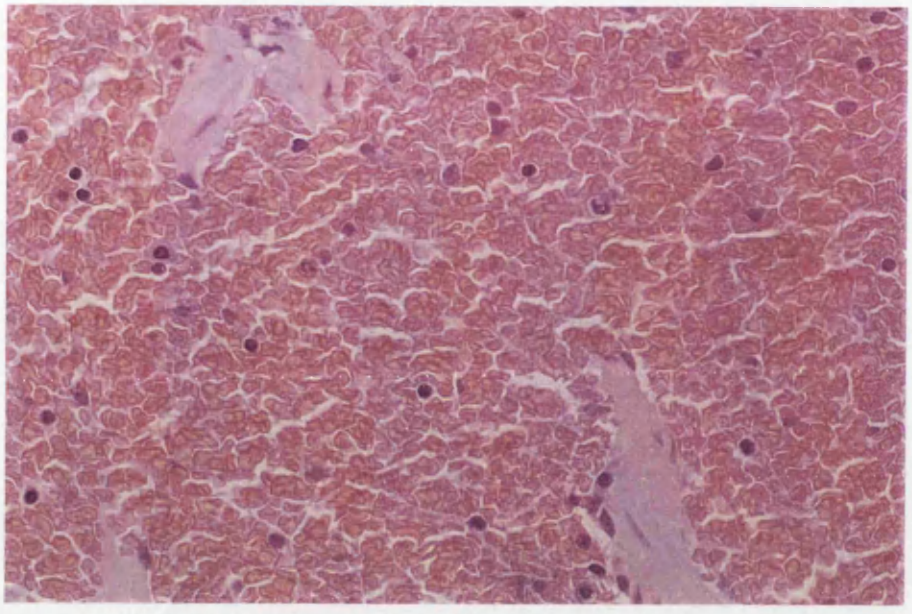


Figure 8.3: Typical colonies derived from circulating colony-forming progenitor cells in **A.** untreated or control cats and **B.** cats receiving 100 - 200 μg/kg/day frSCF after seven days of treatment. Both colonies are shown at an objective magnification of 10 x; colonies are clearly larger in cats receiving frSCF.

A.



B.

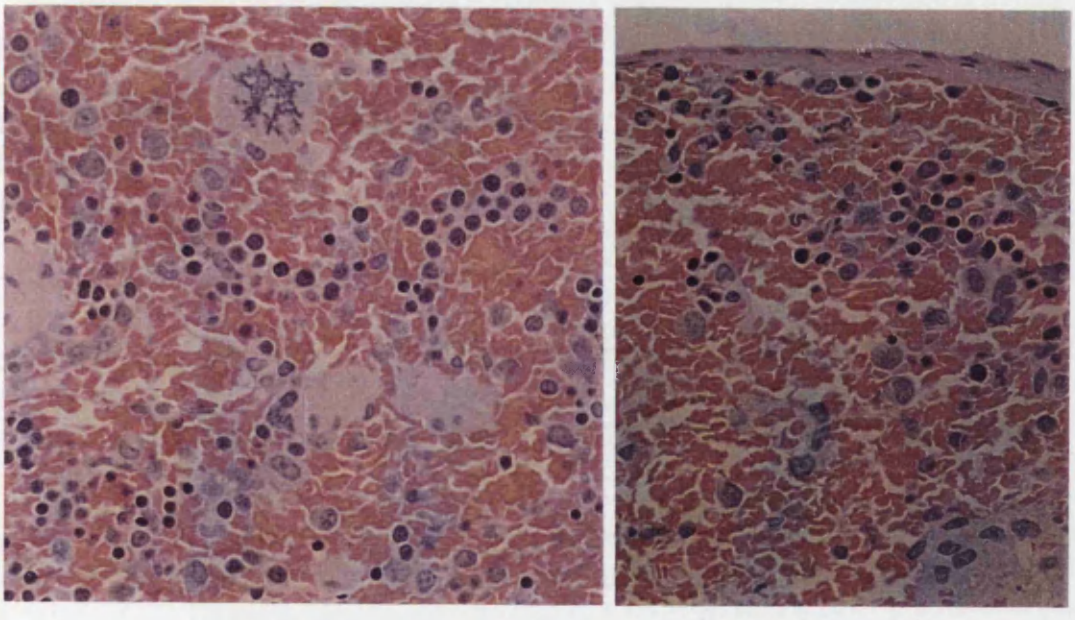
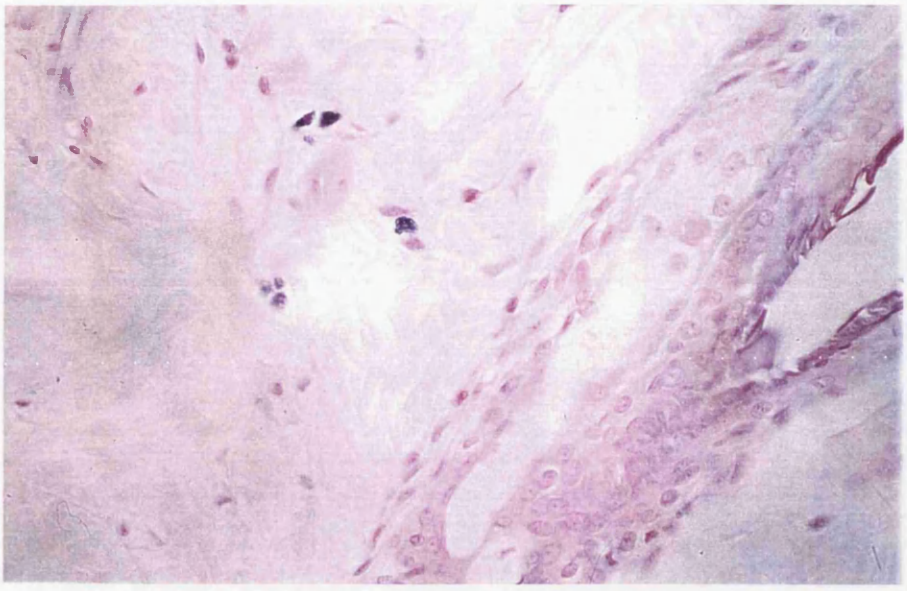


Figure 8.4: Extramedullary splenic haemopoiesis is promoted by frSCF. **A.** spleen of control cat; **B.** spleen following administration of 200 μg/kg/day frSCF for seven days. Increased immature nucleated red blood cells and an occasional foci of granulopoiesis and immature megakaryocytes are seen in B.

Cat ID/treatment Tissue examined	H32 200	H36 200	H31 100	H33 100	H35 25	H34 C	H45 C
Bone Marrow	scant	scant	scant	scant	scant	scant	scant
Spleen	1.0 +/- 1.7	0.4 +/- 0.5	0.4 +/- 0.5	2.2 +/- 1.9	0.8 +/- 1.3	1.0 +/- 1.7	0.2 +/- 0.4
Liver	scant	scant	scant	scant	scant	scant	scant
Stomach	4.8 +/- 1.3	2.6 +/- 0.5	3.8 +/- 0.8	3.6 +/- 1.5	3.4 +/- 1.5	1.8 +/- 1.9	ND
Duodenum	2.4 +/- 2.1	3.4 +/- 1.3	2.2 +/- 1.5	2.6 +/- 1.3	4.6 +/- 1.7	2.0 +/- 1.0	3.2 +/- 3.0
Colon	2.6 +/- 2.3	0.8 +/- 0.4	4.0 +/- 2.5	1.0 +/- 0.7	2.0 +/- 1.9	3.0 +/- 1.9	1.0 +/- 1.0
Lung: bronchial	3.4 +/- 2.2	2.6 +/- 1.8	4.0 +/- 1.2	0.0 +/- 0.0	3.8 +/- 2.6	1.6 +/- 1.8	1.6 +/- 2.3
Lung: alveolar	1.4 +/- 1.3	0.6 +/- 0.9	1.8 +/- 1.6	0.2 +/- 0.4	0.0 +/- 0.0	0.2 +/- 0.4	0.2 +/- 0.4
Skin: injection site	50.6 +/- 8.2	50.4 +/- 4.4	66.8 +/- 8.2	34.3 +/- 4.5	16.4 +/- 4.7	9.8 +/- 3.9	9.0 +/- 4.7
Skin: distant site	10.0 +/- 4.2	6.4 +/- 1.8	12.0 +/- 3.7	13.0 +/- 2.2	12.4 +/- 3.3	9.6 +/- 5.0	9.6 +/- 5.0

Table 8.5: Mast cell counts in the tissues of cats treated with rfSCF and in control cats. The counts shown are the mean of five counts per high power field of view +/- the standard deviation. Numbers of mast cells are significantly increased after seven days in the skin at the site of injection in cats receiving 100 or 200 μg/kg/day frSCF ($p < 0.05$; data analysed using a three way analysis of variance).

A.



B.

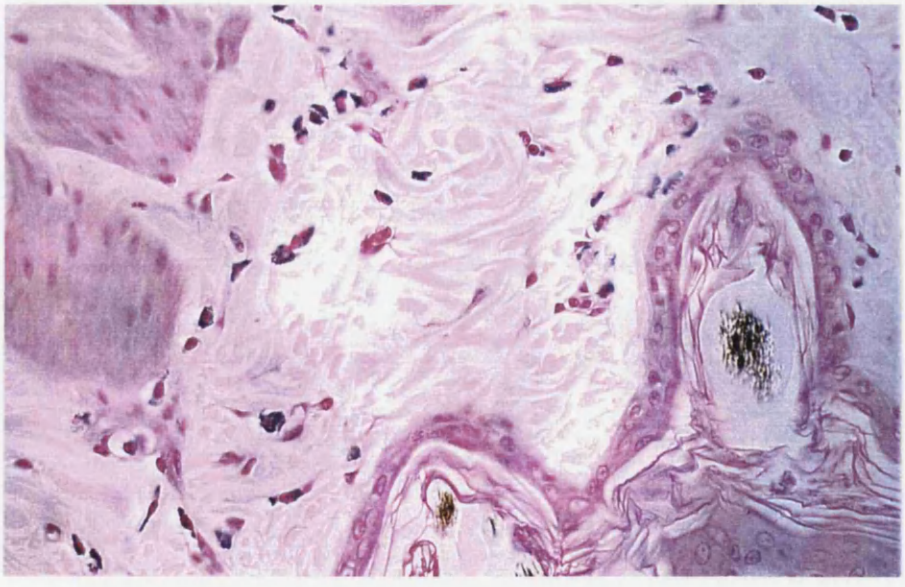


Figure 8.5: Recombinant fSCF increases the number of mast cells in the skin of treated cats at the site of injection. **A.** skin of control cat; **B.** skin of cat treated with 200 μg/kg/day frSCF for seven days. Mast cells appear round with blue cytoplasmic granules.

8.4 DISCUSSION

8.4.1 FELINE RECOMBINANT SCF STIMULATES HAEMOPOEISIS *IN VIVO*

The administration of recombinant cytokines may produce changes in mature blood cell numbers by two distinct mechanisms. Rapid alterations may occur due to the redistribution of blood cells between the circulation and other tissue compartments. More delayed changes in peripheral blood parameters can arise due to alterations in blood cell production i.e. haemopoiesis. Feline rSCF produced a rapid neutrophilia following treatment, an effect also seen in rats given mrSCF (Ulich *et al.*, 1991). Unlike mrSCF, however, feline rSCF does not produce an acute lymphocytosis.

The effects of rSCF upon haemopoiesis have been studied in a number of species, and some differences exist. Baboons given 200 μg/kg/day hrSCF show increases in blood neutrophils after one to two weeks with maximal numbers seen after three to four weeks, thereafter levels fall slightly. RBC counts increase after one to two weeks, peak at three weeks and decrease thereafter; increases in lymphocyte, monocyte, eosinophil, basophil and reticulocyte counts are also seen over a similar time scale (Andrews *et al.*, 1991; Andrews *et al.*, 1992a). Mice treated with rmSCF show increases in neutrophil, lymphocyte, monocyte and reticulocyte numbers after one to two weeks (Molineux *et al.*, 1991). Dogs or rats administered rSCF show similar changes in neutrophil counts but little change in RBC, lymphocyte, monocyte, eosinophil, basophil or reticulocyte counts (Ulich *et al.*, 1991; Schuening *et al.*, 1993). No significant change in platelet counts is seen in any of these species. The failure to demonstrate such increases in mature blood cells following administration of frSCF was probably due to the limited time scale of this experiment. The major stimulatory effect of SCF is upon primitive progenitor cells; increases in mature cell numbers would occur only after these progenitor cells have matured.

In contrast to the variable nature of SCF induced changes in mature blood cell numbers, SCF administration consistently produces increases in bone marrow progenitor cells of multiple lineages (e.g. BFU-E, CFU-GM and CFU-Mix) irrespective of species. More primitive progenitors are also affected, with increases in

marrow HPP-CFC seen in baboons (Andrews *et al.*, 1992a) and increases in marrow CFU-S in mice (Molineux *et al.*, 1991). Despite the lack of increase in blood platelet counts, BM megakaryocytes may be similarly increased (Andrews *et al.*, 1991; Schuening *et al.*, 1993). Similar increases in BM progenitors have been observed in human patients receiving 50 μg/kg/day hrSCF for 15 days (Tong *et al.*, 1993). No changes in the relative number of BM colony forming cells was demonstrated in this study for cats treated with frSCF. This may have been due to the limited period of treatment. Determination of the absolute number of BM progenitors would give a more accurate indication of the ability of SCF to stimulate increases in their number. Feline SCF induced no changes in BM cellularity, in contrast to the increased cellularity seen in dogs (Schuening *et al.*, 1993) and baboons (Andrews *et al.*, 1991) following SCF treatment, albeit for a longer time period.

Increased extramedullary haemopoiesis was evident in the spleen and to a lesser extent the liver of cats receiving 100 to 200 μg/ml/day frSCF for seven days. Developing haemopoietic cells within the spleens of treated animals were mainly of the erythroid series, with lesser numbers of immature cells of granulocyte and megakaryocytes lineages evident. The appearance of normoblasts in the peripheral blood reflects such increased erythropoietic activity. This differential effect is in apparent contrast to previous reports of SCF as a multilineage growth factor but interestingly reflects the major haemopoietic defect seen in mutant *Sl* and *W* mice, that of reduced erythropoiesis. However, this may be an apparent rather than a genuine difference, reflecting the maturation time of the respective cell lineages. The development of reticulocytes from stem cells takes approximately seven days in humans (Thompson, 1979) whilst granulocytes take approximately fourteen days to mature from stem cells (Bainton *et al.*, 1971). Thus a growth factor, such as SCF, acting on multipotential progenitors will likely produce changes in numbers of immature erythroid cells sooner than changes in cells of the granulocyte series, assuming a non-differential effect. It is likely that had frSCF been administered for a longer time period a more profound increase in developing cells of the granulocyte and megakaryocyte series would have been seen.

Increased numbers of haemopoietic progenitor cells in the spleen of frSCF treated cats may have been due to the direct stimulation of progenitor cells normally present within the spleen or may have resulted from the migration of marrow progenitor cells to the spleen and their subsequent proliferation therein. The latter mechanism has been observed during the recovery of mice from haemolytic anaemia induced by phenylhydrazine (Broudy *et al.*, 1996) or treatment with the antibiotic thiamphenicol (Goris *et al.*, 1990). It has been postulated that the splenic microenvironment is more suited to the rapid generation of erythrocytes, compared to the bone marrow, perhaps due to its greater capacity for expansion (Harrison *et al.*, 1994). Conversely, recovery of granulocyte numbers following after a treatment of mice with thiamphenicol is mainly associated with maturation of granulocyte progenitors within the bone marrow rather than the spleen (Goris *et al.*, 1990). Such compartmentalisation of haemopoiesis may provide an alternative explanation for the observation that the spleens of frSCF treated cats mainly showed evidence of increased erythropoiesis. However, this may be a feature peculiar to mice, a species in which the spleen contributes significantly to steady state haemopoiesis in the normal adult, particularly with regard to erythropoiesis (Jain, 1986). Determination of the absolute numbers of primitive CFC in feline BM and spleen would help to clarify their relative contributions to haemopoiesis during periods of increased demand in the cat.

8.4.2 MOBILISATION OF COLONY-FORMING PROGENITOR CELLS

Feline rSCF stimulated increases in both the absolute (per litre) and relative (per 10^5 cells) number of circulating colony-forming progenitor cells in cats given 100 to 200 μg/kg/day frSCF. The numbers of circulating GM-CFC in normal dogs (De Revel *et al.*, 1994) and baboons (Andrews *et al.*, 1992b) are approximately 3 to 5 per 10^5 PBMC and 1 to 8 per 10^5 buffy coat cells cultured, respectively. The number of GM-CFC in normal cats, reported herein, was 0 to 1.4 per 10^5 blood cells cultured. It seems likely that this difference reflects a failure of the clonal assay to support the growth of feline GM-CFC rather than a genuine species difference. The availability of other recombinant cytokines (especially GM-CSF and G-CSF) should enable these

cells to be cultured more effectively and enable more accurate determination of their numbers; alternatively culture supernatants derived from feline cells could be used as a source of colony stimulating activity (e.g. FEA cultures as used by Testa *et al.*, 1983 and Linenberger and Abkowitz, 1992). Recombinant canine SCF increases circulating GM-CFC to 26 to 62 per 10^5 PBMC when given at 200 μg/kg/day for eight days (De Revel *et al.*, 1994). Baboons treated with the same dose of hrSCF for six days show increases to 27 to 56 per 10^5 buffy coat cells cultured (Andrews *et al.*, 1992b). The numbers of circulating GM-CFC in treated cats rose by a similar factor, but absolute numbers remained lower, again probably due to deficiencies in the assay technique.

The mechanisms responsible for the mobilisation of stem and progenitor cells from the bone marrow are poorly understood. It is known, however, that a number of cytokines are able to upregulate or downregulate adhesion molecules on both haemopoietic and endothelial cells, which may provide a mechanism for the release of progenitor cells from the bone marrow. SCF may downregulate progenitor cell expression of the SCF receptor and thus disrupt the attachment mediated by membrane associated SCF and its receptor (Mauch *et al.*, 1995). It is conceivable that the administration of high levels of soluble rSCF may competitively bind to progenitor cell SCF-R and disrupt binding to the endogenous cytokine. SCF and its receptor are believed to direct progenitor cell migration *in vivo* during embryogenesis (Matsui *et al.*, 1990) and SCF is also a potent chemotactic and chemokinetic factor for haemopoietic cells *in vitro* (Okumura *et al.*, 1996). The mobilisation of progenitor cells by SCF may, therefore, be mediated, at least in part, by active induction of their migration.

Stem cell factor has been used in baboons (Andrews *et al.*, 1992b), dogs (De Revel *et al.*, 1994) and mice (Briddell *et al.*, 1993) to mobilise peripheral blood stem cells (PBSC) capable of marrow engraftment. This study suggests that feline SCF may be used to develop methods for peripheral stem cell transplantation in this species. The development of antibodies to progenitor cell markers (e.g. CD34) or improved *in vitro* assays for more primitive feline progenitor cells would help to establish whether frSCF mobilised primitive progenitors are likely to be capable of marrow engraftment.

Prior to considering the use of such techniques in a clinical setting, it would be necessary to demonstrate the ability of mobilised progenitor and stem cells to successfully repopulate the marrow of recipient animals. Protocols for the mobilisation of PBSC in humans generally use stem cell factor in combination with other cytokines such as G-CSF or following chemotherapy due to the ability of such combinations to act synergistically (discussed in chapter nine). The development of other recombinant feline cytokines will allow investigation of such applications in the cat.

8.4.3 POTENTIAL SIDE-EFFECTS OF SCF THERAPY

There were few side effects associated with the administration of frSCF, which is in agreement with other studies using animal models. Adverse effects that have been reported in other species, following treatment with SCF, appear to be largely mediated via its effects upon mast cells. Baboons given 200 μg/kg/day hrSCF, by intravenous infusion showed no adverse effects except one animal which developed transient wheezing, respiratory distress and facial oedema. Its use in dogs at a dose rate of 200 μg/kg/day produced facial oedema during the first few days of therapy, which then subsided (Schuening *et al.*, 1993). When used in the same species at 100 μg/kg/day, no initial adverse effects were seen, but with continued use facial oedema, pyrexia, loss of appetite and general malaise were seen after five weeks (Dale *et al.*, 1995). The most frequent abnormalities reported in a Phase I trial in human patients with advanced breast cancer were dermatological associated, including urticaria at local (injection site) and distant sites. At a dose of 50 μg/kg/day, four out of ten patents developed dose-limiting upper respiratory tract symptoms, including cough, laryngospasm and hoarseness (Demetri *et al.*, 1993). Thus the maximum tolerated dose of rSCF appears less in humans than for animal models. Premedication with histamine receptor antagonists and β-agonists may reduce the incidence of serious adverse effects (Glaspy, 1996).

Humans also appear to show greater sensitivity to the systemic effects of SCF upon mast cell proliferation, exhibiting increased dermal mast cell numbers at sites distant to that of the injected cytokine, following subcutaneous injection of 50 μg/kg/day

hrSCF for fourteen days (Costa *et al.*, 1996). Conversely, administration of 100 μg/kg/day of rhSCF to primates for three weeks produces local but not distant increases in dermal mast cell numbers (Galli *et al.*, 1993). The effect of frSCF, administered to cats, appears similar in this respect, producing only a local increase in mast cell numbers. Unlike rhSCF administered to primates at a similar dose (Galli *et al.*, 1993), frSCF treatment of cats did not produce an increase in mast cells in the liver, spleen or bone marrow. This may reflect a genuine species difference in biological activity or distribution or may be due to the shorter period of administration of the recombinant cytokine in this study. The local increase in mast cells seen may result either from local proliferation of tissue mast cells and/or due to chemoattraction of mast cells to the injection site.

8.4.4 SUMMARY

The limited duration of this experiment likely restricted the extent to which frSCF induced increases in haemopoietic activity were seen. However, it is clear from studies in other species that recombinant SCF is able to act as a multilineage growth factor *in vivo*. That the effects of SCF *in vivo* are so marked in comparison to its effects as a single agent *in vitro*, may be due to its ability to act synergistically with endogenous growth factors or to the permissive effects of other components of the haemopoietic microenvironment (Andrews *et al.*, 1991; Galli *et al.*, 1994). The ability of frSCF to stimulate haemopoiesis and to increase circulating haemopoietic progenitor cells with no serious adverse effects implicates the growth factor as a potentially useful therapeutic agent in the domestic cat. This is considered within the proceeding chapter.

CHAPTER NINE - GENERAL DISCUSSION

9.1 INTRODUCTION

The primary aims of this project were to clone the feline homologue of the cytokine stem cell factor (SCF), express the recombinant protein and characterise its biological activity *in vitro* and *in vivo*. These objectives were largely achieved and provide the background for future work investigating the role of the cytokine in feline haemopoiesis in health and disease. Furthermore, the demonstration that frSCF stimulates haemopoiesis and increases circulating progenitor cells *in vivo* suggests that the cytokine may provide a useful therapeutic agent in domestic cats. This chapter discusses the progress that has been made in the introduction of cytokines to clinical use in veterinary species and then considers the potential clinical applications of SCF in both humans and cats.

9.2 THE CLINICAL USE OF CYTOKINES IN DOMESTIC ANIMALS

There has been a great deal of interest in the potential applications of cytokines in domestic animals, both where the animals are used as models for human disease and in the specific treatment and prevention of animal diseases. Due to the limited or non availability of species specific cytokines, initial studies used human or murine homologues. However, such an approach is limited by two main factors. In the case of a number of cytokines, there is restricted or no activity in the non-native species. This is particularly evident for cytokines which exhibit low inter-species homology. Human and murine interleukin-3 proteins, for example, are only 29% homologous (Yang *et al.*, 1986), and show no cross species activity (Gearing *et al.*, 1994); it is not unexpected, therefore, that administration of human IL-3 to dogs has no significant effects (Ciekot *et al.*, 1991). Where significant cross-species activity does exist, the use of a heterologous cytokine may lead to acute allergic reactions (signs of which include skin rashes, pyrexia and arthralgia) and prolonged administration can result in the production of neutralising antibodies to the cytokine in the recipient animal. Neutralising antibodies are seen in approximately 20% of dogs and 30% of cats following treatment with recombinant human erythropoietin (EPO). Furthermore, these antibodies may cross-react with the animals own EPO, leading to a non-

regenerative anaemia (Gieger, 1992). Similar problems have been reported following the administration of recombinant human granulocyte colony stimulating factor to dogs (Lothrop *et al.*, 1988). There is, however, less tendency for animals on immunosuppressive drugs to develop such antibodies, a situation which can arise when such cytokines are used in combination with conventional chemotherapy for the treatment of neoplastic disease (Goodman *et al.*, 1990; Henry *et al.*, 1990). Heterologous cytokines may also be considered for short term use, where antibody formation is unlikely to occur.

The molecular cloning of species specific cytokines and the subsequent production of recombinant proteins largely overcomes these problems, however it should be remembered that the choice of protein expression system can also influence both the biological activity and immunogenicity of such cytokines, even where a homologous cytokine is utilised (discussed in chapter four). Significant progress has been made within the last decade in the cloning of cytokines in both large and small veterinary species. Following production of the recombinant proteins, a number of experimental trials have been performed to evaluate their potential for prevention and treatment of disease. The potential range of applications of cytokines to clinical veterinary medicine is broad, illustrated by several examples of current interest. Equine interferon γ has been cloned and its use as a vaccine adjuvant and an antiviral agent are being investigated (Nicolson *et al.*, 1994). Recombinant canine G-CSF has been used in the dog to reduce the myelosuppressive effects of chemotherapy associated with drugs such as mitoxantrone (Ogilvie *et al.*, 1992). Bovine cytokines, including IL-2 , IL-1β and G-CSF have been investigated for their potential in the prevention and treatment of mastitis (Nickerson *et al.*, 1989a; Nickerson *et al.*, 1989b; Coyle *et al.*, 1992).

9.3 CLINICAL APPLICATIONS OF STEM CELL FACTOR IN HUMANS

9.3.1 TRANSPLANTATION THERAPY

9.3.1.1 Peripheral blood stem cell transplantation

Peripheral blood stem cells (PBSC) are being increasingly used in humans, as a source of marrow repopulating cells, in preference to traditional BM transplantation techniques. Autologous bone marrow transplantation (ABMT) is frequently used following myelosupressive chemotherapy or radiotherapy with the aim of rapidly restoring normal haemopoietic function, in patients where allotransplantation is not possible. Peripheral blood stem cell transplantation (PSCT) is used in the same setting, with identical objectives. PSCT, however, has a number of distinct advantages over the use of bone marrow for autologous transplantation (Kanz *et al.*, 1993; Kessinger, 1993; Molineux and Dexter, 1995):

i. PSCT is possible in patients with abnormalities that hamper attempts to obtain a marrow sample by aspiration, including hypocellularity, fibrosis or neoplastic infiltration of the bone marrow or tumour metastases within the overlying skeletal bone.
ii. PSCT is often associated with more rapid recovery of normal haemopoietic parameters (especially peripheral blood platelet and neutrophil counts) following transplantation, than is seen with ABMT.
iii. PBSC collection is possible without recourse to general anaesthesia, and can take place in an outpatient setting.

Typical methodologies for PSCT comprise a regime for mobilisation of stem cells followed by cell collections via leukopheresis. The cells may then be frozen and reinfused into the patient following myelosupressive chemotherapy. PBSC may be mobilised following chemotherapy alone, by the use of haemopoietic growth factors or by a combination of the two techniques. It was initially recognised that PBSC were elevated following high dose chemotherapy, coinciding with the recovery of

WBC and/or platelet counts. Increases in circulating CFU-GM of 7 - 25 fold following chemotherapy have been reported, with cyclophosphamide producing higher and more predictable increases. A number of colony stimulating factors have been demonstrated to produce consistent rises in PBSC. G-CSF increases circulating CFU-GM by 4 - 46 fold in healthy humans. GM-CSF produces increases of 3 - 18 fold, but has a higher incidence of side effects such as aching bones, fever and joint pain (reviewed by Craig, 1994). SCF alone produces a variable rise in PBSC or progenitor cells, the effect depending upon dose, duration of therapy, method of administration and interspecies differences, as discussed in chapter eight. Mobilisation of PBSC in humans using SCF alone is constrained by dose-limiting side effects. However, in combination with G-CSF, SCF produces marked increases in PBSC, with minimal side effects. Low-doses of SCF in humans (~10 μg/kg/day), when combined with G-CSF, produce a three fold greater rise in peripheral $CD34^+$ cells than G-CSF alone (Briddell, 1994; Glaspy, 1994). In addition, cells mobilised in this way appear more capable of successfully engrafting animals following lethal radiation than cells mobilised with either agent alone (Andrews *et al.*, 1995). This synergy between SCF and G-CSF has also been reported in mice (Yan *et al.*, 1994), dogs (De Revel *et al.*, 1994) and baboons (Andrews *et al.*, 1994). The use of combinations of growth factors following high dose chemotherapy produces still greater rises in PBSC (Craig, 1994). It is likely that hrSCF will be an important component of future protocols for PSCT in humans.

9.3.1.2 Bone marrow transplantation

Combinations of growth factors, including SCF, may also be used to improve the efficiency of standard bone marrow transplantation procedures. The use of growth factors prior to the harvesting of bone marrow for transplantation results in an expansion of the progenitor and stem cell populations within the donor marrow. Their subsequent utilisation for bone marrow transplantation may facilitate accelerated engraftment (Morstyn *et al.*, 1994; Bodine *et al.*, 1996).

9.3.1.3 *Ex vivo* expansion of progenitor cells

The maintenance and expansion of haemopoietic progenitor and stem cells *in vitro* has been suggested as a method to increase the number of cells available for engraftment. Pluripotent haemopoietic stem cells (PHSC) and progenitor cells, derived from marrow, peripheral blood or umbilical cord blood may be incubated *in vitro* with a combination of growth factors; following expansion of cell numbers they may used for transplantation. Successful transplantation requires sufficient progenitor cells to provide short-term haemopoietic reconstitution and also sufficient cells with long term repopulating ability (PHSC). A primary concern regarding the use of *ex vivo* expanded cells for transplantation has been the loss of PHSCs in culture due to their differentiation into more mature progenitors. However, *ex vivo* expanded cells have been used successfully to produce long term engraftment of mice (Neben *et al.*, 1994). Additionally, the expansion of cells in this way, by producing more mature progenitors, may allow faster recovery of normal haematological parameters following engraftment (Han *et al.*, 1993). SCF has been frequently included in the combination of growth factors used to expand cell populations in this way, although optimum protocols remain to be determined (Molineux and Dexter, 1995). Additionally, cell populations expanded in this way are amenable to infection with retrovirus vectors and may be suitable targets for gene-transfer therapy (Bernad *et al.*, 1994; Dunbar *et al.*, 1996). Future possibilities for *ex vivo* expansion include the large scale production of red blood cells for transplantation or dendritic cells for immunotherapy (McAdams *et al.*, 1996).

9.3.2 Treatment of Cytopenias

The potential use of SCF for the treatment of inherited and acquired bone marrow failure syndromes has been investigated. Inherited BM failure syndromes are rare disorders which are characterised by a cytopenia affecting one or more haemopoietic lineages. They are often associated with an increased risk of myeloid leukaemia. Successful treatment has generally resulted from the use of allogenic bone marrow transplantation, in those patients where this possibility existed. In other cases, treatments such as corticosteroids, androgens and immunosupressive agents have

Erratum

Page 239

Preliminary studies have the cultured bone marrow in vitro that is derived from patients with inherited anaemias including Diamond Blackfan anaemia (DBA), Fanconi's anaemia (FA) and dyskeratosis congenita.

Should read:

Preliminary studies have investigated the ability of rhSCF to support the growth, *in vtro,* of haemopoietic colonies from bone marrow derived from patients with inherited anaemias (including Diamond Blackfan anaemia (DBA), Fanconi's anaemia (FA) and dyskeratosis congenita).

been used, but responses are generally short lived and relapses common (Gillio and Gabrilove, 1993). Preliminary studies have the cultured bone marrow *in vitro* that is derived from patients with inherited anaemias, including Diamond Blackfan anaemia (DBA), Fanconi's anaemia (FA) and dyskeratosis congenita. Despite, the lack of abnormalities associated with SCF and its receptor in DBA, a number of these patients show improved BFU-E colony formation in response to the addition of SCF (Abkowitz *et al.*, 1991; Alter *et al.*, 1992; Gillio and Gabrilove, 1993). Similar studies have been performed using BM cells from patients with aplastic anaemia (AA). SCF increases colony formation *in vitro* (especially BFU-E) in synergy with other growth factors (Wodnar-Filipowicz *et al.*, 1992). Serum SCF concentrations in AA patients show a tendency to low normal levels when compared to controls, additionally patients with better clinical parameters (decreased requirement for transfusions and increased survival) tend to show higher serum SCF levels (Tong *et al.*, 1993). These findings have prompted the suggestion that SCF should be considered for treatment of patients with such congenital and acquired disorders, regardless of the disease aetiology.

Interest has also been shown in the potential use of SCF in the treatment of cytopenias associated with human immunodeficiency virus (HIV) infection. Cytopenias are common in patients with acquired immune deficiency syndrome (AIDS), occurring in up to 70% of individuals (Brandi *et al.*, 1995). The aetiology of such cytopenias is multifactorial. Potential causes include viral infection of progenitor cells or stromal cells, drug related effects (e.g. azidothymidine), secondary infections (e.g. *Mycobacteria spp.*, *Cryptococcus neoformans*), nutritional imbalances and haematological neoplasms (reviewed by Aboulafia and Mitsuyasu, 1991, Calenda and Chermann, 1992). Higher serum levels of SCF have been associated with prolonged survival in patients with HIV infection; furthermore decreases in serum SCF levels are seen with disease progression (Manegold *et al.*, 1995). *In vitro* studies have shown the ability of hSCF to reduce the inhibition of BFU-E formation by azidothymidine, whilst not affecting its inhibition of HIV replication in lymphocytes or monocytes (Miles *et al.*, 1991). A concern over the use of SCF in HIV infected patients would be the potential for the increased susceptibility of haemopoietic stem cells for infection with the retrovirus, due to the induction of cell division. The use of SCF in

combination with anti-retroviral therapy may preclude this possibility (Harbol *et al.*, 1994).

9.3.3 RADIOPROTECTION

Recombinant SCF is able to protect haemopoietic cells against otherwise lethal total body irradiation (TBI) both *in vitro* and *in vivo* (Zsebo *et al.*, 1992; Leigh *et al.*, 1993). The protective effect *in vivo* is most marked when SCF is administered both before and after irradiation. This effect is thought to be mediated by the recruitment of stem cells from G_0 into an active phase of the cell cycle, presumably S phase (McNiece *et al.*, 1993); cells in this phase of the cell cycle (undergoing DNA synthesis) are more resistant to the damaging effects of radiation (Sinclair, 1968). This influence upon cell cycle in haemopoietic cells also renders the cells more sensitive to S phase toxins (e.g. 5-FU) (Molineux *et al.*, 1994); a phenomenon known as chemosensitisation. Unfortunately, unless a differential effect exists between normal and neoplastic tissue then both radioprotection and chemosensitisation are of limited clinical use. However, the use of SCF for chemosensitisation, in combination with BM reconstitution may prove a useful therapeutic modality in the future (Molineux and Dexter, 1995).

9.3.4 ADJUNCTIVE TREATMENT TO CHEMOTHERAPY

Aside from its potential use in chemosensitisation, SCF may find clinical application in the support of standard dose chemotherapy. Its use in such circumstances would likely be in combination with other haemopoietic growth factors that have previously been used alone (e.g. G-CSF, GM-CSF). These colony stimulating factors have been used both before chemotherapy to reduce the incidence of cytopenias and following chemotherapy to treat any cytopenias that develop. Prompt treatment or prevention of severe neutropenia is essential in order to prevent the development of life threatening infections in such patients. Similarly thrombocytopenia, if untreated, can lead to a potentially fatal impairment of haemostasis. Colony stimulating factors may also allow escalation of chemotherapy protocols, potentially resulting in increased remission rates and survival times (Williams, 1994).

9.3.5 OTHER POTENTIAL USES

The pleiotropic effects of SCF upon extra-haemopoietic tissues such as skin melanocytes or gonadal germ cells may present novel therapeutic opportunities in the future. There has been some interest in the use of SCF in the treatment of vitiligo, a skin disease characterised by focal hypopigmentation associated with an absence of melanocytes (Dippel *et al.*, 1995; Glaspy, 1996). SCF and its receptor likely play an important role in normal spermatogenesis and oogenesis (Manova *et al.*, 1993). At present there is limited information regarding the role of the SCF - SCF-R axis in disorders of spermatogenesis or oogenesis; further studies are required before a potential therapeutic role for SCF in their treatment can be suggested. The coexpression of SCF and its receptor may be involved the pathogenesis of certain germ cell tumours (Izquierdo *et al.*, 1995); studies of SCF ligand or receptor expression may help in tumour classification and aid evaluation of prognosis.

9.4 POTENTIAL CLINICAL USES OF RECOMBINANT FELINE STEM CELL FACTOR

Potential clinical applications of SCF in cats are as widespread, in theory, as those suggested for human disease. However, the treatment of veterinary species is governed by economic and practical considerations that are less applicable for human patients. This aside, there are a number of areas which should be considered for further investigation.

Treatment options for defective haemopoiesis in small animals have previously been limited. Lithium carbonate has been used as a non-specific treatment in dogs with cytopenias associated with oestrogen therapy (Hall, 1992), cyclic haemopoiesis (Hammond and Dale, 1980) and suspected megakaryocytic hypoplasia (Murtaugh and Jacobs, 1985). Lithium treatment increases neutrophil and platelet counts in both humans (Lyman *et al.*, 1980) and dogs (Hammond and Dale, 1980). Exposure of long term BM cultures to lithium leads to a sustained increase in granulocyte, monocyte and megakaryocyte production and an increase in colony forming cells (CFU-S, CFU-GM, CFU-Meg and HPP-CFC). This effect has been shown to be

mediated, at least in part, by increases in the stromal cell production of cytokines, including GM-CSF, G-CSF and IL-6 (Quesenberry, 1992, and references therein). However, administration of lithium to healthy cats produces no increase in neutrophil counts, rather it is associated with significant toxicity manifested as anaemia, neutropenia and lymphopenia (Dieringer *et al.*, 1990). The availability of recombinant haemopoietic cytokines, such as SCF, in small animals could provide the veterinary clinician with a new range of treatment options for cytopenias, including those caused by iatrogenic drug effects (e.g. oestrogens, griseofulvin), chemotherapy, infectious agents (e.g. FeLV, FIV or feline parvovirus) and those considered idiopathic (causes of such cytopenias in cats have been reviewed by Baldwin and Ledet, 1994). Since SCF has effects on multiple haemopoietic lineages it may have a broad range of applications, regardless of the affected lineage. Given the demonstration of marked synergy of SCF with other cytokines *in vitro* and *in vivo*, the use of SCF in combination with lineage specific cytokines may provide a more rational approach to therapy than the use of single cytokines to treat cytopenias.

Feline immunodeficiency virus (FIV) is a lentivirus that is morphologically and biochemically related to HIV, but antigenically distinct (Pedersen *et al.*, 1987). The virus infects cats, causing a similar range of clinical signs to those seen in humans infected with HIV. FIV infection in cats is commonly associated with haematological disorders that closely resemble those seen in HIV-seropositive patients. These include anaemia, neutropenia, lymphopenia and thrombocytopenia (Shelton *et al.*, 1989; Shelton *et al.*, 1990; Callanan *et al.*, 1992; Shelton and Linenberger, 1995). It has thus become a valuable experimental model, facilitating studies which are not possible in humans. An area of particular relevance to this discussion, is the potential use of SCF and other cytokines in the treatment of such cytopenias. Clinical trials in experimental or naturally occurring cases of FIV infection could help establish the potential value of SCF in this regard. The model could be extended to investigate the use of SCF in combination with other haemopoietic cytokines. The effect of haemopoietic growth factors upon viral load, alone and in combination with anti-retroviral drugs could also be determined. Feline rSCF could potentially be used as part of combination therapy for clinical cases of FIV with the aim of reducing the haemopoietic toxicity of reverse transcriptase inhibitors such as zidovudine.

Bone marrow transplantation (BMT) has been performed in both dogs and cats. Conditions which have been treated in cats include lysosomal storage diseases, retroviral infections (Gasper *et al.*, 1992) and myeloid leukaemia (Gasper *et al.*, 1996). Although the actual transplantation procedure is relatively simple, preparation of the patient prior to BMT, and management of the immune-compromised patient post-transplantation are both difficult and expensive. This has therefore limited the use of BMT in feline patients to small numbers of animals, largely on an experimental basis. The use of recombinant cytokines such as SCF in BMT protocols may accelerate haemopoietic recovery post-transplantation and so simplify the procedure (Gasper *et al.*, 1992). This may aid the transition of BMT, from being largely an experimental technique, to more widespread use within the clinics. The use of peripheral blood stem cell transplantation in animals has again been limited to experimental models, where SCF has formed an integral part of most mobilisation protocols. The use of both BMT and PBSC in veterinary medicine is restricted by such considerations as cost, availability of specialised equipment (e.g. leucopheresis equipment for PBSC) and technical expertise. This will likely restrict any use of the procedures to limited numbers of referral centres, where the facilities exist and intensive support post-transplantation can be offered.

9.5 FUTURE DIRECTIONS

Since cytokines are pleiotropic and interact in a complex manner, the administration of one cytokine *in vivo* may have wide ranging effects, mediated by alterations in the activity of other cytokines, that cannot be predicted by its *in vitro* actions. The situation becomes even more complex when considering the simultaneous or sequential administration of multiple cytokines. Experimental animal models could prove particularly useful in determining the optimum combination of cytokines for use in a given clinical procedure (e.g. PBSC mobilisation). The use of cytokines in combination will most likely result in the maximisation of their potential therapeutic value and minimise the risk of undesired side effects. In order to fully utilise such models it is important that a wide range of species specific cytokines are developed and also that other necessary reagents are available (e.g. monoclonal antibodies to

specific cell surface markers such as CD34). With this consideration in mind we have been attempting the isolation of a number of other feline cytokines, both haemopoietic and immunomodulatory, and have recently cloned and expressed feline G-CSF (EMBL accession number Y08558; Dunham and Onions, manuscript in preparation).

These considerations are also applicable in the transition of cytokines from the laboratory to useful clinical tools. The development of cytokines as therapeutic agents, however, depends on a number of other factors. The commercial viability of new veterinary drugs depends upon their range of potential clinical applications, efficacy, ease of use, availability of alternative treatments and cost. The commercial application of recombinant DNA techniques to clinical veterinary medicine has hitherto been limited. A recombinant subunit vaccine for FeLV consisting of the viral glycoprotein gp70, expressed in *Escherichia coli*, has been produced and is now in widespread use (Marciani *et al.*, 1991). Vaccines have a wide application, with all pet cats being possible recipients. This can offset the potentially high production and development costs. In the case of individual cytokines, however, potential clinical applications may be more limited, therefore this may constrain the development of cytokines for clinical use. SCF may have a broad range of applications such that it may prove suitable for exploitation in the veterinary clinic, particularly if combined with other cytokines, where lower doses are likely to be effective. Other haemopoietic cytokines that may be considered for commercial exploitation include feline EPO. Human EPO has been used with some success in the treatment of anaemia associated with low levels EPO that arise in cats with chronic renal failure, however acute and chronic side effects are possible due to its heterologous nature (Gieger, 1992). The development of the feline homologue could therefore provide a valuable new agent for the symptomatic treatment of this disease. Furthermore, the prevalence of CRF in older cats is relatively high, affecting 8% of cats aged 10 to 15 years and 30% of cats over 15 years of age in one survey (Krawiec and Gelberg, 1989). This high incidence combined with the tendency for cats to live longer due to improved health care an husbandry may make the cytokine commercially viable to produce.

With regard to feline SCF itself, in order that its potential use in clinical cases can be evaluated it will be necessary to increase the scale of production and optimise expression of the cytokine. Purification techniques would need to be scaled up and validated and the product supplied in a suitable format (likely lyophilised in single use vials). The initial use of frSCF in limited numbers of clinical cases would be carried out under animal test certificates. Whilst frSCF is unlikely to find widespread clinical applications as a single agent, its potential utility in combination with other cytokines, both known and as yet undiscovered is difficult to foresee and will require further investigations.

REFERENCES

REFERENCES

Aarden, L.A., Brunner, T.K., Cerottini, J.C., Dayer, J.-M., de Weck, A.L., Dinarello, C.A., Di Sabato, G., Farrar, J.J., Gery, I., Gillis, S., Handshumacher, R.E., Henney, C.S., Hoffmann, M.K., Koopman, W.J., Krane, S.M., Lachman, L.B., Lefkowits, I., Mishell, R.I., Mizel, S.B., Oppenheim, J.J., Paetkau, V., Plate, J., Rollinghoff, M., Rosenstreich, D., Rosenthal, A.S., Rosenwasser, L.J., Schimpl, A., Shin, H.S., Simon, P.L., Smith, K.A., Wagner, H., Watson, J.D., Wecker, E. and Wood, D.D. (1979) Revised nomenclature for antigen-non-specific T cell proliferation and helper factors. *J. Immunol.* **123**, 2928-2929.

Abkowitz, J.L., Holly, R.D., Segal, G.M. and Adamson, J.W. (1986) Multilineage, non-species specific hematopoietic growth factor(s) elaborated by a feline fibroblast cell line: enhancement by viral infection. *J. Cell. Physiol.* **127**, 189-196.

Abkowitz, J.L., Sabo, K.M., Nakamoto, B., Blau, C.A., Martin, F.H., Zsebo, K.M. and Papayannopoulou, T. (1991) Diamond-Blackfan anemia: In vitro response of erythroid progenitors to the ligand for c-kit. *Blood* **78**, 2198-2202.

Aboulafia, D.M. and Mitsuyasu, R.T. (1991) Hematologic abnormalities in AIDS. *Hematology/Oncology Clinics of North America* **5**, 195-214.

Adachi, S., Ebi, Y., Nishikawa, S., Hayashi, S., Yamazaki, M., Kasugai, T., Yamamura, T., Nomura, S. and Kitamura, Y. (1992) Necessity of extracellular domain of W (c-kit) receptors for attachment of murine cultured mast cells to fibroblasts. *Blood* **79**, 650-656.

Alter, B.P., Knobloch, M.E., He, L., Gillio, A.P., O'Reilly, R.J., Reilly, L.K. and Weinberg, R.S. (1992) Effect of stem cell factor on in vitro erythropoiesis in patients with bone marrow failure syndromes. *Blood* **80**, 3000-3008.

Anderson, D., Koch, C.A., Grey, L., Ellis, C., Moran, M.F. and Pawson, T. (1990) Binding of SH2 domains of phospholipase C(gamma)1, GAP, and Src to activated growth factor receptors. *Science* **250**, 979-982.

Anderson, D.M., Lyman, S.D., Baird, A., Wignall, J.M., Eisenman, J., Rauch, C., March, C.J., Boswell, H.S., Gimpel, S.D., Cosman, D. and Williams, D.E. (1990) Molecular Cloning of Mast Cell Growth Factor, a Hematopoietin That Is Active in Both Membrane Bound and Soluble Forms. *Cell* **63**, 235-243.

Anderson, D.M., Williams, D.E., Tushinski, R., Gimpel, S., Eisenman, J., Cannizzaro, L.A., Aronson, M., Croce, C.M., Huebner, K., Cosman, D. and Lyman, S.D. (1991) Alternate Splicing of mRNAs Encoding Human Mast Cell Growth Factor and Localisation of the Gene to Chromosome 12q22-q24. *Cell Growth and Differentiation* **2**, 373-378.

Anderson, P.H., Berrett, S. and Patterson, D.S.P. (1976) The significance of elevated plasma creatine phosphokinase activity in muscle disease of cattle. *J. Comp. Pathol.* **86**, 531-538.

Andrews, D.F., Bianco, J.A., Moran, D.A. and Singer, J.W. (1991) Inflammatory cytokines and pentoxiffylline (PTX) affect kit ligand (KL) expression by marrow stromal cells (MSC) [Abstract]. *Blood* **78**, 303a

Andrews, R.G., Knitter, G.H., Bartelmez, S.H., Langley, K.E., Farrar, D., Hendren, R.W., Appelbaum, F.R., Bernstein, I.D. and Zsebo, K.M. (1991) Recombinant human stem cell factor, a c-kit ligand, stimulates hematopoiesis in primates. *Blood* **78**, 1975-1980.

Andrews, R.G., Bartelmez, S.H., Knitter, G.H., Myerson, D., Bernstein, I.D., Appelbaum, F.R. and Zsebo, K.M. (1992a) A c-kit ligand, recombinant human stem cell factor, mediates reversible expansion of multiple CD34+ colony-forming cell types in blood and marrow of baboons. *Blood* **80**, 920-927.

Andrews, R.G., Bensinger, W.I., Knitter, G.H., Bartelmez, S.H., Longin, K., Bernstein, I.D., Appelbaum, F.R. and Zsebo, K.M. (1992b) The ligand for c-kit, stem cell factor, stimulates the circulation of cells that engraft lethally irradiated baboons. *Blood* **80**, 2715-2720.

Andrews, R.G., Briddell, R.A., Knitter, G.H., Opie, T., Bronsden, M., Myerson, D., Appelbaum, F.R. and McNiece, I.K. (1994) In vivo synergy between recombinant human stem cell factor and recombinant human granulocyte colony-stimulating factor in baboons: Enhanced circulation of progenitor cells. *Blood* **84**, 800-810.

Andrews, R.G., Briddell, R.A., Knitter, G.H., Rowley, S.D., Appelbaum, F.R. and McNiece, I.K. (1995) Rapid engraftment by peripheral blood progenitor cells mobilized by recombinant human stem cell factor and recombinant human granulocyte colony- stimulating factor in nonhuman primates. *Blood* **85**, 15-20.

Antonelli, G., Currenti, M., Turriziani, O. and Dianzani, F. (1991) Neutralizing antibodies to interferon-alpha: relative frequency in patients treated with different interferon preparations. *J. Infect. Dis.* **163**, 882-885.

Arakawa, T., Yphantis, D.A., Lary, J.W., Narhi, L.O., Lu, H.S., Prestrelski, S.J., Clogston, C.L., Zsebo, K.M., Mendiaz, E.A., Wypych, J. and et al (1991) Glycosylated and unglycosylated recombinant-derived human stem cell factors are dimeric and have extensive regular secondary structure. *J. Biol. Chem.* **266**, 18942-18948.

Argyle, D.J., Smith, K., McBride, K., Fulton, R. and Onions, D.E. (1995) Nucleotide and predicted peptide sequence of feline interferon- gamma (IFN-gamma). *DNA Seq.* **5**, 169-171.

Aruffo, A. (1994) Transient expression of protein using COS cells. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (Eds.) *Current Protocols in Molecular Biology*, pp. 16.13.1-16.13.7. New York: Greene Publishing Associates Inc. and John Wiley and Sons Inc.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1994) *Current Protocols in Molecular Biology*, New York: Greene Publishing Associates Inc. and John Wiley and Sons Inc.

Aviv, H. and Leder, P. (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc.Natl.Acad.Sci.U.S.A.* **69**, 1408-1412.

Bainton, D.F., Ullyot, J.L. and Farquhar, M.G. (1971) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J. Exp. Med.* **134**, 907-934.

Baker, T.A., Grossman, D. and Gross, C.A. (1984) A gene regulating the heat shock response in *Escherichia coli* also affects proteolysis. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6779-6783.

Baltensperger, K., Kozma, L.M., Cherniack, A.D., Klarlund, J.K., Chawla, A., Banerjee, U. and Czech, M.P. (1993) Binding of the Ras activator Son of sevenless to insulin receptor substrate-1 signaling complexes. *Science* **260**, 1950-1952.

Banner, D.W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H.J., Broger, C., Loetscher, H. and Lesslauer, W. (1993) Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* **73**, 431-445.

Bazan, J.F. (1990a) Haemopoietic receptors and helical cytokines. *Immunol. Today* **11**, 350-354.

Bazan, J.F. (1990b) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6934-6938.
Bazan, J.F. (1991a) Neurotropic cytokines in the hematopoietic fold. *Neuron* 7, 197-208.

Bazan, J.F. (1991b) Genetic and structural homology of stem cell factor and macrophage colony-stimulating factor. *Cell* **65**, 9-10.

Bazan, J.F. (1993) Emerging families of cytokines and receptors. *Current Biology* **3**, 603-606.
Becker, A.J., McCulloch, E.A. and Till, J.E. (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* **197**, 452-454.

Bennett, D. (1956) Developmental analysis of a mutation with pleiotropic effects in the mouse. *J. Morphol.* **98**, 199-234.

Bernad, A., Varas, F., Gallego, J.M., Almendral, J.M. and Bueren, J.A. (1994) Ex vivo expansion and selection of retrovirally transduced bone marrow: An efficient methodology for gene-transfer to murine lympho- haemopoietic stem cells. *Brit. J. Haematol.* **87**, 6-17.

Bernstein, S.E. (1960) Steel Dickie. *Mouse News Lett.* **23**, 33.

Bernstein, S.E. (1970) Tissue transplantation as an analytic and therapeutic tool in hereditary anaemias. *Am. J. Surg.* **119**, 448-451.

Bertoncello, I. (1992) Status of High Proliferative Potential Colony-Forming Cells in the Hematopoietic Stem Cell Hierarchy. *Current Topics in Microbiology and Immunology* **177**, 83-94.

Besmer, P., Murphy, J.E., George, P.C., Qiu, F., Bergold, P.J., Lederman, L., Snyder, H.W., Brodeur, D., Zuckerman, E.E. and Hardy, W.D. (1986) A new acute transforming feline retrovirus and relationship of its oncogene *v-kit* with the protein kinase gene family. *Nature* **320**, 415-421.

Besmer, P., Manova, K., Duttlinger, R., Huang, E.J., Packer, A., Gyssler, C. and Bachvarova, R.F. (1993) The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. *Dev. Suppl.* 125-137.

Billman-Jacobe, H. (1996) Expression in bacteria other than *Escherichia coli*. *Curr. Opin. in Biotech.* 7, 500-504.

Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7, 1513-1523.

Blechman, J.M., Lev, S., Barg, J., Eisenstein, M., Vaks, B., Vogel, Z., Givol, D. and Yarden, Y. (1995) The fourth immunoglobulin domain of the stem cell factor receptor couples ligand binding to signal transduction. *Cell* **80**, 103-113.

Blume-Jensen, P., ClaessonWelsh, L., Siegbahn, A., Zsebo, K.M. and Westermark B Heldin, C.H. (1991) Activation of the human c-kit product by ligand-induced dimerization mediates circular actin reorganization and chemotaxis. *EMBO Journal* **10**, 4121-4128.

Blume-Jensen, P., Siegbahn, A., Stabel, S., Heldin, C.H. and Ronnstrand, L. (1993) Increased Kit/SCF receptor induced mitogenicity but abolished cell motility after inhibition of protein kinase C. *EMBO Journal* **12**, 4199-4209.

Blume-Jensen, P., Ronnstrand, L., Gout, I., Waterfield, M.D. and Heldin, C.H. (1994) Modulation of Kit/stem cell factor receptor-induced signaling by protein kinase C. *J. Biol. Chem.* **269**, 21793-21802.

Blume-Jensen, P., Wernstedt, C., Heldin, C.H. and Ronnstrand, L. (1995) Identification of the major phosphorylation sites for protein kinase C in kit/stem cell factor receptor in vitro and in intact cells. *J. Biol. Chem.* **270**, 14192-14200.

Bocci, V., Pacini, A., Pessina, G.P., Paulesu, L., Muscettola, M. and Lunghetti, G. (1985) Catabolic sites of human interferon-gamma. *J. Gen. Virol.* **66**, 887-891.

Bodine, D.M., Crosier, P.S. and Clark, S.C. (1991) Effects of hematopoietic growth factors on the survival of primitive stem cells in liquid suspension cultures. *Blood* **78**, 914-920.

Bodine, D.M., Seidel, N.E., Zsebo, K.M. and Orlic, D. (1993) In vivo administration of stem cell factor to mice increases the absolute number of pluripotent hematopoietic stem cells. *Blood* **82**, 445-455.

Bodine, D.M., Seidel, N.E. and Orlic, D. (1996) Bone-marrow collected 14 days after in vivo administration of granulocyte colony-stimulating factor and stem cell factor to mice has 10-fold repopulating ability than in untreated bone marrow. *Blood* **88**, 89-97.

Bonfini, L., Migliaccio, E., Pelicci, G., Lanfrancone, L. and Pelicci, P.G. (1996) Not all Shc's roads lead to Ras. *Trends in Biochemical Sciences* **21**, 257-261.

Bonnerjea, J., Oh, S. and Hoare, M. (1986) Protein-purification - the right step at the right time. *Bio/Technology* **4**, 954

Boulay, J. and Paul, W.E. (1993) Hemapoietin sub-family classification based on size, gene organisation and sequence homology. *Current Biology* **3**, 573-581.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1990) The GTPase superfamily: A conserved switch for diverse cell functions. *Nature* **348**, 125-132.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) The GTPase superfamily: Conserved structure and molecular mechanism. *Nature* **349**, 117-127.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* **72**, 248-254.

Bradley, T.R. and Metcalf, D. (1966) The growth of mouse bone marrow cells in vitro. *Australian Journal of Experimental Biology and Medical Science* **44**, 287-300.

Brake, A.J., Merryweather, J.P., Coit, D.G., Heberlein, U.A., Masiarz, F.R., Mullenbach, G.T., Urdea, M.S., Valenzuela, P. and Barr, P.J. (1984) Alpha-factor-directed synthesis and secretion of mature foreign proteins in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4642-4646.

Brandi, G., Carnevali, A., Rossi, L., Fraternale, A., Schiavano, G.F. and Magnani, M. (1995) Effect of antiviral treatments on the bone marrow in murine aids. Running head: Antiviral drug effects on bone marrow. *Blood Cells, Molecules, and Diseases* **21**, 109-118.

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

Brannan, C.I., Lyman, S.D., Williams, D.E., Eisenman, J., Anderson, D.M., Cosman, D., Bedell, M.A., Jenkins, N.A. and Copeland, N.G. (1991) Steel-Dickie mutation encodes a c-kit ligand lacking transmembrane and cytoplasmic domains. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4671-4674.

Brannan, C.I., Bedell, M.A., Resnick, J.L., Eppig, J.J., Handel, M.A., Williams, D.E., Lyman, S.D., Donovan, P.J., Jenkins, N.A. and Copeland, N.G. (1992) Developmental abnormalities in *Steel*^{17H} mice result from a splicing defect in the steel factor cytoplasmic tail. *Genes and Development* **6**, 1832-1842.

Brent, R. (1994) Protein Expression: Introduction. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (Eds.) *Current Protocols in Molecular Biology*, pp. 16.05-16.06. New York: Greene Publishing Associates Inc. and John Wiley and Sons Inc.

Briddell, R.A., Hartley, C.A., Smith, K.A. and McNiece, I.K. (1993) Recombinant Rat Stem Cell Factor Synergises With Recombinant Human Granulocyte Colony-Stimulating Factor In Vivo in Mice to Mobilize Peripheral Blood Progenitor Cells That Have Enhanced Repopulating Potential. *Blood* **82**, 1720-1723.

Briddell, R.A. (1994) Recombinant human stem cell factor (rhSCF) and filgastrim (rhG-CSF) synergise to mobilise myeloid, erythroid and megakaryocyte progenitors in patients with breast cancer [Abstract]. *Brit. J. Haematol.* **87 (Suppl. 1)**, 92.

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

Broudy, V.C., Lin, N.L., Priestley, G.V., Nocka, K. and Wolf, N.S. (1996) Interaction of stem-cell factor and its receptor c-kit mediates lodgment and acute expansion of hematopoietic-cells in the murine spleen. *Blood* **88**, 75-81.

Brown, J., Greaves, M.F. and Molgaard, H.V. (1991) The gene encoding the stem cell antigen, CD34, is conserved in mouse and expressed in haemopoietic progenitor cell lines, brain, and embryonic fibroblasts. *Int. Immunol.* **3**, 175-184.

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

Broxmeyer, H.E., Hangoc, G., Cooper, S., Anderson, D., Cosman, D., Lyman, S.D. and Williams, D.E. (1991b) Influence of murine mast cell growth factor (*c-kit* ligand) on colony formation by mouse marrow hematopoietic progenitor cells. *Exp. Hematol.* **19**, 143 146.

Broxmeyer, H.E., Maze, R., Miyazawa, K., Carow, C., Hendrie, P.C., Cooper, S., Hangoc, G., VadhanRaj, S. and Lu, L. (1991c) The kit receptor and its ligand, steel factor, as regulators of hemopoiesis. *Cancer Cells* **3**, 480-487.

Buckholz, R.G. and Gleeson, M.A. (1991) Yeast systems for the commercial production of heterologous proteins. *Biotechnology* **9**, 1067-1072.

Burnette, W.N. (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**, 195-203.

Calenda, V. and Chermann, J.C. (1992) The effects of HIV on hematopoiesis. *Eur. J. Haematol.* **48**, 181-186.

Callanan, J.J., Thompson, H., Toth, S.R., O'Neil, B., Lawrence, C.E., Willett, B. and Jarrett, O. (1992) Clinical and pathological findings in feline immunodeficiency virus experimental infection. *Vet. Immunol. Immunop.* **35**, 3-13.

Cannon, J.G., Tompkins, R.G., Gelfand, J.A., Michie, H.R., Stanford, G.G., Vandermeer, J.W.M., Endress, S., Lonnemann, G., Corsetti, J., Chernow, B., Wilmore, D.W., Wolff, S.M., Burke, J.F. and Dinarello, C.A. (1990) Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever. *Journal of Infectious Diseases* **161**, 79-84.

Chabot, B., Stephenson, D.A., Chapman, V.M., Besmer, P. and Bernstein, A. (1988) The proto-oncogene *c-kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus. *Nature* **335**, 88-89.

Chardin, P., Camonis, J.H., Gale, N.W., Van Aelst, L., Schlessinger, J., Wigler, M.H. and BarSagi, D. (1993) Human Sos1: A guanine nucleotide exchange factor for Ras that binds to GRB2. *Science* **260**, 1338-1343.

Chardin, P., Cussac, D., Maignan, S. and Ducruix, A. (1995) The Grb2 adaptor. *FEBS Lett.* **369**, 47-51.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299.

Chou, P.Y. and Fasman, G.D. (1978) Prediction of the secondary structure of proteins from their amino acid sequence. *Advances in Enzymology* **47**, 45-147.

Chung, C.T., Niemela, S.L. and Miller, R.H. (1989) One-step preparation of competant *Esherichia coli*: Transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2172-2175.

Ciekot, P.E., Ogilvie, G.K., Fettman, M.J. and et al (1991) Evaluation of GM-CSF, IL-3, and Gm-CSF/IL-3 fusion protein (pIXY 321) as a multilineage colony stimulating factors in the dog [Abstract]. *Veterinary Cancer Society Annual Meeting, Minniapolis, Minn.* 41-43.

Cohen, D.R., Hapel, A.J. and Young, I.G. (1986) Cloning and expression of the rat interleukin-3 gene. *Nucleic. Acids. Res.* **14**, 3641-3658.

Cohen, S., Bigazzi, P.E. and Yoshida, T. (1974) Similarities of T cell function in cell mediated immunity and antibody production. *Cell. Immunol.* **12**, 150-159.

Coleman, J.W., Holliday, M.R., Kimber, I., Zsebo, K.M. and Galli, S.J. (1993) Regulation of mouse peritoneal mast cell secretory function by stem cell factor, IL-3 or IL-4. *J. Immunol.* **150**, 556-562.

Copeland, N.G., Gilbert, D.J., Cho, B.C., Donovan, P.J., Jenkins, N.A., Cosman, D., Anderson, D., Lyman, S.D. and Williams, D.E. (1990) Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. *Cell* **63**, 175-183.

Costa, J.J., Demetri, G.D., Harrist, T.J., Dvorak, A.M., Hayes, D.F., Merica, E.A., Menchaca, D.M., Gringeri, A.J., Schwartz, L.B. and Galli, S.J. (1996) Recombinant human stem-cell factor (kit-ligand) promotes human mast- cell and melanocyte hyperplasia and functional activation *in vivo*. *J. Exp. Med.* **183**, 2681-2686.

Coutinho, L.H., Gilleece, M.H., De Wynter, E.A., Will, A. and Testa, N.G. (1993) Cultures using human marrow. In: Testa, N.G. and Molineux, G. (Eds.) *Haemopoiesis: a practical approach*, pp. 75-106. New York: Oxford University Press

Coyle, P., Furda, G.J., Tao, W., Daley, M.J. and Johnston, P.A. (1992) r-BoIL-1beta as a preventative and therapeutic for Staphylococcus aureus mastitis: correlation with its effects upon polymorphonuclear neutrophil function. *J. Dairy Sci.* Supplement

Cozzi, P.J., Padrid, P.A., Takeda, J., Alegre, M.L., Yuhki, N. and Leff, A.R. (1993) Sequence and functional-characterization of feline interleukin-2. *Biochem. Biophys. Res. Comm.* **194**, 1038-1043.

Craig, J.I.O. (1994) Peripheral Blood Stem Cells: Mobilisation and Engraftment. *Bone Marrow Transplantation* **14**, S10-S11.

Curran, J.A., Argyle, D.J., Cox, P., Onions, D.E. and Nicolson, L. (1994) Nucleotide-sequence of the equine interferon-gamma cDNA. *DNA Seq.* **4**, 405-407.

Curry, J.L. and Trentin, J.J. (1967) Hemopoietic spleen colony studies. I. Growth and differentiation. *Developmental Biology* **15**, 395-413.

Dai, C.H., Krantz, S.B. and Zsebo, K.M. (1991) Human burst-forming units-erythroid need direct interaction with stem cell factor for further development. *Blood* **78**, 2493-2497.

Dale, D.C., Lau, S., Nash, R., Boone, T. and Osborne, W. (1992) Effect of endotoxin on serum granulocyte and granulocyte-macrophage colony-stimulating factor levels in dogs. *J. Infect. Dis.* **165**, 689-694.

Dale, D.C., Rodger, E., Cebon, J., Ramesh, N., Hammond, W.P. and Zsebo, K.M. (1995) Long-term treatment of canine cyclic hematopoiesis with recombinant canine stem cell factor. *Blood* **85**, 74-79.

Daniel, S.L., Brenner, C.A., Legendre, A.M., Soloman, A. and Rouse, B.T. (1992) Feline cytokines TNF alpha and IL-1 beta: PCR cloning and sequencing of DNA. *Anim. Biotechnol.* **3**, 281-297.

Darnell J.E. Jr., Kerr, I.M. and Stark, G.R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**, 1415-1421.

DaSilva, L., Howard, O.M., Rui, H., Kirken, R.A. and Farrar, W.L. (1994) Growth signaling and JAK2 association mediated by membrane-proximal cytoplasmic regions of prolactin receptors. *J. Biol. Chem.* **269**, 18267-18270.

Dastych, J. and Metcalfe, D.D. (1994) Stem cell factor induces mast cell adhesion to fibronectin. *J. Immunol.* **152**, 213-219.

Dawson, M. (1985) The Biology of the Horseshoe Crab. *LAL Update* **3**, 4.

De Alberle, S.B. (1927) A study of the hereditary anaemias of mice. *Am. J. Anat.* **40**, 219-247.

De Maeyer, E. and De Maeyer-Guignard, J. (1988) *Interferons and other Regulatory cytokines*, New York: John Wiley and Sons.

De Maeyer, E. and De Maeyer-Guignard, J. (1994) Interferons. In: Thomson, A. (Ed.) *The Cytokine Handbook*, 2nd edn. pp. 265-288. San Diego,CA. Academic Press Ltd.

De Revel, T., Appelbaum, F.R., Storb, R., Schuening, F., Nash, R., Deeg, J., McNiece, I., Andrews, R. and Graham, T. (1994) Effects of granulocyte colony-stimulating factor and stem cell factor, alone and in combination, on the mobilization of peripheral blood cells that engraft lethally irradiated dogs. *Blood* **83**, 3795-3799.

De Vos, A.M., Ultsch, M. and Kossiakoff, A.A. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **255**, 306-311.

Demetri, G., Costa, J., Hayes, D., Sledge, G., Galli, S., Hoffman, R., Merica, E., Rich, W., Harkins, B., McGuire, B. and Gordon, M. (1993) A phase I trial of recombinant methionyl human stem cell factor (SCF) in patients with advanced breast carcinoma pre- and post- chemotherapy (chemo) with cyclophosphamide (C) and doxyrubicin (A) [Abstract]. *Proceedings of ASCO* **12**, 142.

Derynck, R., Content, J., DeClerq, E., Volckaert, G., Tavernier, J., Devos, R. and Fiers, W. (1980) Isolation and structure of a human fibroblast interferon gene. *Nature* **285**, 542-547.

Deryugina, E.I. and MullerSieburg, C.E. (1993) Stromal cells in long-term cultures: Keys to the elucidation of hematopoietic development? *Critical Reviews in Immunology* **13**, 115-150.

Dexter, T.M., Garland, J., Scott, D., Scolnick, E. and Metcalf, D. (1980) Growth of factor-dependent hemopoietic precursor cell lines. *J. Exp. Med.* **152**, 1036-1047.

Dexter, T.M., Spooncer, E., Simmons, P. and Allen, T.D. (1984) Long-term Marrow Culture: An overview of techniques and experience. In: Wright, D.G. and Greenberger, J.S. (Eds.) *Long Term Bone Marrow Culture*, pp. 57-96. Alan R. Liss, Inc. New York, USA.

Dexter, T.M. (1989) Hematopoietic growth-factors. *Brit. Med. Bull.* **45**, 337-349.

Dieringer, T.M., Rogers, K.S., Brown, S.A., Whitney, M.S. and Weeks, B.R. (1990) Evaluation of lithium carbonate as a bone marrow stimulant in healthy cats [Abstract]. *J. Vet. Intern. Med.* **4**, 111

Dippel, E., Haas, N., Grabbe, J., Schadendorf, D., Hamann, K. and Czarnetzki, B.M. (1995) Expression of the c-kit receptor in hypomelanosis: A comparative study between piebaldism, naevus depigmentosus and vitiligo. *British Journal of Dermatology* **132**, 182-189.

Dumonde, D.C., Wolstencroft, R.A., Panayi, G.S., Mathew, M., Marley, J. and Howson, W.T. (1969) 'Lymphokines': Non antibody mediators of cellular immunity generated by lymphocyte activation. *Nature* **224**, 38-42.

Dunbar, C.E., Seidel, N.E., Doren, S., Sellers, S., Cline, A.P., Metzger, M.E., Agricola, B.A., Donahue, R.E. and Bodine, D.M. (1996) Improved retroviral gene transfer into murine and rhesus peripheral blood or bone marrow repopulating cells primed in vivo with stem cell factor and granulocyte colony-stimulating factor. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11871-11876.

Dunham, S.P., Argyle, D.J. and Onions, D.E. (1995) The isolation and sequence of canine interleukin-2. *DNA Seq.* **5**, 177-180.

Dunham, S.P. and Onions, D.E. (1996) The cloning and sequencing of cDNAs encoding two isoforms of feline stem cell factor. *DNA Seq.* **6**, 233-237.

Duronio, V., Welham, M.J., Abraham, S., Dryden, P. and Schrader, J.W. (1992) $p21^{ras}$ activation via hemopoietin receptors and c-kit requires tyrosine kinase activity but not tyrosine phosphorylation of $p21^{ras}$ GTPase-activating protein. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1587-1591.

Durrant, I. (1990) Light-based detection of biomolecules. *Nature* **346**, 297-298.

Ebbe, S., Phalen, E. and Stohlman, F., Jr. (1972) Some abnormalities of megakaryocytes and platelets in *W/W^v* and *Sl/Sl^d* mice [Abstract]. *Blood* **40**, 964.

Ebbe, S., Phalen, E. and Stohlman, F., Jr. (1973) Abnormalities of megakaryocytes in *W/W^v* mice. *Blood* **42**, 857-864.

Ebbe, S., Phalen, E., D'Amore, P. and Howard, D. (1978) Megakaryocytic responses to thrombocytopenia and thrombocytosis in *Sl/Sl^d* mice. *Exp. Hematol.* **6**, 201-212.

Ebbe, S. and Phalen, E. (1978) Regulation of megakaryocytes in*W/W^v* mice. *J. Cell Physiol.* **96**, 73-80.

Eckart, M.R. and Bussineau, C.M. (1996) Quality and authenticity of heterologous proteins synthesized in yeast. *Current Opinion in Biotechnology* **7**, 525-530.

Egan, S.E., Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M. and Weinberg, R.A. (1993) Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363**, 45-51.

Egan, S.E. and Weinberg, R.A. (1993) The pathway to signal achievement. *Nature* **365**, 781-783.

Ennis, P.D., Zemmour, J., Salter, R.D. and Parham, P. (1996) Rapid cloning of HLA-A,B cDNA by using the polymerase chain reaction: Frequency and nature of errors produced in amplification. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2833-2837.

Fahlman, C., Blomhoff, H.K., Veiby, O.P., McNiece, I.K. and Jacobsen, S.E.W. (1994) Stem cell factor and interleukin-7 synergize to enhance early myelopoiesis in vitro. *Blood* **84**, 1450-1456.

Fairbairn, L.J., Cowling, G.J., Reipert, B.M. and Dexter, T.M. (1993) Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. *Cell* **74**, 823-832.

Flanagan, J.G., Chan, D.C. and Leder, P. (1991) Transmembrane Form of the *kit* Ligand Growth Factor is determined by Alternative Splicing and Is Missing in the Sl^d Mutant. *Cell* **64**, 1025-1035.

Fleming, W.H., Alpern, E.J., Uchida, N., Ikuta, K. and Weissman, I.L. (1993) Steel factor influences the distribution and activity of murine hematopoietic stem cells in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3760-3764.

Freund, J., Casals, J. and Hismer, E.P. (1937) Sensitisation and antibody formation after injection of tubercle bacilli and paraffin oil. *Proceedings of the Society for Experimental Biology and Medicine* **37**, 509

Frolova, E.I., Dolganov, G.M., Mazo, I.A., Smirnov, D.V., Copeland, P., Stewart, C., OBrien, S.J. and Dean, M. (1991) Linkage mapping of the human CSF2 and IL3 genes. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4821-4824.

Fu, X.Y., Kessler, D.S., Veals, S.A., Levy, D.E. and Darnell, J.E., Jr. (1990) ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8555-8559.

Fujii, H., Nakagawa, Y., Schindler, U., Kawahara, A., Mori, H., Gouilleux, F., Groner, B., Ihle, J.N., Minami, Y., Miyazaki, T. and et al (1995) Activation of Stat5 by interleukin 2 requires a carboxyl-terminal region of the interleukin 2 receptor beta chain but is not essential for the proliferative signal transmission. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5482-5486.

Fujita, J., Onoue, H., Ebi, Y., Nakayama, H. and Kanakura, Y. (1989) In vitro duplication and in vivo cure of mast-cell deficiency of Sl/Sl(d) mutant mice by cloned 3T3 fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2888-2891.

Fulton, R., Gasper, P.W., Ogilvie, G.K., Boone, T.C. and Dornsife, R.E. (1991) Effect of recombinant human granulocyte colony-stimulating factor on hematopoiesis in normal cats. *Exp. Hematol.* **19**, 759-767.

Funk, P.E., Varas, A. and Witte, P.L. (1993) Activity of stem cell factor and IL-7 in combination on normal bone marrow B lineage cells. *J. Immunol.* **150**, 748-752.

Galli, S.J., Dvorak, A.M., Marcum, J.A., Ishizaka, T., Nabel, G., Simonian, H.D., Pyne, K., Goldin, J.M., Rosenberg, R.D., Cantor, H. and Dvorak, H.F. (1982) Mast cell clones: A model for the analysis of cellular maturation. *J. Cell Biol.***95**, 435-444.

Galli, S.J., Tsai, M., Langley, K.E., Zsebo, K.M. and Geissler, E.N. (1991) Stem-cell factor (scf), a ligand for c-kit, induces mediator release from some populations of mouse mast-cells. *FASEB J.* **5**, A1092

Galli, S.J., Iemura, A., Garlick, D.S., Gamba Vitalo, C., Zsebo, K.M. and Andrews, R.G. (1993) Reversible expansion of primate mast cell populations in vivo by stem cell factor. *J Clin. Invest.* **91**, 148-152.

Galli, S.J., Zsebo, K.M. and Geissler, E.N. (1994) The kit ligand, stem cell factor. *Adv. Immunol.* **55**, 1-96.

Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120**, 97-120.

Gasper, P.W., Fulton, R. and Thrall, M.A. (1992) Bone marrow transplantation: update and current considerations. In: Kirk, R.W. (Ed.) *Current Veterinary Therapy XI*, pp. 466-469. Philadelphia: WB Saunders.

Gasper, P.W., Rosen, D.K. and Fulton, R. (1996) Allogeneic marrow transplantation in a cat with acute myeloid leukemia. *J. Am. Vet. Med. Assoc.* **208**, 1280-1284.

Gearing, A.J.H., Cartwright, J.E. and Wadhwa, M. (1994) Biological and immunological assays for cytokines. In: Thomson, A. (Ed.) *The Cytokine Handbook*, 2nd edn. pp. 507-524. San Diego,CA. Academic Press Ltd.

Gearing, D.P. and Ziegler, S.F. (1993) The haemopoietic growth factor receptor family. *Current Opinion in Hematology* 138-148.

Geissler, E.N., McFarland, E.C. and Russell, E.S. (1981) Analysis of pleiotropism at the dominant white-spotting (*W*) locus of the house mouse: a description of ten new alleles. *Genetics* **97**, 337-361.

Geissler, E.N., Cheng, S.V., Gusella, J.F. and Housman, D.E. (1988a) Genetic analysis of the dominant white-spotting (W) region on mouse chromosome 5: Identification of cloned DNA markers near *W*. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9635-9639.

Geissler, E.N., Ryan, M.A. and Housman, D.E. (1988b) The Dominant-White Spotting (*W*) Locus of the Mouse Encodes the *c-kit* Proto-Oncogene. *Cell* **55**, 185-192.

Geissler, E.N., Liao, M., Brook, J.D., Martin, F.H., Zsebo, K.M., Housman, D.E. and Galli, S.J. (1991) Stem cell factor (SCF), a novel hematopoietic growth factor and ligand for c-kit tyrosine kinase receptor, maps on human chromosome 12 between 12q14.3 and 12qter. *Somatic Cell and Molecular Genetics* **17**, 207-214.

Giebel, L.B., Strunk, K.M., Holmes, S.A. and Spritz, R.A. (1992) Organization and nucleotide-sequence of the human kit (mast stem-cell growth-factor receptor) protooncogene. *Oncogene* **7**, 2207-2217.

Giebel, L.B. and Spritz, R.A. (1991) Mutation of the kit (mast stem-cell growth-factor receptor) protooncogene in human piebaldism. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8696-8699.

Gieger, U. (1992) Erythropoietin and its clinical use. *Compendium of Continuing Education for the Practicing Veterinarian* **14**, 25-34.

Gillespie, P.G. and Hudspeth, A.J. (1991) Chemiluminescence detection of proteins from single cells. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2563-2567.

Gillio, A.P. and Gabrilove, J.L. (1993) Cytokine Treatment of Inherited Bone Marrow Failure Syndromes. *Blood* **81**, 1669-1674.

Glaspy, J. (1994) Efffects of stem cell factor (rhSCF) and filgastrim (rhG-CSF) on the mobilisation of peripheral blood progenitor cells (PBPC) and hematological recovery post transplant: preliminary phase I/II study results [Abstract]. *Brit. J. Haematol.* **87 (Suppl. 1)**, 156.

Glaspy, J. (1996) Clinical applications of stem cell factor. *Current Opinion in Hematology* **3**, 223-229.

Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175-182.
Goeddel, D.V. (1990) Systems for heterologous gene expression. *Methods Enzymol.* **185**, 3-7.

Goodman, J.W. and Hodgson, G.S. (1962) Evidence for stem cells in the peripheral blood of mice. *Blood* **19**, 702-714.

Goodman, S.A., Brewer, W.G., Henry, C.J., McPhearson, C.J., Murray, S., Abraha, T. and Lothrop, C.D., Jr. (1990) Antibody formation to rhG-CSF is delayed in dogs treated with chemotherapeutic drugs [Abstract]. *J. Vet. Intern. Med.* **4**, 111.

Gordon, M.S. (1994) Stem Cell Factor: Biological actions and potential role in therapy. *Clin. Immunotherapeutics* **2**, 161-166.

Gordon, M.Y. (1991) Hemopoietic growth factors and receptors: bound and free. *Cancer Cells* **3**, 127-133.

Goris, H., Bungart, B., Loeffler, M., Schmitz, S. and Nijhof, W. (1990) Migration of stem cells and progenitors between marrow and spleen following thiamphenicol treatment of mice. *Exp. Hematol.* **18**, 400-407.

Gotoh, A., Takahira, H., Mantel, C., Litz-Jackson, S., Boswell, H.S. and Broxmeyer, H.E. (1996) Steel factor induces serine phosphorylation of Stat3 in human growth factor-dependent myeloid cell lines. *Blood* **88**, 138-145.

Grabarek, J., Groopman, J.E., Lyles, Y.R., Jiang, S., Bennett, L., Zsebo, K. and Avraham, H. (1994) Human kit ligand (stem cell factor) modulates platelet activation in vitro. *J. Biol. Chem.* **269**, 21718-21724.

Grant, S.E. (1995) Studies on haemopoiesis in early feline immunodeficiency virus infection. University of Glasgow. Ph.D. Thesis.

Greenwood, P.J., Seamer, C. and Tisdall, D.J. (1996) Cloning, sequencing and expression of stem-cell factor (*c-kit* ligand) cDNA of brushtail possum (*Trichosurus-vulpecula*). R*eprod. Fert. Dev.* **8**, 789-795.

Gribskov, M., Burgess, R.R. and Devereux, J. (1986) PEPPLOT, a protein secondary structure analysis program for the UWGCG sequence analysis software package. *Nucleic. Acids. Res.* **14**, 327-334.

Grodberg, J. and Dunn, J.J. (1988) *omp T* encodes the *Escherichia coli* outer mambrane protease that cleaves T7 RNA polymerase during purification. *J. Bacteriol.* **170**, 2212-2220.

Gruss, H.J. and Dower, S.K. (1995) Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. *Blood* **85**, 3378-3404.

GST Gene Fusion System Manual (1994) 2nd Edn, Uppsala, Sweden: Pharmacia Biotech, Inc.

Guenet, J.L. and Mercier-Balaz, M. (1975) A fertile allele of the *W* series (W^f). *Mouse News Lett.* **53**, 57.

Gupta, P., McCarthy, J.B. and Verfaillie, C.M. (1996) Stromal fibroblast heparan sulfate is required for cytokine-mediated ex vivo maintenance of human long-term culture-initiating cells. *Blood* **87**, 3229-3236.

Gupta, S., Yan, H., Wong, L.H., Ralph, S., Krolewski, J. and Schindler, C. (1996) The SH2 domains of Stat1 and Stat2 mediate multiple interactions in the transduction of IFN-alpha signals. *EMBO J.* **15**, 1075-1084.

Habig, W.J., Pabst, M.J. and Jakoby, W.B. (1974) Glutathione S-transferases. *J. Biol. Chem.* **249**, 7130-7139.

Hack, C.E., De Groot, E.R., Felt-Bersma, R.J., Nuijens, J.H., Strack Van Schijndel, R.J., Eerenberg-Belmer, A.J., Thijs, L.G. and Aarden, L.A. (1989) Increased plasma levels of interleukin-6 in sepsis. *Blood* **74**, 1704-1710.

Hall, E.J. (1992) Use of lithium for treatment of estrogen-induced bone marrow hypoplasia in a dog. *J. Am. Vet. Med. Assoc.* **200**, 814-816.

Hammond, W.P. and Dale, D.C. (1980) Lithium therapy of canine cyclic hematopoiesis. *Blood* **55**, 26-28.

Han, M., Kobayashi, M., Imamura, M., Hashino, S., Kobayashi, H., Maeda, S., Iwasaki, H., Fujii, Y., Musashi, M., Sakurada, K., Kasai, M., Hapel, A.J. and Miyazaki, T. (1993) In vitro expansion of murine hematopoietic progenitor cells in liquid cultures for bone marrow transplantation: Effects of stem cell factor. *Int. J. Hematol.* **57**, 113-120.

Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) The protein kinase family; conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.

Harbol, A.W., Liesveld, J.L., SimpsonHaidaris, P.J. and Abboud, C.N. (1994) Mechanisms of cytopenia in human immunodeficiency virus infection. *Blood Reviews* **8**, 241-251.

Harlow, E. and Lane, D. (1988) *Antibodies: a laboratory manual*, Cold Spring Habor Laboratory, USA.

Harrison, D.E. (1972) Lifesparing ability (in lethally irradiated mice) of *W/W^v* mouse marrow with no macroscopic colonies. *Radiation Research* **52**, 553-563.

Harrison, D.E., Zsebo, K.M. and Astle, C.M. (1994) Splenic primitive hematopoietic stem cell (PHSC) activity is enhanced by steel factor because of PHSC proliferation. *Blood* **83**, 3146-3151.

Harrison, D.E. and Astle, C.M. (1976) Population of lymphoid tissues in cured *W* anaemic mice by donor cells. *Transplantation* **22**, 42-46.

Harrison, D.E. and Cherry, M. (1975) Survival of allografts in *W/W^v* anaemic mice: effect of disparity at the *Ea-2* locus. *Immunogenetics* **2**, 219-229.

Harrison, D.E. and Russell, E.S. (1972) The response of *W/W^v* and *Sl/Sl^d* anaemic mice to haemopoietic stimuli. *Brit. J. Haematol.* **22**, 155-168.

Hayashi, K. (1994) Manipulation of DNA by PCR. In: Mullis, K.B., Ferre, F. and Gibbs, R.A. (Eds.) *The Polymerase Chain Reaction*, pp. 3-13. Boston: Birkhauser.

Heaney, M.L. and Golde, D.W. (1996) Soluble cytokine receptors. *Blood* **87**, 847-857.

Heath, D.S., Axelrad, A.A., McLeod, D.L. and Shreeve, M.M. (1976) Separation of the erythropoietin-responsive progenitors BFU-E and CFU-E in mouse bone marrow by unit gravity sedimentation. *Blood* **47**, 777-792.

Heim, M.H. (1996) The Jak-STAT pathway: Specific signal transduction from the cell membrane to the nucleus. *Eur. J. Clin. Invest.* **26**, 1-12.

Heinrich, M.C., Dooley, D.C. and Keeble, W.W. (1995) Transforming growth factor beta1 inhibits expression of the gene products for steel factor and its receptor (c-kit). *Blood* **85**, 1769-1780.

Heldin, C.H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell* **80**, 213-223.

Henry, C.J., Goodman, S. and Lothrop, C.D., Jr. (1990) Dogs receiving mitoxantrone and cyclophosphamide do not produce clinically significant antibody titres to rhG-CSF [Abstract]. *J. Vet. Intern. Med.* **4**, 111.

Herbst, R., Shearman, M.S., Obermeier, A., Schlessinger, J. and Ullrich, A. (1992) Differential effects of W mutations on p145c-kit tyrosine kinase activity and substrate interaction. *J. Biol. Chem.* **267**, 13210-13216.

Hilton, D.J. (1994) Introduction to the cytokine receptors. In: Nicola, N.A. (Ed.) *Guidebook to the cytokines and their receptors*, pp. 9-16. New York. Oxford University Press.

Hilton, D.J., Watowich, S.S., Katz, L. and Lodish, H.F. (1996) Saturation mutagenesis of the WSXWS motif of the erythropoietin receptor. *J. Biol. Chem.* **271**, 4699-4708.

Hirota, S., Ito, A., Morii, E., Wanaka, A., Tohyama, M., Kitamura, Y. and Nomura, S. (1992) Localization of mRNA for c-kit receptor and its ligand in the brain of adult rats: an analysis using in situ hybridization histochemistry. *Mol. Brain Res.* **15**, 47-54.

Hochstein, H.D. (1987) The LAL test versus the rabbit pyrogen test for endotoxin detection. *Pharmaceutical Technology* 124-129.

Hodgson, J. (1993) Expression Systems: A User's Guide. *Bio/Technology* **11**, 887-893.

Howe, L.R., Leevers, S.J., Gomez, N., Nakielny, S., Cohen, P. and Marshall, C.J. (1992) Activation of the MAP kinase pathway by the protein kinase raf. *Cell* **71**, 335-342.

Hsiung, H.M., Mayne, N.G. and Becker, G.W. (1986) High level expression, efficient secretion, and folding of human growth hormone in *Escherichia coli*. *Bio/Technology* **4**, 991-995.

Huang, E., Nocka, K., Beier, D.R., Chu, T., Buck, J., Lahm, H., Wellner, D., Leder, P. and Besmer, P. (1990) The Hematopoietic Growth Factor KL is Encoded by the *Sl* Locus and Is the Ligand of the *c-kit* Receptor, the Gene Product of the *W* Locus. *Cell* **63**, 225-233.

Huang, E.J., Nocka, K., H., Buck, J. and Besmer, P. (1992) Differential Expression and Processing of Two Cell Associated Forms of the Kit-Ligand: KL-1 and KL-2. *Mol. Biol. Cell* **3**, 349-362.

Hubank, M. and Schatz, D.G. (1994) Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res.* **22**, 5640-5648.

Hughes, D.A., Ashworth, A. and Marshall, C.J. (1993) Complementation of byr1 in fission yeast by mammalian MAP kinase kinase requires coexpression of Raf kinase. *Nature* **364**, 349-352.

Hunter, T. and Karin, M. (1992) The regulation of transcription by phosphorylation. *Cell* **70**, 375-387.

Iemura, A., Tsai, M., Ando, A., Wershil, B.K. and Galli, S.J. (1994) The c-kit ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. *American Journal of Pathology* **144**, 321-328.

Ihle, J.N. (1995) The Janus protein tyrosine kinase family and its role in cytokine signaling. *Adv. Immunol.* **60**, 1-35.

Ihle, J.N. and Kerr, I.M. (1995) Jaks and Stats in signaling by the cytokine receptor superfamily. *Trends in Genetics* **11**, 69-74.

Ikuta, K. and Weissman, I.L. (1992) Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1502-1506.

Innis, M.A., Myambo, K.B., Gelfand, D.H. and Brow, M.A. (1988) DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9436-9440.

Innis, M.A., Gelfland, D.H. and White, T.J. (1990) *PCR Protocols. A Guide to Methods and Applications*, San Diego, CA: Academic Press Inc.

Innis, M.A. and Gelfland, D.H. (1990) Optimisation of PCRs. In: Innis, M.A., Gelfland, D.H., Sninsky, J.J. and White, T.J. (Eds.) *PCR Protocols. A Guide to Methods and Applications*, pp. 3-12. Academic Press Inc. San Diego, CA.

Irani, A.M.A., Nilsson, G., Miettinen, U., Craig, S.S., Ashman, L.K., Ishizaka, T., Zsebo, K.M. and Schwartz, L.B. (1992) Recombinant human stem cell factor stimulates differentiation of mast cells from dispersed human fetal liver cells. *Blood* **80**, 3009-3021.

Isaacs, A. and Lindenmann, J. (1957) Virus interference. I. The interferon. *Proc. Roy. Soc. (London),* **B147**, 258-267.

Izquierdo, M.A., Van Der Valk, P., Van ArkOtte, J., Rubio, G., GermaLluch, J.R., Ueda, R., Scheper, R.J., Takahashi, T. and Giaccone, G. (1995) Differential expression of the c-kit proto-oncogene in germ cell tumours. *Journal of Pathology* **177**, 253-258.

Jacobsen, F.W., Stokke, T. and Jacobsen, S.E.W. (1995) Transforming growth factor-beta potently inhibits the viability-promoting activity of stem cell factor and other cytokines and induces apoptosis of primitive murine hematopoietic progenitor cells. *Blood* **86**, 2957-2966.

Jain, N.C. (1986) The Haemopoietic System. In: *Schalm's Veterinary Hematology*, 3rd edn. pp. 350-387. Philadelphia: Lea and Febinger.

Jain, N.C. (1993a) *Essentials of Veterinary Hematology*, Malvern, Pennsylvania, USA. Lea and Febinger.

Jain, N.C. (1993b) The neutrophils. In: *Essentials of Veterinary Hematology*, pp. 222-246. Malvern, Pennsylvania, USA. Lea and Febinger.

Jarrett, O., Laird, H.M. and Hay, D. (1973) Determinants of the host range of feline leukaemia viruses. *J. Gen. Virol.* **20**, 169-175.

Jones, R.J. (1992) Spleen colony-forming unit: a myeloid stem cell. *Current Topics in Microbiology and Immunology* **177**, 75-82.

Kan, O., Whetton, A.D. and Heyworth, C.M. (1993) Development of haemopoietic cells in liquid culture. In: Testa, N.G. and Molineux, G. (Eds.) *Haemopoiesis: a practical approach*, pp. 123-137. New York: Oxford University Press.

Kanz, L., Brugger, W. and Mertelsmann, R. (1993) Hematopoietic Growth Factors and Peripheral Blood Stem Cells as supportive agents in dose intensification. *Eur. J Cancer* **29A**, S23-S26.

Katayama, N., Shih, J.P., Nishikawa, S.I., Kina, T., Clark, S.C. and Ogawa, M. (1993) Stage-specific expression of c-kit protein by murine hematopoietic progenitors. *Blood* **82**, 2353-2360.

Keller, G. (1992) Clonal analysis of Haemopoietic Stem Cell Development in-vivo. *Current Topics in Microbiology and Immunology* **177**, 41-57.

Keller, J.R., Ortiz, M. and Ruscetti, F.W. (1995) Steel factor (c-kit ligand) promotes the survival of hematopoietic stem/progenitor cells in the absence of cell division. *Blood* **86**, 1757-1764.

Keshet, E., Lyman, S.D., Williams, D.E., Anderson, D.M., Jenkins, N.A., Copeland, N.G. and Parada, L.F. (1991) Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. *EMBO J.* **10**, 2425-2435.

Kessinger, A. (1993) Utilisation of peripheral blood stem cells in autotransplantation. *Hematology/Oncology Clinics of North America* **7**, 535-545.

Kessler, D.S., Veals, S.A., Fu, X.Y. and Levy, D.E. (1990) Interferon-alpha regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. *Genes Dev.* **4**, 1753-1765.

Khoury, E., Andre, C., PontvertDelucq, S., Drenou, B., Baillou, C., Guigon, M., Najman, A. and Lemoine, F.M. (1994) Tumor necrosis factor alpha (TNFalpha) downregulates c-kit proto-oncogene product expression in normal and acute myeloid leukemia CD34+ cells via p55 TNFalpha receptors. *Blood* **84**, 2506-2514.

Kinashi, T., Harada, N., Severinson, E. and et al (1986) Cloning of complementary DNA encoding T-cell replacing factor and identity with B-cell growth factor II. *Nature* **324**, 70-73.

Kinoshita, T., Yokota, T., Arai, K. and Miyajima, A. (1995) Suppression of apoptotic death in hematopoietic cells by signalling through the IL-3/GM-CSF receptors. *EMBO J.* **14**, 266-275.

Kishimoto, T., Taga, T. and Akira, S. (1994) Cytokine signal transduction. *Cell* **76**, 253-262.

Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., Miyagawa, K., Piao, Y., Miyazono, K., Urabe, A. and Takaku, F. (1989) Establishment and characterisation of a unique human cell line that proliferates dependently on GM-CSF, Il-3, or erythropoietin. *J. Cell. Physiol.* **140**, 323-334.

Kitamura, Y., Go, S. and Hatanaka, K. (1978) Decrease of mast cells in *W/W^v* mice and their increase by bone marrow transplantation. *Blood* **52**, 447-452.

Kitamura, Y. and Go, S. (1979) Decreased production of mast cells in *Sl/Sl^d* anaemic mice. *Blood* **53**, 492-497.

Koch, C.A., Anderson, D., Moran, M.F., Ellis, C. and Pawson, T. (1991) SH2 and SH3 signalling domains: elements that control of cytoplasmic signalling proteins. *Science* **252**, 668-674.

Kodama, H., Nose, M., Yamaguchi, Y., Tsunoda, J., Suda, T. and Nishikawa, S. (1992) In vitro proliferation of primitive hemopoietic stem cells supported by stromal cells: evidence for the presence of a mechanism(s) other than that involving c-kit receptor and its ligand. *J. Exp. Med.* **176**, 351-361.

Kohno, T., Carmichael, D.F., Sommer, A. and Thompson, R.C. (1990) Refolding of recombinant proteins. *Methods Enzymol.* **185**, 187-195.

Krause, D.S., Fackler, M.J., Civin, C.I. and May, W.S. (1996) CD34: Structure, biology, and clinical utility. *Blood* **87**, 1-13.

Krawiec, D.R. and Gelberg, H.B. (1989) Chronic renal disease in cats. In: Kirk, R.W. (Ed.) *Current Veterinary Therapy X*, pp. 1170-1173. Philadelphia: WB Saunders Co.

Kurland, C. and Gallant, J. (1996) Errors of heterologous protein expression. *Current Opinion in Biotechnology* **7**, 489-493.

Kwok, S., Mack, D.H., Mullis, K.B., Poiesz, B., Ehrlich, G., Blair, D., Friedman-Kien, A. and Sninsky, J.J. (1987) Identification of human immunodeficiency virus sequences by using in vitro enzymatic amplification and oligomer cleavage detection. *J. Virol.* **61**, 1690-1694.

Kyriakis, J.M., App, H., Zhang, X.F., Banerjee, P., Brautigan, D.L., Rapp, U.R. and Avruch, J. (1992) Raf-1 activates MAP kinase-kinase. *Nature* **358**, 417-421.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Langley, K.E., Wypych, J., Mendiaz, E.A., Clogston, C.L., Parker, V.P., Farrar, D.H., Brothers, M.O., Satygal, V.N., Leslie, I., Birkett, N.C. and et al (1992) Purification and characterization of soluble forms of human and rat stem cell factor recombinantly expressed by Escherichia coli and by Chinese hamster ovary cells. *Arch. Biochem. Biophys.* **295**, 21-28.

Langley, K.E., Bennett, L.G., Wypych, J., Yancik, S.A., Liu, X.D., Westcott, K.R., Chang, D.G., Smith, K.A. and Zsebo, K.M. (1993) Soluble stem cell factor in human serum. *Blood* **81**, 656-660.

Langley, K.E., Mendiaz, E.A., Liu, N., Narhi, L.O., Zeni, L., Parseghian, C.M., Clogston, C.L., Leslie, I., Pope, J.A., Lu, H.S. and et al (1994) Properties of variant forms of human stem cell factor recombinantly expressed in Escherichia coli. *Arch. Biochem. Biophys.* **311**, 55-61.

Lardon, F., Snoeck, H.W., Nijs, T.G., Lenjou, M., Peetermans, M.E., Rodrigus, I., Berneman, Z.N. and Bockstaele, D.R.V. (1994) Transforming growth factor-beta regulates the cell cycle status of interleukin-3 (IL-3) plus IL-1, stem cell factor, or IL-6 stimulated CD34+ human hematopoietic progenitor cells through different cell kinetic mechanisms depending on the applied stimulus. *Exp. Hematol.***22**, 903-909.

Lefkowitz, R.J. and Caron, M.G. (1988) Adrenergic receptors. Models for the study of receptors coupled to guanine nucleotide regulatory proteins. *J. Biol. Chem.* **263**, 4993-4996.

Leftwich, J.A., Westin, E.H. and Huff, T.F. (1992) Expression of c-kit by mesenteric lymph node cells from Nippostrongylus brasiliensis-infected mice and by mast cell colonies developing from these cells in response to 3T3 fibroblast-conditioned medium. *J. Immunol.* **148**, 2894-2898.

Leigh, B.R., Hancock, S.L. and Knox, S.J. (1993) The effect of stem cell factor on irradiated human bone marrow. *Cancer Research* **53**, 3857-3859.

Lev, S., Yarden, Y. and Givol, D. (1992) Dimerization and activation of the kit receptor by monovalent and bivalent binding of the stem cell factor. *J. Biol. Chem.* **267**, 15970-15977.

Lewis, J.P., O'Grady, L.F., Bernstein, S.E., Russell, E.S. and Trobaugh, F. (1967) Growth and differentiation of transplanted *W/W*^{v} marrow. *Blood* **30**, 601-616.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., BarSagi, D., Margolis, B. and Schlessinger, J. (1993) Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature* **363**, 85-88.

Lichtman, M.A. (1984) The relationship of stromal cells to haemopoietic cells in the marrow. In: Wright, D.G. and Greenberger, J.S. (Eds.) *Long-term bone marrow culture*, pp. 3-29. New York: Alan R. Liss, Inc.

Linenberger, M.L., Beebe, A.M., Pedersen, N.C., Abkowitz, J.L. and Dandekar, S. (1995) Marrow accessory cell infection and alterations in hematopoiesis accompany severe neutropenia during experimental acute infection with feline immunodeficiency virus. *Blood* **85**, 941-951.

Linenberger, M.L. and Abkowitz, J.L. (1992) Studies in feline long-term marrow culture: Hematopoiesis on normal and feline leukemia virus infected stromal cells. *Blood* **80**, 651-662.

Linnekin, D., Weiler, S.R., Mou, S., DeBerry, C.S., Keller, J.R., Ruscetti, F.W., Ferris, D.K. and Longo, D.L. (1996) JAK2 is constitutively associated with c-Kit and is phosphorylated in response to stem cell factor. *Acta Haematologica* **95**, 224-228.

Lisitsyn, N., Lisitsyn, N. and Wigler, M. (1993) Cloning the differences between two complex genomes. *Science* **259**, 946-951.

Little, C.C. and Cloudman, A.M. (1937) The occurrence of a dominant spotting mutation in the house mouse. *Proc. Natl. Acad. Sci. U.S.A.* **23**, 535-537.

Lothrop, C.D., Warren, D.J., Souza, L.M., Jones, J.B. and Moore, M.A.S. (1988) Correction of Canine Cyclic Hematopoiesis With Recombinant Human Granulocyte Colony-Stimulating Factor. *Blood* **72**, 1324-1328.

Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., BarSagi, D. and Schlessinger, J. (1992) The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* **70**, 431-442.

Lu, H.S., Clogston, C.L., Wypych, J., Fausset, P.R., Lauren, S., Mendiaz, E.A., Zsebo, K.M. and Langley, K.E. (1991) Amino acid sequence and post-translational modification of stem cell factor isolated from buffalo rat liver cell-conditioned medium. *J. Biol. Chem.* **266**, 8102-8107.

Luckow, V.A. and Summers, M.D. (1988) Trends in the development of Baculovirus vectors. *Bio/Technology* **6**, 47-55.

Luckow, V.A. and Summers, M.D. (1989) High level expression of nonfused foreign genes with Autographa californica nuclear polyhedrosis virus expression vectors. *Virology* **170**, 31-39.

Lyman, G.H., Williams, C.C. and Preston, D. (1980) The use of lithium carbonate to reduce infection and leukopenia during systemic chemotherapy. *N. Engl. J. Med.* **302**, 257-260.

Majumdar, M.K., Feng, L., Medlock, E., Toksoz, D. and Williams, D.A. (1994) Identification and mutation of primary and secondary proteolytic cleavage sites in murine stem cell factor cDNA yields biologically active, cell-associated protein. *J. Biol. Chem.* **269**, 1237-1242.

Manegold, C., Jablonowski, H., Armbrecht, C., Strohmeyer, G. and Pietsch, T. (1995) Serum levels of stem cell factor are increased in asymptomatic human immunodeficiency virus-infected patients and are associated with prolonged survival. *Blood* **86**, 243-249.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory.

Manova, K., Nocka, K., Besmer, P. and Bachvarova, R.F. (1990) Gonadal expression of c-kit encoded at the w-locus of the mouse. *Development* **110**, 1057-1069.

Manova, K., Huang, E.J., Angeles, M., DeLeon, V., Sanchez, S., Pronovost, S.M., Besmer, P. and Bachvarova, R.F. (1993) The expression pattern of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in oocyte growth and in proliferation of spermatogonia. *Dev. Biol.* **157**, 85-99.

Manova, K. and Bachvarova, R.F. (1991) Expression of c-kit encoded at the W locus of mice in developing embryonic germ cells and presumptive melanoblasts. *Dev. Biol.* **146**, 312-324.

Marciani, D.J., Kensil, C.R., Beltz, G.A. and et al (1991) Genetically-engineered subunit vaccine against feline leukaemia virus. *Vaccine* **9**, 89

Martin, F.H., Suggs, S.V., Langley, K.E., Lu, H.S., Ting, J., Okino, K.H., Morris, C.F., McNiece, I.K., Jacobsen, F.W., Mendiaz, E.A., Birkett, N.C., Smith, K.A., Johnson, M.J., Parker, V.P., Flores, J.C., Patel, A.C., Fisher, E.F., Erjavec, H.O., Herrera, C.J., Wypych, J., Sachdev, R.K., Pope, J.A., Leslie, I., Wen, D., Lin, C., Cupples, R.L. and Zsebo, K.M. (1990) Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* **63**, 203-211.

Mason, D.Y., Cordell, J., Brown, M., Pallesen, G., Ralfkiaer, E., Rothbard, J., Crumpton, M. and Gatter, K.C. (1989) Detection of T cells in paraffin wax embedded tissue using antibodies against a peptide sequence from the CD3 antigen. *J. Clin. Pathol.* **42**, 1194-1200.

Mason, D.Y., Cordell, J.L., Gaulard, P., Tse, A.G. and Brown, M.H. (1992) Immunohistological detection of human cytotoxic/suppressor T cells using antibodies to a CD8 peptide sequence. *J. Clin. Pathol.* **45**, 1084-1088.

Matos, M.E., Schnier, G.S., Beecher, M.S., Ashman, L.K., Williams, D.E. and Caligiuri, M.A. (1993) Expression of a functional c-kit receptor on a subset of natural killer cells. *J. Exp. Med.* **178**, 1079-1084.

Matous, J.V., Langley, K. and Kaushansky, K. (1996) Structure-function-relationships of stem-cell factor - an analysis based on a series of human-murine stem-cell factor chimera and the mapping of a neutralizing monoclonal-antibody. *Blood* **88**, 437-444.

Matsuguchi, T., Inhorn, R.C., Carlesso, N., Xu, G., Druker, B. and Griffin, J.D. (1995) Tyrosine phosphorylation of p95Vav in myeloid cells is regulated by GM- CSF, IL-3 and steel factor and is constitutively increased by p210BCR/ABL. *EMBO J.* **14**, 257-265.

Matsui, Y., Zsebo, K.M. and Hogan, B.L. (1990) Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. *Nature* **347**, 667-669.

Mauch, P., Lamont, C., Neben, T.Y., Quinto, C., Goldman, S.J. and Witsell, A. (1995) Hematopoietic stem cells in the blood after stem cell factor and interleukin-11 administration: Evidence for different mechanisms of mobilization. *Blood* **86**, 4674-4680.

Mayer, T.C. and Green, M.C. (1968) An experimental analysis of the pigment defect caused by mutations at the *W* and *Sl* loci in mice. *Developmental Biology* **18**, 62-75.

McAdams, T.A., Winter, J., Miller, W.M. and Papoutsakis, E.T. (1996) Hemopoietic cell culture therapies (Part II): clinical aspects and applications. *Trends in Biotechnology* **14**, 388-396.

McCredie, K.B., Hersh, E.M. and Freireich, E.J. (1971) Cells capable of colony formation in the peripheral blood of man. *Science* **171**, 293-294.

McCulloch, E.A., Siminovitch, L., Till, J.E., Russell, E.S. and Bernstein, S.E. (1964) The cellular basis of the genetically determined hemopoietic defect in anemic mice. *Blood* **26**, 399-410.

McGlade, C.J., Ellis, C., Reedijk, M., Anderson, D., Mbamalu, G., Reith, A.D., Panayotou, G., End, P., Bernstein, A., Kazlauskas, A., Waterfield, M.D. and Pawson, T. (1992) SH2 domains of the p85-alpha subunit of phosphatidylinositol 3-kinase regulate binding to growth-factor receptors. *Mol. Cell. Biol.* **12**, 991-997.

McInnes, C.J., Haig, D. and Logan, M. (1993) The cloning and expression of the gene for ovine interleukin-3 (multi CSF) and a comparison of the in vitro hematopoietic activity of ovine Il-3 with ovine GM-CSF and human M-CSF. *Exp. Hematol.* **21**, 1528-1532.

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

McNiece, I.K., Briddell, R.A., Hartley, C.A. and Andrews, R.G. (1993) The role of stem cell factor in mobilization of peripheral blood progenitor cells: synergy with G-CSF. *Stem. Cells Dayt.* **11** Suppl 3, 83-88.

McNiece, I.K. and Briddell, R.A. (1995) Stem cell factor. *Journal of Leukocyte Biology* **58**, 14-22.

McSweeney, P.A., Rouleau, K.A., Storb, R., Bolles, L., Wallace, P.M., Beauchamp, M., Krizanac-Bengez, L., Moore, P., Sale, G., Sandmaier, B., de Revel, T., Appelbaum, F.R. and Nash, R.A. (1996) Canine CD34: cloning of the cDNA and evaluation of an antiserum to recombinant protein. *Blood* **88**, 1992-2003.

Medlock, E.S., Migita, R.T., Trebasky, L.D., Greiner, D., Hendren, W. and DePrince, R. (1992) *Inst. Lab. Anim. Resour.* **34**, S22

Meininger, C.J., Yano, H., Rottapel, R., Bernstein, A., Zsebo, K.M. and Zetter, B.R. (1992) The c-kit receptor ligand functions as a mast cell chemoattractant. *Blood* **79**, 958-963.

Metcalf, D. (1989) The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature* **339**, 27-30.

Metcalf, D. (1991a) Lineage commitment of hemopoietic progenitor cells in developing blast cell colonies: Influence of colony-stimulating factors. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11310-11314.

Metcalf, D. (1991b) The leukemia inhibitory factor (LIF). *Int. J. Cell Cloning* **9**, 95-108.

Metcalf, D. (1993a) Hematopoietic Regulators: Redundancy or Subtlety? *Blood* **12**, 3515-3523.

Metcalf, D. (1993b) The Cellular Basis for Enhancement Interactions Between Stem Cell Factor and the Colony Stimulating Factors. *Stem Cells* **11**, 1-11.

Metcalf, D. and Nicola, N.A. (1991) Direct proliferative actions of stem cell factor on murine bone marrow cells in vitro: effects of combination with colony-stimulating factors. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6239-6243.

Miles, S.A., Lee, K., Hutlin, L., Zsebo, K.M. and Mitsuyasu, R.T. (1991) Potential use of human stem cell factor as adjunctive therapy for human immunodeficiency virus-related cytopenias. *Blood* **78**, 3200-3208.

Miller, L.K. (1988) Baculoviruses as gene expression vectors. *Annu. Rev. Microbiol.* **42**, 177-199.

Mitsui, H., Furitsu, T., Dvorak, A.M., Irani, A.M., Schwartz, L.B., Inagaki, N., Takei, M., Ishizaka, K., Zsebo, K.M., Gillis, S. and et al (1993) Development of human mast cells from umbilical cord blood cells by recombinant human and murine c-kit ligand. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 735-739.

Miura, O., Nakamura, N., Quelle, F.W., Witthuhn, B.A., Ihle, J.N. and Aoki, N. (1994) Erythropoietin induces association of the JAK2 protein tyrosine kinase with the erythropoietin receptor in vivo. *Blood* **84**, 1501-1507.

Miura, Y., Komatsu, N. and Suda, T. (1990) Growth and differentiation of two human megakaryoblastic cell lines; CMK and UT-7. *Prog. Clin. Biol. Res. Prog. Clin. Biol. Res.* **356:** 259-270.

Miyajima, A., Hara, T. and Kitamura, T. (1992a) Common subunits of cytokine receptors and the functional redundancy of cytokines. *Trends in Biochemical Sciences* **17**, 378-382.

Miyajima, A., Kitamura, T., Harada, N., Yokota, T. and Arai, K. (1992b) Cytokine receptors and signal transduction. *Annu. Rev. Immunol.* **10**, 295-331.

Miyazawa, K., Hendrie, P.C., Mantel, C., Wood, K., Ashman, L.K. and Broxmeyer, H.E. (1991) Comparative analysis of signaling pathways between mast cell growth factor (c-kit ligand) and granulocyte-macrophage colony-stimulating factor in a human factor-dependent myeloid cell line involves phosphorylation of Raf-1, GTPase-activating protein and mitogen- activated protein kinase. *Exp. Hematol.* **19**, 1110-1123.

Miyazawa, K., Williams, D.A., Gotoh, A., Nishimaki, J., Broxmeyer, H.E. and Toyama, K. (1995) Membrane-bound steel factor induces more persistent tyrosine kinase activation and longer life span of c-kit gene-encoded protein than its soluble form. *Blood* **85**, 641-649.

Molineux, G., Migdalska, A., Szmitkowski, M., Zsebo, K. and Dexter, T.M. (1991) The effects on hematopoiesis of recombinant stem cell factor (ligand for c-kit) administered in vivo to mice either alone or in combination with granulocyte colony-stimulating factor. *Blood* **78**, 961-966.

Molineux, G., Migdalska, A., Haley, J., Evans, G.S. and Dexter, T.M. (1994) Total marrow failure induced by pegylated stem-cell factor administered before 5-fluorouracil. *Blood* **83**, 3491-3499.

Molineux, G. and Dexter, T.M. (1995) The therapeutic potential of stem cell factor. *FORUM - Trends in Experimental and Clinical Medicine* **5**, 166-172.

Moodie, S.A., Willumsen, B.M., Weber, M.J. and Wolfman, A. (1993) Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* **260**, 1658-1661.

Moore, M.A.S. (1991) Clinical Implications of Positive and Negative Hematopoietic Stem Cell Regulators. *Blood* **78**, 1-19.

Morrison, D.C. and Ulevitch, R.J. (1978) The effects of bacterial endotoxins on host mediation systems. A review. *Am. J. Pathol.* **93**, 526-617.

Morstyn, G., Brown, S., Gordon, M., Crawford, J., Demetri, G., Rich, W., McGuire, B., Foote, M. and McNiece, I. (1994) Stem cell factor is a potent synergistic factor in hematopoiesis. *Oncology* **51**, 205-214.

Muller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A.G., Barbieri, G., Witthuhn, B.A., Schindler, C. and et al (1993) The protein tyrosine kinase JAK1 complements defects in interferon- alpha/beta and -gamma signal transduction. *Nature* **366**, 129-135.

Mullis, K., Faloona, F., Sharf, S., Saiki, R., Horn, G. and Erlich, H. (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb. Symp. Quant. Biol.* **51**, 263-273.

Mullis, K.B. and Faloona, F.A. (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155**, 335-350.

Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T. and Kishimoto, T. (1991) Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11349-11353.

Murtaugh, R.J. and Jacobs, R.M. (1985) Suspected immune-mediated megakaryocytic hypoplasia or aplasia in a dog. *J. Am. Vet. Med. Assoc.* **186**, 1313-1315.

Nabel, G., Galli, S.J., Dvorak, A.M., Dvorak, H.F. and Cantor, H. (1981) Inducer T lymphocytes synthesise a factor that stimulates growth of cloned mast cells. *Nature* **291**, 332-334.

Nagata, S., Tsuchiya, M., Asano, S., Kaziro, Y., Yamazaki, T., Yamamoto, O., Hirata, Y., Kubota, N., Oheda, M., Nomura, H. and Ono, M. (1986) Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. *Nature* **319**, 415-418.

Nakagawa, Y., Kosugi, H., Miyajima, A., Arai, K.I. and Yokota, T. (1994) Structure of the gene encoding the alpha subunit of the human granulocyte- macrophage colony stimulating factor receptor. Implications for the evolution of the cytokine receptor superfamily. *J. Biol. Chem.* **269**, 10905-10912.

Nakahata, T. and Ogawa, M. (1982) Hemopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono- and multipotential hemopoietic progenitors. *J. Clin. Invest.* **70**, 1324-1328.

Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T. and Akira, S. (1993) Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2207-2211.

Nakajima-Iijima, S., Hamada, H., Reddy, P., and Kakunaga, T. 1985 Molecular structure of the human cyoplasmic β-actin gene: interspecies homology of sequences in the introns. *Proc.Natl.Acad.Sci.U.S.A.* **82,** 6133-6137.

Nakamura, N., Sudo, T., Matsuda, S. and Yanai, A. (1992) Molecular-cloning of feline interferon cDNA by direct expression. *Bioscience Biotechnology and Biochemistry* **56**, 211-214.

Narazaki, M., Witthuhn, B.A., Yoshida, K., Silvennoinen, O., Yasukawa, K., Ihle, J.N., Kishimoto, T. and Taga, T. (1994) Activation of JAK2 kinase mediated by the interleukin 6 signal transducer gp130. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2285-2289.

Natali, P.G., Nicotra, M.R., Sures, I., Santoro, E., Bigotti, A. and Ullrich, A. (1992) Expression of c-kit receptor in normal and transformed human nonlymphoid tissues. *Cancer Research* **52**, 6139-6143.

Neben, S., Donaldson, D., Sieff, C., Mauch, P., Bodine, D., Ferrara, J., Yetzaldape, J. and Turner, K. (1994) Synergistic effects of interleukin-11 with other growth-factors on the expansion of murine hematopoietic progenitors and maintenance of stem-cells in liquid culture. *Exp. Hematol.* **22**, 353-359.

Necas, E., Sefc, L., Brecher, G. and Bookstein, N. (1995) Hematopoietic reserve provided by spleen colony-forming units (CFU-S). *Exp. Hematol.* **23**, 1242-1246.

Nickerson, S.C., Baker, P.A. and Trinidad, P. (1989a) Local immunostimulation of the bovine mammary gland with interleukin-2. *J. Dairy Sci.* **72**, 1764-1773.

Nickerson, S.C., Owens, W.E. and Watts, J.L. (1989b) Effects of recombinant granulocyte colony-stimulating factor on Staphylococcus aureus mastitis in lactating dairy cows. *J. Dairy Sci.* **72**, 3286-3294.

Nicola, N.A., Peterson, L., Hilton, D.J. and Metcalf, D. (1988) Cellular processing of murine colony-stimulating factor (multi-CSF, GM-CSF, G-CSF) receptors by normal haemopoietic cells and cell lines. *Growth Factors* **1**, 41-49.

Nicola, N.A. (1994) An introduction to the cytokines. In: Nicola, N.A. (Ed.) *Guidebook to cytokines and their receptors*, pp. 1-7. New York: Oxford University Press]

Nicola, N.A. and Metcalf, D. (1991) Subunit promiscuity among haemopoietic growth factor receptors. *Cell* **67**, 1-4.

Nicolson, L., Curran, J.A., Argyle, D.J., Cox, P. and Onions, D.E. (1994) Potential use of recombinant cytokines in prevention and treatment of equine infectious disease. In: Nakajima, H. and Plowright, W. (Eds.) *Equine infectious disease*, VII edn. pp. 139-142. Newmarket,UK. R & W Publications Ltd.

Nilsson, G., Butterfield, J.H., Nilsson, K. and Siegbahn, A. (1994) Stem cell factor is a chemotactic factor for human mast cells. *J. Immunol.* **153**, 3717-3723.

Nishikawa, S., Kusakabe, M., Yoshinaga, K., Ogawa, M., Hayashi, S., Kunisada, T., Era, T. and Sakakura, T. (1991) In utero manipulation of coat color formation by a monoclonal anti-c- kit antibody: two distinct waves of c-kit-dependency during melanocyte development. *EMBO J.* **10**, 2111-2118.

Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A. and Besmer, P. (1989) Expression of c-kit gene-products in known cellular targets of w- mutations in normal and w-mutant mice - evidence for an impaired c- kit kinase in mutant mice. *Genes Dev.* **3**, 816-826.

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

Nocka, K., Tan, J.C., Chiu, E., Chu, T.Y., Ray, P., Traktman, P. and Besmer, P. (1990b) Molecular-bases of dominant negative and loss of function mutations at the murine c-kit white spotting locus - W^{37}, W^{V}, W^{41} AND *W. EMBO J.* **9**, 1805-1813.

Noma, Y., Sideras, P., Naito, T., Bergsedt-Lindquist, S., Azuma, C., Severinson, E., Tanabe, T., Kinashi, T., Matsuda, F., Yaoita, Y. and Honjo, T. (1986) Cloning of cDNA encoding the murine IgG1 induction factor by a novel strategy using SP6 promoter. *Nature* **319**, 640-646.

Novitsky, T.J. (1983) Choosing a Method. *LAL Update* **1**, 1.

O'Farrell, A.M., Ichihara, M., Mui, A.L.F. and Miyajima, A. (1996) Signaling pathways activated in a unique mast cell line where interleukin- 3 supports survival and stem cell factor is required for a proliferative response. *Blood* **87**, 3655-3668.

Ogawa, M., Porter, P.N. and Nakahata, T. (1983) Renewal and commitment to differentiation of hemopoietic stem cells (an interpretive review). *Blood* **61**, 823-829.

Ogawa, M., Matsuzaki, Y., Nishikawa, S., Hayashi, S., Kunisada, T., Sudo, T., Kina, T. and Nakauchi, H. (1991) Expression and function of c-kit in hemopoietic progenitor cells. *J. Exp. Med.* **174**, 63-71.

Ogawa, M. (1993) Differentiation and Proliferation of Hematopoietic Stem Cells. *Blood* **81**, 2844-2853.

Ogilvie, G.K., Obradovich, J.E., Cooper, M.F., Walters, L.M., Salman, M.D. and Boone, T.C. (1992) Use of recombinant canine granulocyte colony-stimulating factor to decrease myelosuppression associated with the administration of mitoxantrone in the dog. *J. Vet. Intern. Med.* **6**, 44-47.

Ohashi, T., Matsumoto, Y., Watari, T., Goitsuka, R., Tsujimoto, H. and Hasegawa, A. (1993) Molecular-cloning of feline interleukin-6 cDNA. *J. Vet. Med. Sci.* **55**, 941-944.

Okayama, H. and Berg, P. (1982) High-efficiency cloning of full-length cDNA. *Molecular and Cellular Biology* **2**, 161-170.

Okuda, K., Sanghera, J.S., Pelech, S.L., Kanakura, Y., Hallek, M., Griffin, J.D. and Druker, B.J. (1992) Granulocyte-macrophage colony-stimulating factor, interleukin-3, and steel factor induce rapid tyrosine phosphorylation of p42 and p44 MAP kinase. *Blood* **79**, 2880-2887.

Okumura, N., Tsuji, K., Ebihara, Y., Tanaka, I., Sawai, N., Koike, K., Komiyama, A. and Nakahata, T. (1996) Chemotactic and chemokinetic activities of stem-cell factor on murine hematopoietic progenitor cells. *Blood* **87**, 4100-4108.

Olins, P.O. (1996) Quantity versus authenticity of heterologously produced proteins: An inevitable compromise? Editorial overview. *Current Opinion in Biotechnology* **7**, 487-488.

Olivier, J.P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E. and Pawson, T. (1993) A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell* **73**, 179-191.

Orlic, D. and Bodine, D.M. (1994) What defines a pluripotent hematopoietic stem cell (PHSC): Will the real PHSC please stand up! *Blood* **84**, 3991-3994.

Orr-Urtreger, A., Avivi, A., Zimmer, Y., Givol, D., Yarden, Y. and LONAI, P. (1990) Developmental expression of c-kit, a proto-oncogene encoded by the W locus. *Development* **109**, 911-923.

Pandiella, A., Bosenberg, M.W., Huang, E.J., Besmer, P. and Massague, J. (1992) Cleavage of membrane-anchored growth factors involves distinct protease activities regulated through common mechanisms. *J. Biol. Chem.* **267**, 24028-24033.

Parry, A.D., Minasian, E. and Leach, S.J. (1991) Cytokine conformations: predictive studies. *J. Molec. Recog.* **4**, 63-75.

Paulson, R.F., Vesely, S., Siminovitch, K.A. and Bernstein, A. (1996) Signalling by the W/Kit receptor tyrosine kinase is negatively regulated in vivo by the protein tyrosine phosphatase Shp1. *Nature Genet.* **13**, 309-315.

Pawson, T. (1994) SH2 and SH3 domains in signal transduction. *Advances in Cancer Research* **64**, 87-110.

Pedersen, N.C., Ho, E., Brown, M.L. and et al (1987) Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science* **235**, 790-793.

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Pawson, T. and Pelicci, P.G. (1992) A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70**, 93-104.

Perlman, D. and Halvorson, H.O. (1983) A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J. Mol. Biol.* **167**, 391-409.

Petitte, J.N. and Kulik, M.J. (1996) Cloning and characterization of cDNAs encoding two forms of avian stem cell factor. *Biochim. Biophys. Acta-* **1307**, 149-151.

Pharmacia Biotech Booklet (1994) Ion Exchange Chromatography: Principles and Methods. Uppsala, Sweden. Pharmacia Biotech.

Pluznik, D.H. and Sachs, L. (1965) The cloning of normal "mast" cells in tissue culture. *J. Cell Comp. Physiol.* **66**, 319-324.

Poo, W.J., Conrad, L. and Janeway, C.A., Jr. (1988) Receptor-directed focusing of lymphokine release by helper T cells. *Nature* **332**, 378-380.

Porath, J. and Flodin, P. (1959) Gel filtration: A method for desalting and group separation. *Nature* **183**, 1657-1659.

Prasse, K.W., Kaeberle, M.L. and Ramsey, F.R. (1973) Blood neutrophilic granulocyte kinetics in cats. *Am. J. Vet. Res.* **34**, 1021-1025.

Qiu, F., Ray, P., Brown, K., Barker, P.E., Jhanwar, S., Ruddle, S.H. and Besmer, P. (1988) Primary structure of *c-kit* - relationship with the CSF-1/PDGF receptor kinase family oncogenic activation of *v-kit* involves deletion of extracellular domain and C-terminus. *EMBO J.* **7**, 1003-1011.

Quelle, F.W., Shimoda, K., Thierfelder, W., Fischer, C., Kim, A., Ruben, S.M., Cleveland, J.L., Pierce, J.H., Keegan, A.D., Nelms, K. and et al (1995) Cloning of murine Stat6 and human Stat6, Stat proteins that are tyrosine phosphorylated in responses to IL-4 and IL-3 but are not required for mitogenesis. *Mol. Cell Biol.* **1555**, 3336-3343.

Quelle, F.W., Wang, D., Nosaka, T., Thierfelder, W.E., Stravopodis, D., Weinstein, Y. and Ihle, J.N. (1996) Erythropoietin induces activation of Stat5 through association with specific tyrosines on the receptor that are not required for a mitogenic response. *Mol. Cell. Biol.* **16**, 1622-1631.

Quesenberry, P.J. (1992) Stroma-dependent hematolymphopoietic stem cells. *Current Topics in Microbiology and Immunology* **177**, 151-166.

R & D Systems Bulletin. (1995) Cytokines in neurohumoral regulation. *Cytokine Bulletin* 1-3.

Rameh, L.E., Chen, C.S. and Cantley, L.C. (1995) Phosphatidylinositol (3,4,5)P3 interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. *Cell* **83**, 821-830.

Reagan, W.J., Murphy, D., Battaglino, M., Bonney, P. and Boone, T.C. (1995) Antibodies to canine granulocyte colony-stimulating factor induce persistent neutropenia. *Vet. Pathol.* **32**, 374-378.

Reith, A.D., Ellis, C., Lyman, S.D., Anderson, D.M., Williams, D.E., Bernstein, A. and Pawson, T. (1991) Signal transduction by normal isoforms and *W*-mutant variants of the kit receptor tyrosine kinase. *EMBO J.* **10**, 2451-2459.

Rennick, D., Hunte, B., Holland, G. and Thompson-Snipes, L. (1995) Cofactors are essential for stem cell factor-dependent growth and maturation of mast cell progenitors: comparative effects of interleukin- 3 (IL-3), IL-4, IL-10, and fibroblasts. *Blood* **85**, 57-65.

Riggs, P. (1994) Introduction to protein expression by fusion protein vectors. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (Eds.) *Current Protocols in Molecular Biology*, pp. 16.4.1-16.4.3. New York: Greene Publishing Associates Inc. and John Wiley and Sons Inc.

Robinson, W.A., Metcalf, D. and Bradley, T.R. (1967) Stimulation by normal and leukaemic mouse sera of colony formation *in-vitro* by mouse bone marrow cells. *J. Cell Comp. Physiol.* **69**, 83

Rojko, J.L., Cheney, C.M., Gasper, P.W., Hamilton, K.L., Hoover, E.A., Mathes, L.E. and Kociba, G.J. (1986) Infectious feline leukaemia virus is erythrosuppressive in vitro. *Leuk. Res.* **10**, 1193-1199.

Ross, E.M. (1989) Signal sorting and amplification through G protein-coupled receptors. *Neuron* **3**, 141-152.

Rossi, P., Marziali, G., Albanesi, C., Charlesworth, A., Geremia, R. and Sorrentino, V. (1992) A novel c-kit transcript, potentially encoding a truncated receptor, originates within a kit gene intron in mouse spermatids. *Developmental Biology* **152**, 203-207.

Roth, M.J., Tanese, N. and Goff, S.P. (1985) Purification and characterization of murine retroviral reverse transcriptase expressed in Escherichia coli. *J. Biol. Chem.* **260**, 9326-9335.

RozakisAdcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P.G., Schlessinger, J. and Pawson, T. (1992) Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* **360**, 689-692.

RozakisAdcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993) The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature* **363**, 83-85.

Ruscetti, F., Boggs, D.R., Torok, B.J. and Boggs, S.S. (1976) Reduced blood and marrow neutrophils and granulocytic colony forming cells in *Sl/Sl*^{d} mice. *Exp. Biol. Med.* **152**, 398-402.

Russell, E.S., Bernstein, S.E., Lawson, S.A. and Smith, M. (1959) Long-continued function of normal blood-forming tissue transplanted into genetically anemic hosts. *J. Natl. Cancer Inst.* **23**, 557-566.

Russell, E.S. (1979) Hereditary anemias of the mouse: a review for geneticists. *Advances in Genetics* **20**, 357-459.

Saiki, R.K., Sharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**, 1350-1354.

Saiki, R.K., Bugawan, T.L., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1986) Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. *Nature* **324**, 163-166.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491.

Sanger, F., Niklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467.

Sareneva, T., Pirhonen, J., Cantell, K. and Julkunen, I. (1995) N-glycosylation of human interferon-gamma: glycans at Asn-25 are critical for protease resistance. *Biochem. J.* **308**, 9-14.

Schall, T.J., Jongstra, J., Dyer, B.J., Jorgensen, J., Clayberger, C., Davis, M.M. and Krensky, A.M. (1988) A human T cell-specific molecule is a member of a new gene family. *J. Immunol.* **141**, 1018-1025.

Scharf, S.J. (1990) Cloning with PCR. In: Innis, M.A., Gelfland, D.H., Sninsky, J.J. and White, T.J. (Eds.) *PCR Protocols. A Guide to Methods and Applications*, pp. 84-91. San Diego, CA. Academic Press Inc.

Schijns, V.E.C.J., Wierda, C.M.H., Van Dam, E.J.M., Vahlenkamp, T.W. and Horzinek, M.C. (1995) Molecular cloning of feline interleukin-4. Unpublished Work.

Schindler, C. and Darnell, J.E.J. (1995) Transcriptional responses to polypeptide ligands: The JAK-STAT pathway. *Ann. Rev. Biochem.* **64**, 621-651.

Schlessinger, J. (1988) Signal transduction by allosteric receptor oligomerisation. *Trends in Biochemical Sciences* **13**, 443-447.

Schlessinger, J. (1994) SH2/SH3 signaling proteins. *Current Opinion in Genetics and Development* **4**, 25-30.

Schuening, F.G., Appelbaum, F.R., Deeg, H.J., Sullivan Pepe, M., Graham, T.C., Hackman, R., Zsebo, K.M. and Storb, R. (1993) Effects of recombinant canine stem cell factor, a c-kit ligand, and recombinant granulocyte colony-stimulating factor on hematopoietic recovery after otherwise lethal total body irradiation. *Blood* **81**, 20-26.

Seidman, J.G. (1994) Construction of recombinant DNA libraries. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (Eds.) *Current Protocols in Molecular Biology*, pp. 5.0.3-5.0.4. New York: Greene Publishing Associates Inc. and John Wiley and Sons Inc.

Serra, R. and Moses, H.L. (1996) Tumor suppressor genes in the TGF-β sigalling pathway? *Nature Med.* **2**, 390-391.

Shein, C.H. and Noteborn, M.H.M. (1988) Formation of soluble recombinant proteins in *Escherichia coli* is favoured by lower growth temperature. *Bio/Technology* **6**, 291-294.

Shelton, G.H., Abkowitz, J.L., Linenberger, M.L., Russell, R.G. and Grant, C.K. (1989) Chronic leukopenia associated with feline immunodeficiency virus infection in a cat. *J. Am. Vet. Med. Assoc.* **194**, 253-255.

Shelton, G.H., Linenberger, M.L., Grant, C.K. and Abkowitz, J.L. (1990) Hematologic manifestations of feline immunodeficiency virus infection. *Blood* **76**, 1104-1109.

Shelton, G.H. and Linenberger, M.L. (1995) Hematologic abnormalities associated with retroviral infections in the cat. *Semin. Vet. Med. Surg. (Small. Anim).* **10**, 220-233.

Shuai, K., Schindler, C., Prezioso, V.R. and Darnell, J.E., Jr. (1992) Activation of transcription by IFN-gamma: tyrosine phosphorylation of a 91-kD DNA binding protein. *Science* **258**, 1808-1812.

Shuai, K., Stark, G.R., Kerr, I.M. and Darnell, J.E., Jr. (1993a) A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. *Science* **261**, 1744-1746.

Shuai, K., Ziemiecki, A., Wilks, A.F., Harpur, A.G., Sadowski, H.B., Gilman, M.Z. and Darnell, J.E. (1993b) Polypeptide signaling to the nucleus through tyrosine phosphorylation of JAK and STAT proteins. *Nature* **366**, 580-583.

Shuai, K., Horvath, C.M., Huang, L.H., Qureshi, S.A., Cowburn, D. and Darnell, J.E., Jr. (1994) Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell* **76**, 821-828.

Shull, R.M., Suggs, S.V., Langley, K.E., Okino, K.H., Jacobsen, F.W. and Martin, F.H. (1992) Canine stem cell factor (c-kit ligand) supports the survival of hematopoietic progenitors in long-term canine marrow culture. *Exp. Hematol.* **20**, 1118-1124.

Silvennoinen, O., Schindler, C., Schlessinger, J. and Levy, D.E. (1993) Ras-independent growth factor signaling by transcription factor tyrosine phosphorylation. *Science* **261**, 1736-1739.

Simmons, D.L., Satterthwaite, A.B., Tenen, D.G. and Seed, B. (1992) Molecular cloning of a cDNA encoding CD34, a sialomucin of human hematopoietic stem cells. *J. Immunol.* **148**, 267-271.

Sinclair, W.K. (1968) Cyclic X-ray responses in mammalian cells *in-vitro*. *Radiat. Res.* **33**, 620-643.

Sitnicka, E., Ruscetti, F.W., Priestley, G.V., Wolf, N.S. and Bartelmez, S.H. (1996) Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells. *Blood* **88**, 82-88.

Skolnik, E.Y., Batzer, A., Li, N., Lee, C.H., Lowenstein, E., Mohammadi, M., Margolis, B. and Schlessinger, J. (1993a) The function of GRB2 in linking the insulin receptor to Ras signaling pathways. *Science* **260**, 1953-1955.

Skolnik, E.Y., Lee, C.H., Batzer, A., Vicentini, L.M., Zhou, M., Daly, R., Myers, M.J., Jr., Backer, J.M., Ullrich, A., White, M.F. and et al (1993b) The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine- phosphorylated IRS1 and Shc: implications for insulin control of ras signalling. *EMBO J.* **12**, 1929-1936.

Smith, C.A., Farrah, T. and Goodwin, R.G. (1994) The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* **76**, 959-962.

Smith, D.B. and Corcoran, L.M. (1994) Expression and Purification of Glutathione S-Transferase Fusion Proteins. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (Eds.) *Current Protocols in Molecular Biology*, pp. 16.7.1-16.7.8. New York: Greene Publishing Associates Inc. and John Wiley and Sons Inc.

Smith, D.B. and Johnson, K.S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31-40.

Smith, K.A. and Zsebo, K.M. (1993) Measurement of Human and Murine Stem Cell Factor (*c-kit* ligand). In: Coligan, J.E., Krusibeck, A.M., Margulies, D.H., Shevach, E.M. and Strober, W. (Eds.) *Current Protocols in Immunology*, pp. 16.17.1-16.17.11. New York: Greene Publishing Associates Inc. and John Wiley and Sons Inc.

Smith, T.F. and Waterman, M.S. (1981) Comparison of Bio-sequences. *Advances in Applied Mathematics* **2**, 482-489.

Somers, W., Ultsch, M., De Vos, A.M. and Kossiakoff, A.A. (1994) The X-ray structure of a growth hormone-prolactin receptor complex. *Nature* **372**, 478-481.

Sorrentino, V., Giorgi, M., Geremia, R., Besmer, P. and Rossi, P. (1991) Expression of the c-kit protooncogene in the murine male germ-cells. *Oncogene* **6**, 149-151.

Spiegel, A.M. (1992) G proteins in cellular control. *Curr. Opin. Cell Biol.* **4**, 203-211.

Spooncer, E., Heyworth, C.M., Dunn, A. and Dexter, T.M. (1986) Self-renewal and differentiation of interleukin-3-dependent multipotent stem cells are modulated by stromal cells and serum factors. *Differentiation* **31**, 111-118.

Sprang, S.R. and Bazan, J.F. (1993) Cytokine structural taxonomy and mechanisms of receptor engagement. *Current Opinion in Structural Biology* **3**, 815-827.

Spritz, R.A., Giebel, L.B. and Holmes, S.A. (1992) Dominant negative and loss of function mutations of the c-kit (mast stem-cell growth-factor receptor) protooncogene in human piebaldism. *Amer. J. Hum. Gen.* **50**, 261-269.

Steis, R.G., Smith, J.W., 2d, Urba, W.J., Clark, J.W., Itri, L.M., Evans, L.M., Schoenberger, C. and Longo, D.L. (1988) Resistance to recombinant interferon alfa-2a in hairy-cell leukemia associated with neutralizing anti-interferon antibodies. *N. Engl. J. Med.* **318**, 1409-1413.

Steis, R.G., Smith, J.W., 2d, Urba, W.J., Venzon, D.J., Longo, D.L., Barney, R., Evans, L.M., Itri, L.M. and Ewel, C.H. (1991) Loss of interferon antibodies during prolonged continuous interferon-alpha 2α therapy in hairy cell leukemia. *Blood* 77, 792-798.

Stephenson, J.R., Axelrad, A.A., McLeod, D.L. and Shreeve, M.M. (1971) Induction of colonies of hemoglobin-synthesising cells by erythropoietin *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1542-1546.

Stormo, G.D., Schneider, T.D. and Gold, L. (1982) Characterisation of translation initiation sites in *E.coli*. *Nucleic Acids Res.* **10**, 2971-2996.

Strader, C.D., Fong, T.M., Tota, M.R., Underwood, D. and Dixon, R.A.F. (1994) Structure and function of G protein-coupled receptors. *Ann. Rev. Biochem.* **63**, 101-132.

Suda, T., Suda, J. and Ogawa, M. (1984) Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2520-2524.

Sutcliffe, J.G., Shinnick, T.M., Green, N., Liu, F.T., Niman, H.L. and Lerner, R.A. (1980) Chemical synthesis of a polypeptide predicted from nucleotide sequence allows detection of a new retroviral gene product. *Nature* **287**, 801-805.

Szczylik, C., Skorski, T., Ku, D.H., Nicolaides, N.C., Wen, S.C., Rudnicka, L., Bonati, A., Malaguarnera, L. and Calabretta, B. (1993) Regulation of proliferation and cytokine expression of bone marrow fibroblasts: Role of c-myb. *J. Exp. Med.* **178**, 997-1005.

Tabor, S. and Richardson, C.C. (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4767-4771.

Tabor, S. and Richardson, C.C. (1989) Selective inactivation of the exonuclease activity of bacteriophage T7 DNA polymerase by *in vitro* mutagenesis. *J. Biol. Chem.* **264**, 6447-6458.

Tam, J.P. (1988) Synthetic peptide vaccine design: synthesis and properties of a high- density multiple antigenic peptide system. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5409-5413.

Tam, J.P. and Shao, J. (1993) Synthesis and use of multiple antigen peptide (MAP) systems. In: Coligan, J.E., Krusibeck, A.M., Margulies, D.H., Shevach, E.M. and Strober, W. (Eds.) *Current Protocols in Immunology*, pp. 9.6.1-9.6.19. New York: Greene Publishing Associates Inc. and John Wiley and Sons Inc.

Tan, J.C., Nocka, K., Ray, P., Traktman, P. and Besmer, P. (1990) The dominant W42 spotting phenotype results from a missense mutation in the c-kit receptor kinase. *Science* **247**, 209-212.

Tang, B., Mano, H., Yi, T. and Ihle, J.N. (1994) Tec kinase associates with c-kit and is tyrosine phosphorylated and activated following stem cell factor binding. *Mol. Cell Biol.* **14**, 8432-8437.

Taniguchi, H., Toyoshima, T., Fukao, K. and Nakauchi, H. (1996) Presence of hematopoietic stem cells in the adult liver. *Nat. Med.* **2**, 198-203.

Taniguchi, T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R. and Hamuro, J. (1983) Structure and expression of a cloned cDNA for human interleukin-2. *Nature* **302**, 305-310.

Tanner, J.W., Chen, W., Young, R.L., Longmore, G.D. and Shaw, A.S. (1995) The conserved box 1 motif of cytokine receptors is required for association with JAK kinases. *J. Biol. Chem.* **270**, 6523-6530.

Tartaglia, L.A., Ayres, T.M., Wong, G.H. and Goeddel, D.V. (1993) A novel domain within the 55 kd TNF receptor signals cell death. *Cell* **74**, 845-853.

Testa, N.G., Onions, D.E., Jarrett, O. and et, a. (1983) Haemopoietic colony formation (BFU-E, GM-CFC) during the development of pure red cell hypoplasia induced in the cat by feline leukaemia virus. *Leuk. Res.* **7**, 103-116.

Testa, N.G., Heyworth, C.M., Lord, B.I. and De Wynter, E.A. (1995) Biological assays for haemopoietic growth factors. In: Balkwill, F.R. (Ed.) *Cytokines: a practical approach*, 2nd edn. pp. 247-268. New York: Oxford University Press

Thompson, R.B. (1979) Haematopoiesis: The Erythrocyte: Erythrocytosis. In: Thompson, R.B. (Ed.) *A Short Textbook of Haematology*, 5th edn. pp. 1-24. Tunbridge Wells, Kent, UK. Pitman Medical Publ. Co. Ltd.

Thoreau, E., Petridou, B., Kelly, P.A., Djiane, J. and Mornon, J.P. (1991) Structural symmetry of the extracellular domain of the cytokine/growth hormone/prolactin receptor family and interferon receptors revealed by hydrophobic cluster analysis. *FEBS Letters* **282**, 26-31.

Till, J.E., McCulloch, E.A. and Siminovitch, L. (1964) A stochastic model of stem cell proliferation based on the growth of spleen colony forming cells. *Proc. Natl. Acad. Sci. U.S.A.* **51**, 29-36.

Till, J.E. and McCulloch, E.A. (1961) Direct Measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* **14**, 213-222.

Toksoz, D., Zsebo, K.M., Smith, K.A., Hu, S., Brankow, D., Suggs, S.V., Martin, F.H. and Williams, D.A. (1992) Support of human hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expressing the membrane-bound and secreted forms of the human homolog of the steel gene product, stem cell factor. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7350-7354.

Tong, J., Gordon, M.S., Srour, E.F., Cooper, R.J., Orazi, A., McNiece, I. and Hoffman, R. (1993) In vivo administration of recombinant methionyl human stem cell factor expands the number of human marrow hematopoietic stem cells. *Blood* **82**, 784-791.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350-4754.

Tsai, M., Shih, L.S., Newlands, G.F.J., Takeishi, T., Langley, K.E., Zsebo, K.M., Miller, H.R.P., Geissler, E.N. and Galli, S.J. (1991a) The rat c-kit ligand, stem cell factor, induces the development of connective tissue-type and mucosal mast cells in vivo. Analysis by anatomical distribution, histochemistry, and protease phenotype. *J. Exp. Med.* **174**, 125-131.

Tsai, M., Takeishi, T., Thompson, H., Langley, K.E., Zsebo, K.M., Metcalfe, D.D., Geissler, E.N. and Galli, S.J. (1991b) Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6382-6386.

Tsujimura, T., Koshimizu, U., Katoh, H., Isozaki, K., Kanakura, Y., Tono, T., Adachi, S., Kasugai, T., Tei, H., Nishimune, Y., Nomura, S. and Kitamura, Y. (1993) Mast cell number in the skin of heterozygotes reflects the molecular nature of c-kit mutation. *Blood* **81**, 2530-2538.

Tushinski, R.J., Oliver, I.T., Guilbert, L.J., Tynan, P.W., Warner, J.R. and Stanley, E.R. (1982) Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. *Cell* **28**, 71-81.

Ueda, Y., Sakurai, T. and Yanai, A. (1993) Homogeneous production of feline interferon in silkworm by replacing single amino-acid code in signal peptide region in recombinant baculovirus and characterization of the product. *J. Vet. Med. Sci.* **55**, 251-258.

Ulich, T.R., del Castillo, J., Yi, E.S., Yin, S., McNiece, I., Yung, Y.P. and Zsebo, K.M. (1991) Hematologic effects of stem cell factor in vivo and in vitro in rodents. *Blood* **78**, 645-650.

Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212.

Valent, P., Spanblochl, E., Sperr, W.R., Sillaber, C., Zsebo, K.M., Agis, H., Strobl, H., Geissler, K., Bettelheim, P. and Lechner, K. (1992) Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/kit-ligand in long-term culture. *Blood* **80**, 2237-2245.

Van Die, I., van Tetering, A., Bakker, H., van den Eijnden, D.H. and Joziasse, D.H. (1996) Glycosylation in lepidopteran insect cells: identification of a beta 1-4-N-acetylgalactosaminyltransferase involved in the synthesis of complex-type oligosaccharide chains. *Glycobiology.* **6**, 157-164.

Van Zant, G. and Goldwasser, E. (1977) Simultaneous effects of erythropoietin and colony-stimulating factor on bone marrow cells. *Science* **198**, 733-735.

Vandenbark, G.R., Chen, Y., Friday, E., Pavlik, K., Anthony, B., DeCastro, C. and Kaufman, R.E. (1996) Complex regulation of human c-kit transcription by promoter repressors, activators, and specific myb elements. *Cell Growth and Differentiation* **7**, 1383-1392.

Veres, G., Gibbs, R.A., Scherer, S.E. and Caskey, C.T. (1987) The molecular basis of the sparse fur mouse mutation. *Science* **237**, 415-417.

Verfaillie, C.M. (1992) Direct contact between human primitive hematopoietic progenitors and bone marrow stroma is not required for long-term in vitro hematopoiesis. *Blood* **79**, 2821-2826.

Verfaillie, C.M., Catanzarro, P.M. and Li, W.N. (1994) Macrophage inflammatory protein 1 alpha, interleukin 3 and diffusible marrow stromal factors maintain human hematopoietic stem cells for at least eight weeks in vitro. *J. Exp. Med.* **179**, 643-649.

Vilcek, J. and Le, J. (1994) Immunology of the cytokines: an introduction. In: Thomson, A. (Ed.) *The Cytokine Handbook*, 2nd edn. pp. 1-19. San Diego, CA. Academic Press Ltd.

Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**, 205-214.

von Heijne, G. (1983) Patterns of amino acids near signal-sequence cleavage sites. *Eur. J. Biochem.* **133**, 17-21.

von Heijne, G. (1986) A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14**, 4683-4690.

Wadhwa, M., Bird, C., Page, L., Mire-Sluis, A. and Thorpe, R. (1995) Quantitative biological assays for individual cytokines. In: Balkwill, F.R. (Ed.) *Cytokines: a practical approach*, 2nd edn. pp. 357-391. New York: Oxford University Press.

Wadhwa, M., Bird, C., Fagerberg, J., Gaines-Das, R., Ragnhammar, P., Mellstedt, H. and Thorpe, R. (1996) Production of neutralizing granulocyte-macrophage colony-stimulating factor (GM-CSF) antibodies in carcinoma patients following GM-CSF combination therapy. *Clin. Exp. Immunol.* **104**, 351-358.

Wagner, R., Geyer, H., Geyer, R. and Klenk, H.D. (1996) N-acetyl-beta-glucosaminidase accounts for differences in glycosylation of influenza virus hemagglutinin expressed in insect cells from a baculovirus vector. *J. Virol.* **70**, 4103-4109.

Walter, G., Scheidtmann, K.H., Carbone, A., Laudano, A.P. and Doolittle, R.F. (1980) Antibodies specific for the carboxy- and amino-terminal regions of simian virus 40 large tumor antigen. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5197-5200.

Walter, P. and Lingappa, V.R. (1986) Mechanism of protein translocation across the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* **2**, 499-516.

Warne, P.H., Viciana, P.R. and Downward, J. (1993) Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. *Nature* **364**, 352-355.

Watling, D., Guschin, D., Muller, M., Silvennoinen, O., Witthuhn, B.A., Quelle, F.W., Rogers, N.C., Schindler, C., Stark, G.R., Ihle, J.N. and et al (1993) Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. *Nature* **366**, 166-170.

Weiler, S.R., Mou, S., DeBerry, C.S., Keller, J.R., Ruscetti, F.W., Ferris, D.K., Longo, D.L. and Linnekin, D. (1996) JAK2 is associated with the c-kit proto-oncogene product and is phosphorylated in response to stem cell factor. *Blood* **87**, 3688-3693.

Wen, Z., Zhong, Z. and Darnell, J.E., Jr. (1995) Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* **82**, 241-250.

Wershil, B.K., Tsai, M., Geissler, E.N., Zsebo, K.M. and Galli, S.J. (1992) The rat c-kit ligand, stem cell factor, induces c-kit receptor-dependent mouse mast cell activation in vivo. Evidence that signaling through the c-kit receptor can induce expression of cellular function. *J. Exp. Med.* **175**, 245-255.

Wheelock, E.F. (1965) Interferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin. *Science* **149**, 310-311.

Whitlock, C.A. and Witte, O.N. (1982) Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3608-3612.

Williams, D.E., DeVries, S.P., Namen, A.E., Widmer, M.B. and Lyman, S.D. (1992) The steel factor. *Dev. Biol.* **151**, 368-376.

Williams, D.E. (1994) Optimizing the effectiveness of hematopoietic growth factors. *J. Clin. Immunol.* **14**, 215-223.

Williams, G.T., Smith, C.A., Spooncer, E., Dexter, T.M. and Taylor, D.R. (1990) Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature* **343**, 76-79.

Williams, L.T. (1989) Signal transduction by the platelet-derived growth factor receptor. *Science* **243**, 1564-1570.

Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Yi, T., Tang, B., Miura, O. and Ihle, J.N. (1993) JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* **74**, 227-236.

Wodnar-Filipowicz, A., Tichelli, A., Zsebo, K.M., Speck, B. and Nissen, C. (1992) Stem cell factor stimulates the in vitro growth of bone marrow cells from aplastic anemia patients. *Blood* **79**, 3196-3202.

Wolf, H., Modrow, S., Motz, M., Jameson, B., Hermann, G. and Fortsch, B. (1987) An Integrated Family of Amino Acid Sequence Analysis Programs. *Computer Applications in the Biosciences* **4**, 187-191.

Wong, G.G., Witek, J.A.S., Temple, P.A., Wilkens, K.M., Leary, A.C., Luxenberg, D.P., Jones, S.S., Brown, E.L., Kay, R.M., Orr, E.C., Shoemaker, C., Golde, D.W., Kaufman, R.J., Hewick, R.M., Wang, E.A. and Clark, S.C. (1985) Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science* **228**, 810-815.

Wu, A.M., Till, J.E., Siminovitch, L. and McCulloch, E.A. (1967) A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cellls. *J. Cell Physiol.* **69**, 177-184.

Wu, A.M., Till, J.E., Siminovitch, L. and McCulloch, E.A. (1968) Cytological evidence for a relationship between normal haematopoietic colony forming cells and cells of the lymphoid system. *J. Exp. Med.* **127**, 455-464.

Wypych, J., Bennett, L.G., Schwartz, M.G., Clogston, C.L., Lu, H.S., Broudy, V.C., Bartley, T.D., Parker, V.P. and Langley, K.E. (1995) Soluble Kit receptor in human serum. *Blood* **85**, 66-73.

Yamamoto, K., Tojo, A., Aoki, N. and Shibuya, M. (1993) Characterization of the promoter region of the human c-kit proto-oncogene. *Japanese Journal of Cancer Research* **84**, 1136-1144.

Yamazaki, K., Roberts, R.A., Spooncer, E., Dexter, T.M. and Allen, T.D. (1989) Cellular interactions between 3T3 cells and interleukin-3-dependent multipotent haemopoietic cells: a model system for stromal-cell- mediated haemopoiesis. *J. Cell. Physiol.* **139**, 301-312.

Yan, X.Q., Briddell, R., Hartley, C., Stoney, G., Samal, B. and McNiece, I. (1994) Mobilization of long-term hematopoietic reconstituting cells in mice by the combination of stem cell factor plus granulocyte colony- stimulating factor. *Blood* **84**, 795-799.

Yang, Y.C., Ciarletta, A.B., Temple, P.A., Chung, M.P., Kovacic, S., Witek Giannotti, J.S., Leary, A.C., Kriz, R., Donahue, R.E., Wong, G.G. and Clark, S.C. (1986) Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell* **47**, 3-10.

Yarden, Y., Kuang, W.J., YangFeng, T., Coussens, L., Munemitsu, S., Dull, T.J., Chen, E., Schlessinger, J., Francke, U. and Ullrich, A. (1987) Human proto-oncogene c-kit - a new cell-surface receptor tyrosine kinase for an unidentified ligand. *EMBO Journal* **6**, 3341-3351.

Yarden, Y. and Ullrich, A. (1988) Growth-factor receptor tyrosine kinases. *Ann. Rev. Biochem.* **57**, 443-478.

Yasuda, H., Galli, S.J. and Geissler, E.N. (1993) Cloning and functional analysis of the mouse c-kit promoter. *Biochem. Biophys. Res. Comm.* **191**, 893-901.

Yokota, T., Lee, F., Rennick, D., Hall, C., Arai, N., Mosmann, T., Nabel, G., Cantor, H. and Arai K, I. (1984) Isolation and characterisation of a mouse cDNA clone that expresses mast-cell growth-factor activity in monkey cells. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1070-1074.

Yoshimura, A., Zimmers, T., Neumann, D., Longmore, G., Yoshimura, Y. and Lodish, H.F. (1992) Mutations in the Trp-Ser-X-Trp-Ser motif of the erythropoietin receptor abolish processing, ligand binding, and activation of the receptor. *J. Biol. Chem.* **267**, 11619-11625.

Young, P.R. (1992) Protein hormones and their receptors. *Current Opinion in Biotechnology* **3**, 408-421.

Zhang, X.F., Settleman, J., Kyriakis, J.M., Takeuchi-Suzuki, E., Elledge, S.J., Marshall, M.S., Bruder, J.T., Rapp, U.R. and Avruch, J. (1993) Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature* **364**, 308-313.

Zhang, Z. and Anthony, R.V. (1994) Porcine stem cell factor/c-kit ligand: its molecular cloning and localization within the uterus. *Biol. Reprod.* **50**, 95-102.

Zhou, J.H., Ohtaki, M. and Sakurai, M. (1993) Sequence of a cDNA encoding chicken stem cell factor. *Gene* **127**, 269-270.

Zhou, J.H., Hikono, H., Ohtaki, M., Kubota, T. and Sakurai, M. (1994) Cloning and characterization of cDNAs encoding two normal isoforms of bovine stem cell factor. *Biochim. Biophys. Acta* **1223**, 148-150.

Zon, L.I. (1995) Developmental Biology of Hematopoiesis. *Blood* **86**, 2876-2801.

Zsebo, K.M., Williams, D.A., Geissler, E.N., Broudy, V.C., Martin, F.H., Atkins, H.L., Hsu, R.Y., Birkett, N.C., Okino, K.H., Murdock, D.C., Jacobsen, F.W., Langley, K.E., Smith, K.A., Takeishi, T., Cattenach, B.M., Galli, S.J. and Suggs, S.V. (1990a) Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* **63**, 213-224.

Zsebo, K.M., Wypych, J., McNiece, I.K., Lu, H.S., Smith, K.A., Karkare, S.B., Sachdev, R.K., Yuschenkoff, V.N., Birkett, N.C., Williams, L.R., Satyagal, V.N., Tung, W., Bosselman, R.A., Mendiaz, E.A. and Langley, K.E. (1990b) Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver--conditioned medium. *Cell* **63**, 195-201.

Zsebo, K.M., Smith, K.A., Hartley, C.A., Greenblatt, M., Cooke, K., Rich, W. and McNiece, I.K. (1992) Radioprotection of mice by recombinant rat stem cell factor. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9464-9468.

Zuckerman, K.S., Quesenberry, P.J., Levin, J. and Sullivan, R. (1979) Contamination of erythropoietin by endotoxin: in vivo and in vitro effects on murine erythropoiesis. *Blood* **54**, 146-158.

GLOSSARY

GENERAL ABREVIATIONS

°C	degrees celsius
µl	microlitre(s)
µM	micromolar
2-ME	β-mercaptoethanol
A	adenine or adenosine; one letter code for alanine
$A_{260/280/600}$	absorbance at 260, 280 or 600 nm
AA	aplastic anaemia
Ab	antibody
ABC	avidin-biotin complex
ABMT	autologous bone marrow transplantation
ACK	ammonium chloride/potassium
Ag	antigen
AP	alkaline phosphatase
APS	ammonium persulphate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AUFS	absorbance units, full scale
BFU-E	burst forming unit erythroid
BM	bone marrow
BMDMC	bone marrow derived mast cells
BMMC	bone marrow mononuclear cells
bp	base pair
Bq	Becquerel
BSA	bovine serum albumin
C	cytosine or cytidine; one letter code for cysteine
CD	cluster of differentiation
cDNA	complimentary deoxyribonucleic acid
CFA	complete Freund's adjuvant
CFC	colony forming cell
CFU	colony forming unit
CFU-Bas	colony forming unit - basophil
CFU-Eo	colony forming unit - eosinophil
CFU-E	colony forming unit - erythroid
CFU-GEMM	colony forming unit -granulocyte erythroid macrophage monocyte
CFU-GM	colony forming unit - granulocyte macrophage
CFU-Meg	colony forming unit - megakaryocyte

CFU-Mono	colony forming unit - monocyte
Ci	curie
CM	conditioned medium
Con-A	concanavalin A
CPA	cell proliferation assay
cpm	counts per minute
Da	dalton
dATP	deoxyadenosine triphosphate
DBA	Diamond Blackfan anaemia
dCTP	deoxycytidine triphosphate
ddATP	dideoyadenosine triphosphate
DDBJ	DNA Data Bank of Japan
ddCTP	dideoycytidine triphosphate
ddGTP	dideoyguanosine triphosphate
ddNTP	dideoynucleoside triphosphate
ddTTP	dideoythymidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle medium
DMF	dimethyformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EtBr	ethidium bromide
FA	Fanconi's anaemia
FBS/FCS	fetal bovine serum/fetal calf serum
FCS	fetal calf serum
FeLV	feline leukaemia virus
FITC	fluorescein isothiocyanate
FIV	feline immunodeficiency virus
FPLC	fast protein, peptide or polynucleotide liquid chromatography
G	gauge; guanine or guanosine; one letter code for glycine
g	gravity; gram(s)

GAP	GTPase activating protein
GF	gel filtration
GST	glutathine S-transferase
GTP	guanosine 5'- triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
IEX	ion exchange
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
IMDM	Iscove's modified Dulbecco's medium
IPTG	isopropyl-1-thio-β-D-galactoside
IU	international unit
kb	kilobase
kDa	kilodalton
KoAc	potassium acetate
KLH	keyhole limpet haemocyanin
L	litre
LB	Luria Bertani medium
M	molar
MAP	multiple antigenic peptide; mitogen-associated kinase
mcg	microgram
mg	milligram(s)
mM	micromolar
M-MuLV	Moloney murine leukaemia virus
mRNA	messenger ribonucleic acid
MWCO	molecular weight cut off
NEAA	non essential amino acids
nm	nanometre
nt	nucleotide
NTP	nucleoside triphosphate
oligo(dT)	oligodeoxythymidylic acid
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBSC	peripheral blood stem cell

PCR	polymerase chain reaction
PFA	paraformaldehyde
PGC	primordial germ cell
PHA	phytohaemagglutinin
PHSC	pluripotent haemopoietic stem cell
PMSF	phenylmethylsulphonyl fluoride
poly(A)$^+$	polyadenylated (mRNA)
PSCT	peripheral blood stem cell transplantation
RBC	red blood cell
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RPMI	Rosewell Park Memorial Institute
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sl	Steel locus
SPF	specific pathogen free
STWS	Scott's tap water substitute
T	thymine or thymidine; one letter code for threonine
TBE	Tris/borate/EDTA
TBS-T	tris buffered saline-tween solution
TE	Tris-EDTA buffer
TEA	Tris/EDTA/acetate
TEMED	*N*,*N*,*N'*,*N'*- tetramethyl-ethylenediamine
T_m	melting (or midpoint) temperature
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	Tris hydrochloride
tRNA	transfer ribonucleic acid
TSS	transformation and storage solution
UV	ultraviolet
UWGCG	University of Wisconsin Genetics Computer Group
W	dominant white spotting locus
WBC	white blood cell
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CYTOKINES

BDNF	brain-derived neurotrophic factor
CNTF	ciliary neurotrophic factor
CSF	colony stimulating factor
CSF-1	colony stimulating factor-1 (M-CSF)
EGF	epidermal growth factor
EPO	erythropoietin
FGF	fibroblast growth factor
Flt	fms-like tyrosine kinase
frSCF	feline recombinant stem cell factor
G-CSF	granulocyte colony stimulating factor
GGF	glial growth factor
GH	growth hormone
GM-CSF	granulocyte macrophage colony stimulating factor
GRO	growth-related oncogene
HGF	hepatocyte growth factor
HRG	heregulin
IFN	interferon
IGF	insulin like growth factor
IL	interleukin
KGF	keratinocyte growth factor
LIF	leukaemia inhibitory factor
M-CSF	macrophage colony stimulating factor
MCP	macrophage chemoattractant protein
MIP	macrophage inhibitory protein
NDF	Neu differentiation factor
NGF	nerve growth factor
NT	neurotopin
OSM	oncostatin-M
PDGF-A/B	platelet derived growth factor
PRL	prolactin
RANTES	regulated upon activation, normal T cell expressed and secreted
SCF	stem cell factor
SCF-R	stem cell factor receptor
TGF	transforming growth factor
TNF	tumour necrosis factor
TPO	thrombopoietin

VEGF vascular endothelial growth factor

APPENDICES

A 1 MATERIALS

A 1.1 RADIOCHEMICALS

[α-^{35}S]-dATP (specific activity of > 37 TBq/mmol at reference date), for DNA sequencing and methyl-^{3}H thymidine (specific activity of 74 Gbq/mmol at reference date), for cell proliferation assays were supplied by Amersham Life Science (Bucks, UK). [α-^{35}S]-dATP was stored in 2 µl aliquots, in screw-top eppendorf tubes, at -70°C, until use. Methyl-^{3}H thymidine was stored at +4°C.

A 1.2 GENERAL CHEMICALS

Chemicals used were of analytical or ultrapure quality and were supplied by Sigma Chemical Company (Dorset, England), Fisons Scientific Equipment (Loughborough, UK) or BDH Ltd. (Poole, England), unless stated otherwise.

Caesium Chloride: Boehringer Mannheim, UK.

Bacterial agar and *tryptone*: Oxoid.

Yeast extract and *bactopeptone*: Difco.

A 1.3 COMPLETE KITS

Sequenase Version 2.0 DNA Sequencing Kit (USB, Cleveland, Ohio) distributed in the UK by Amersham Life Science. Stored at -20°C.

First-Strand cDNA synthesis kit, QuickPrep mRNA purification kit and Bulk GST purification module supplied by Pharmacia Biotech (Herts, UK).

TA cloning kit supplied by Invitrogen (NV Leek, The Netherlands).

Gene Amp PCR Core Reagents (Roche Molecular Systems Inc., New Jersey, USA) supplied in the UK by Applied Biosystems Ltd.

Sequitherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, WI), distributed in the UK by Cambio (Cambridge, England).

Wizard™ Minipreps DNA Purification System supplied by Promega (Madison, WI).

A 1.4 BACTERIAL STRAINS

E.coli INVαF' cells (Invitrogen): F' *end*A1 *rec*A1 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R^+ λ^-.

E.coli DH5α cells (Gibco BRL): F' ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1.

Both of the above strains have the ϕ80*lac*ZΔM15 marker, enabling blue-white screening by α-complementation of β-galactosidase encoded by vector DNA (e.g. pCR™ II). The genotypes *end*A1 and *hsd*R17 give improved quality of miniprep DNA; *rec*A1 denotes recombination negative, recommended for stable replication of high copy number plasmids.

E.coli JM105 cells (Pharmacia Biotech): *thi rps*L *end*A *sbc*B15 *hsd*R4 *Sup*E Δ(*lac-pro*AB)/F' [*tra*D36 *ro*AB^+ *Lacl*^{q} *Lac*ZΔM15]. Host restriction minus, modification plus.

E.coli BL21 cells (Pharmacia Biotech): F^- *omp*T *hsd*S (r_B^-, m_B^-) *gal.*

JM105 were used for cloning and maintenance of the pGEX-4T-1 plasmid whilst BL21 were transformed with the pGEX plasmid for protein expression.

A 1.5 DNA

Plasmid, molecular weight marker and oligonucleotide DNAs were stored at -20°C.

A 1.5.1 Plasmid Vectors

pGEX-4T-1 (Pharmacia Biotech): Plasmid designed for inducible, high level intracellular expression of genes as soluble fusion proteins with *Schistosoma japonicum* glutathione S-transferase (GST).

pCR™ II Vector (Invitrogen): Plasmid designed for direct cloning of PCR products with 3' deoxyadenonosine residues (A - overhangs), generated by the non-template dependent activity of *Taq* polymerase. The vector is supplied as linearised DNA with single 3' deoxythymidine (T) residues allowing for efficient ligation of target sequence to vector.

pUC18 (Invitrogen): Plasmid supplied with TA cloning kit for use as positive control for verifying the transformation efficiency of competent bacteria; concentration of 0.1 μg/ml.

A 1.5.2 Molecular Size Standards

ϕX174 RF DNA/Hae III fragments (size range 72-1,353 bp) and λ DNA/HindIII fragments (size range 125-23,130 bp) were supplied by Gibco BRL.

A 1.5.3 Oligonucleotide Primers

Oligonucleotide primers for use in PCR amplification reactions and chain termination sequencing (other than M13 universal primers) were synthesised by Alta Biosciences (Birmingham, UK). They were reverse phase purified and supplied as lyophilised DNA. Primers were reconstituted in dH_2O, quantified by spectrophotometry and diluted to a concentration of 20 μM.

β actin primers (Clontech) for use in PCR amplification reactions were supplied by Cambridge Bioscience. Primers supplied at 20 μM concentration.

M13 universal primers (USB) for chain termination sequencing were supplied by Amersham Life Science.

IRD41 labelled primers for use with the Licor Model 4000 automated sequencer were supplied by Hybaid UK Ltd.

A 1.6 ENZYMES

All enzymes were stored at -20°C, being removed immediately before use.

Restriction enzymes and their associated reaction buffers were supplied by Gibco BRL.

T4 DNA Ligase was provided by Gibco BRL or Invitrogen (as part of the TA Cloning Kit).

Taq DNA polymerase was provided by Perkin Elmer Cetus, Norwalk, C.T.

Pfu DNA polymerase was provided by Stratagene Ltd, Cambridge, UK.

Murine Moloney Virus Reverse Transcriptase Enzyme was supplied by Pharmacia Biotech (as part of the cDNA cloning kit).

Sequitherm thermostable DNA polymerase (Epicentre Technologies) was supplied by Cambio.

RNAse A was supplied by Sigma.

A 1.7 PROTEIN SDS-PAGE STANDARDS

Prestained SDS-PAGE low range standard (20.5 - 112 KDa) was supplied by Biorad (Hercules, CA). MultiMark multi-colored standard (4 - 250 kDa) and Mark12 wide range protein standard (2.5 - 200 kDa) were supplied by Novel Experimentation Technology, San Diego, CA. In each case 5 µl of standard were heated to 40°C for one minute prior to use to dissolve any precipitated solids.

A 1.8 EQUIPMENT

A 1.8.1 Major Equipment

Benchtop centrifuges: Omnifuge 2.0 RS and Megafuge 1.0 (Heraeus Sepatech - Germany).

Microcentrifuge: Biofuge 13 (Heraeus Sepatech).

Incubators for tissue culture: supplied by Heraeus Sepatech and Leec Ltd. (Nottingham, UK).

Water baths: supplied by Grant Instruments (Cambridge) Ltd.(England).

Spectrophotometer: Model DU640, Beckman.

Vacuum dessicator: Hetovac, Heto Laboratory Equipment, Denmark.

Manual Sequencing Apparatus: Flowgen.

Automatic Sequencing Apparatus: Licor Model 4000 sequencer, sequencing plates etc. - Licor Inc., Lincoln, Nebraska.

Automated Processor: Kodak X-omat processor, model ME-3, Eastman Kodak Co., New York, USA.

Gel drier: Model 583 gel dryer, Biorad, Hercules, CA.

Pippetteman (P20, P200, P1000): supplied by Gilson Medical Electronics (Villiers-le-Bel, France).

Automatic Sarpette: supplied by Sarstedt.

Ultraviolet Transilluminator: supplied by UV Products Inc. (San Gabriel, CA).

Autoradiography (Film) cassettes: with intensifying screens (Cronex), supplied by Dupont.

A 1.8.2 Consumables

Bottle top filters (0.22μm pore size) were supplied by Sigma; for sterilisation of tissue culture media and degassing and sterilisation of FPLC buffer solutions.

Screw top 1.5 ml eppendorf tubes, 0.5 ml and 1.5 ml flip top tubes were supplied by Treff AG (Degersheim, Switzerland).

Pipette tips were supplied by Sarstedt.

Syringes (two, five, 10, 20 and 50 ml) were supplied by Becton Dickinson.

Flat ended gel loading tips were supplied by Sorenson Bioscience Ltd.

Filter tip pipette tips (30 μl and 200 μl) were supplied by Rainin Instrument Co. (Woburn, MA); for use in setting up PCR reactions.

Acrodisc syringe filters (0.22 and 0.4 μm) were supplied by Gelman Sciences (Ann Abor, MI); used for sterilising of filtering small volumes of solutions.

Petri dishes, bijoux and universals were supplied by Greiner (Stonehouse, Glos., UK).

Disposable, sterile scalpels were supplied by Swann-Morton (Sheffield, England).

A 1.9 Experimental Animals

Cats: Specific pathogen free (SPF) cats were obtained from a commercial breeding unit, housed at Glasgow University and fed a commercial diet. All procedures were carried out in accordance with Home Office regulations.

Rabbits: SPF Rabbits, used to raise polyclonal antisera, were obtained from a breeding unit and maintained on a commercial diet in the University of Glasgow,

Department of Biological Services. All procedures were carried out in accordance with Home Office Regulations.

Rats: Ten rats were used to prepare spleen cell conditioned medium for supplementation of MC/9 growth medium, rats were aged 6 - 10 weeks at the time of spleen removal. They were obtained from a breeding unit and maintained on a commercial diet in the University of Glasgow, Department of Biological Services. All procedures were carried out in accordance with Home Office Regulations.

A 1.10 CELL CULTURE MATERIALS

A 1.10.1 Cell Lines

TF-1 cell line was kindly provided by Dr T. Mire-Sluis of NIBSC, Herts, UK.
MC/9 cells were supplied by ATCC (Rockville, Maryland, USA)
FEA cells were kindly supplied by M. Golder (Feline Virus Unit, Department of Veterinary Pathology, University of Glasgow)

A 1.10.2 Recombinant growth factors

Recombinant human GM-CSF (Sargramostim - Immunex, Seattle, WA) used for maintaining the TF-1 cell line was a kind gift from Dr T. Mire-Sluis of NIBSC, Herts, UK.
Recombinant murine stem cell factor was supplied by Sigma.
Recombinant human G-CSF was supplied by R&D Systems Europe Ltd. (Abingdon, Oxon, UK).

Growth factors were typically stored at dilutions of 1 μg/ml in sterile PBS/0.1% BSA at -70°C.

A 1.10.3 Plasticware etc.

Tissue culture flasks, 96 well plates etc. were supplied by Costar (Cambridge, MA).
Cryotubes and 35 mm petri dishes were supplied by Nunc (DK 400, Roskilde, Denmark).

Falcon conical centrifuge tubes (15 and 50 ml) were supplied Becton Dickinson UK Ltd. (Oxford, UK).

A 1.10.4 Solutions, media and supplements

All solutions and media for cell culture were supplied by Gibco BRL.

A 1.10.4.1 Media.

All media were supplied as sterile solutions and stored at +4°C.

RPMI 1640 medium: without L-glutamine.

Iscove's modified Dulbecco's Medium (IMDM): with L-glutamine, 25 mM HEPES.

Dulbecco's Modified Eagle's Medium (DMEM): with L-glutamine, 4500 mg/L D-glucose, 25 mM HEPES.

A 1.10.4.2 Supplements.

Fetal Bovine Serum (FBS): virus screened, mycoplasma screened. FBS was heat inactivated at 56°C for 30 minutes then stored in 50 ml aliquots at -20°C until use.

L-glutamine: Supplied 200 mM (100 x) stock solution. This was stored in five millilitre aliquots at -20°C and routinely added to culture media prior to use.

Penicillin/streptomycin: Supplied as a 100 x stock solution of 10,000 units penicillin and 10,000 units streptomycin per millilitre. Stored in five millilitre aliquots at -20°C.

MEM Non Essential Amino Acids (NEAA): supplied as 100 x solution containing L-alanine (890 mg/L), L-asparagine (1320 mg/L), L-aspartic acid (1330 mg/L), L-glutamic acid (1470 mg/L), glycine (750 mg/L), L-proline (1150 mg/L) and L-serine (1050 mg/L). Stored at +4°C.

L-arginine: Supplied as lyophilised powder; reconstituted in 10 ml of culture medium to make solution of 20 mg/ml and stored at +4°C.

L-asparagine: Supplied as lyophilised powder; reconstituted in 10 ml of culture medium to make solution of 5 mg/ml and stored at +4°C.

Folic acid: supplied as USP grade powder. Stored at room temperature; dissolved in culture medium and filter sterilised prior to use.

Trypsin-EDTA: Supplied as 10 x liquid, stored at -20°C. This was diluted 1:10 in sterile PBS prior to use and stored at +4°C.

2-Mercaptoethanol (2-ME): Supplied as 50 mM solution in Dulbecco's PBS.

A 1.11 Buffers, Solutions and Growth Media

A 1.11.1 Water

Tissue culture grade distilled water was supplied by Gibco BRL. Ultrapure water (for procedures involving recombinant DNA, PCR etc.) was provided by a Millipore Q50 water purification system (Millipore (UK) Ltd., Watford, UK). A Millipore RO10 system was used to supply water for preparation of general solutions and media.

A 1.11.2 Antibiotics

Ampicillin (Penbritin™ - Beecham Research (Herts, England)): Solution of 100 mg/ml prepared by addition of 5 ml of dH_2O to vial of ampicillin; filter sterilised and stored in aliquots at -20°C until use.

Spectinomycin (Sigma): Supplied as powder containing 654μg active base per mg; stored at +4°C.

A 1.11.3 Buffers and solutions

10 x TBE Buffer: Tris base 216 g, boric acid 110 g, EDTA 16.9 g. pH 8.2/8.3, made up to 2 L.

50 x TAE Buffer Solution: Tris base 484.5g, NaOAc 272.15 g, NaCl 116.8 g, Na_2EDTA 74.45 g. pH adjusted to 8.15 with glacial acetic acid and made up to 2 L volume.

SDS-PAGE Protein Gel Fix-Stain Solution: dH_2O 45 ml, methanol 45 ml, HAc 10 ml, Coomassie Brilliant Blue R250 0.25g. Filtered through Whatman No. 1 filter paper.

Lysis Buffer: 25 mM Tris HCl pH 8.0, 10 mM EDTA, 50 mM Glucose.

1% SDS/ 0.2M NaOH: Made up immediately prior to use by combining equal volumes of 2% SDS and 0.4M NaOH.

KoAc: 60 ml 5M Potassium acetate, 11.5 ml acetic acid, 28.5 ml dH_2O.

1M Tris HCl: 121g Tris base, 800ml dH_2O. Adjusted to desired pH with concentrated HCl and made up to 1L.

TE Buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

1 x PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 (pH 7.3).

10 x DNA Gel Loading Buffer: 20% w/v Ficoll 400, 0.1 M Na_2EDTA, pH 8, 1.0% w/v sodium dodecyl sulphate, 0.25% bromophenol blue, 0.25% xylene cyanol.

Ethidium bromide: made to a working solution of 3mg/ml with dH_2O in a fume cupboard. Stored away from light.

10 x SDS-PAGE Electrode (Running) Buffer: tris base 60 g, glycine 288 g, SDS 20 g. Made up to 2 L by addition of dH_2O.

Protein Sample Loading Buffer: SDS Reducing Buffer: 62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol. dH_2O 3.0 ml, 0.5M Tris-HCl, pH 6.8 1.0 ml, glycerol, 1.6 ml, 10% SDS 1.6 ml, β-mercaptoethanol 0.4 ml, 0.5% (w/v) bromophenol blue (in dH_2O) 0.4 ml; stored at 4°C. Sample diluted at least 1:4 with buffer and heated at 100°C for 5 minutes prior to loading gel.

10 x Tris Buffered Saline (TBS): Tris base 24.2 g, NaCl 80.0 g, HCl 38.0 ml. pH 7.6. dH_2O to 1L.

10 x Semi Dry Transfer Buffer: Tris base (48mM) 58 g, glycine (39mM) 29 g, SDS (0.01%) 1 g. dH_2O to 1L. Working stock was prepared prior to use by the addition of 100 ml 10 x stock to 200 ml methanol and 700 ml dH_2O.

ACK lysis buffer: 8.29 g NH_4Cl (0.15 M), 1 g $KHCO_3$ (1.0 mM), 37.2 mg Na_2EDTA (0.1 mM). Dissolved in 800 ml dH_2O, pH adjusted to 7.2 - 7.4 with 1 M HCl, then dH_2O added to one litre.

X-gal solution: prepared as 40 mg/ml stock in dimethylformamide; stored at -20°C in the dark.

Sequencing gel solution (6%; for manual sequencing): prepared with 21 g ultrapure urea, five millilitres 10 x TBE buffer, six millilitres Long Ranger Gel solution (AT Biochem, Malvern, PA) and dH_2O to 50 ml. This was filtered through a 0.4 μm syringe filter, and stored at +4°C for no longer than one week.

A 1.11.4 Bacteriological Media

Media was sterilised by autoclaving at 121°C for 15 minutes, unless stated otherwise.

LB Medium: 20 g tryptone, 20 g NaCl, 10 g Yeast Extract, to 2 L with dH_2O, pH adjusted to 7.0 with NaOH.

SOC Medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.

2YT medium (Gibco BRL): SELECT peptone 140 16 g/L, yeast extract 10 g/L, NaCl 5 g/L.

TSS (Transformation and storage solution) Buffer: tryptone 1.0 g, yeast extract 0.5 g, NaCl 0.5 g, polyethylene glycol (MW 3350) 0.8 g, DMSO 5.0 ml, $MgCl_2$ (1 M) 5.0 ml. Combined in 70 ml dH_2O, pH adjusted to 6.5 with HCl or NaOH, volume made up to 100 ml with dH_2O and then filter sterilised. Stored at 4°C for up to six months.

A 2 FPLC PURIFICATION PROGRAMS

A 2.1 ANION EXCHANGE CHROMATOGRAPHY

Buffer A = 20 mM TrisHCl (pH 8.0).

Buffer B = 20 mM TrisHCl 1.00 M NaCl (pH 8.0).

A 2.1.1 Column Equilibration

```
 0.0 CONC B%          0.0
 0.0 ML/MIN          1.00
 0.0 CM/MIN          0.25
 0.0 VALVE.POS        1.1
 0.0 MONITOR            1
 5.0 CONC B%          0.0
 5.0 CONC B%          100
15.0 CONC B%          100
15.0 CONC B%          0.0
20.0 CONC B%          0.0
```

A 2.1.2 SCF Purification

```
 0.0 CONC B%          0.0
 0.0 ML/MIN          1.00
 0.0 CM/MIN          0.50
 0.0 MONITOR            1
10.0 CONC B%          0.0
10.0 VALVE.POS        1.1
10.0 HOLD            (sample loaded into superloop then CONT pressed).
15.0 VALVE.POSN       1.2
10.0 CM/MIN         1.00
40.0 CONC B%          0.0
40.0 VALVE.POS        1.1
40.0 PORT.SET         6.1              (starts Fraction Collector).
70.0 CONC B%         35.0
```

The Fraction collector (FRAC-100 - Pharmacia) was programmed with a delay of 10 minutes and a fraction size of 0.5 ml. The chart recorder was programmed to give 2 AU_{280} (absorbance units at 280 nm) at full scale deflection.

A 2.2 GEL FILTRATION CHROMATOGRAPHY

Buffer A (storage buffer) = 20 % ethanol.

Buffer B (Eluent) = 0.15 M NaCl, 12.3 mM KH_2PO_4, 37.7 mM Na_2HPO_4 (pH 7.0).

METHOD NO. 1

(equilibrates column with elution buffer prior to use)

```
  0.0      CONC B%          100
  0.0      ML/MIN          0.75
  0.0      CM/MIN          0.00
  0.0      VALVE.POS        1.1
160.0      ML/MIN          1.00
400.0      CONC B%          100
```

METHOD NO. 2

(purification/calibration protocol)

```
  0.0      CONC B%          100
  0.0      ML/MIN          1.00
  0.0      CM/MIN          0.50
  0.0      VALVE.POS        1.1
  0.0      HOLD       (sample loaded into superloop then CONT pressed).
  0.0      VALVE.POS        1.2
  0.0      PORT.SET         6.1
  0.0      MONITOR            1
200.0      CONC B%          100
200.0      PORT.SET         6.0
200.0      CALL METH          0
```

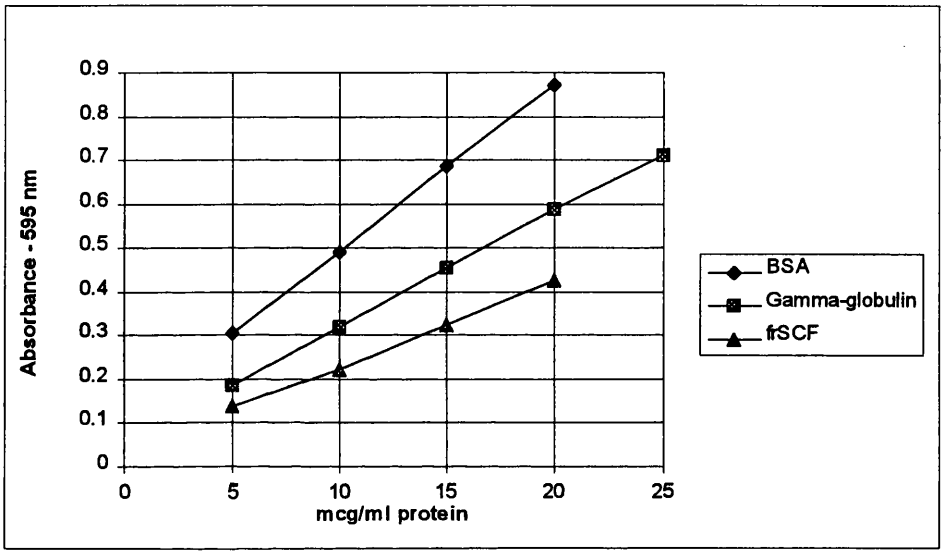
METHOD NO. O

(equilibrates column in 20% EtOH for storage).

```
    0.0    CONC B%          0.0
    0.0    ML/MIN          0.50
    0.0    CM/MIN          0.00
  400.0    CONC B%          0.0
```

A 3 BRADFORD ASSAY CALIBRATION RESULTS

The graph below shows a typical calibration curve obtained by the Bradford assay as detailed in 2.2.6.1. It can be seen that the use of BSA as a standard results in the under estimation of the concentration of frSCF in a sample by a factor of approximately two, within the concentration range shown.



A 4 PREDICTED ANTIGENICITY OF THE HUMAN CD34 PROTEIN

The antigenicity of the deduced hCD34 protein was predicted, by the method of Jameson-Wolfe, using the UNIX - version 7 of the GCG program "PeptideStructure". The output from the program, which is shown below, also contains predictions of hydrophilicity using the method of Kyte and Doolittle, surface probability according to Emini, chain flexibility according to Karplus-Schulz, secondary structure according to Chou-Fasman, and secondary structure according to Garnier-Osguthorpe-Robson. Secondary structure abbreviations: T = turn; B = beta sheet; H = alpha helix.

Pos	AA	GlycoS	HyPhil	SurfPr	FlexPr	CF-Pred	GORPred	AI-Ind ..
1	M	.	1.150	1.111	1.000	.	.	0.900
2	P	.	1.100	0.914	1.000	T	T	1.550
3	R	.	1.033	1.032	1.000	T	T	1.700
4	G	.	0.629	1.053	1.000	T	T	1.700
5	W	.	0.357	0.562	0.975	B	T	0.700
6	T	.	-0.229	0.154	0.952	B	B	-0.300
7	A	.	-1.414	0.128	0.938	B	B	-0.600
8	L	.	-2.014	0.100	0.931	B	B	-0.600
9	C	.	-2.029	0.093	0.931	B	B	-0.600
10	L	.	-2.671	0.076	0.934	B	B	-0.600
11	L	.	-2.957	0.076	0.939	B	B	-0.600
12	S	.	-2.186	0.220	0.952	B	B	-0.600
13	L	.	-1.714	0.357	0.980	B	B	-0.600
14	L	.	-1.114	0.428	1.011	B	.	-0.450
15	P	.	-0.971	0.277	1.036	T	.	-0.050
16	S	.	-1.357	0.332	1.039	T	.	-0.050
17	G	.	-0.700	0.540	1.012	T	B	-0.050
18	F	.	-0.700	0.288	0.975	.	B	-0.600
19	M	.	-0.429	0.359	0.952	.	B	-0.600
20	S	.	-0.043	0.583	0.959	.	B	-0.300
21	L	.	0.400	1.083	0.993	.	.	0.450
22	D	.	0.857	1.083	1.043	t	T	1.500
23	N	.	1.229	1.166	1.080	T	T	1.700
24	N	G	0.857	1.428	1.096	T	.	1.300
25	G	.	1.500	1.234	1.095	T	.	1.300
26	T	.	1.229	1.187	1.079	.	.	0.900
27	A	.	1.229	1.278	1.064	.	.	0.900
28	T	.	0.186	1.065	1.048	.	.	0.600
29	P	.	0.357	1.141	1.040	.	.	0.600
30	E	.	0.357	1.630	1.037	.	.	0.600
31	L	.	1.114	1.957	1.044	.	.	0.900
32	P	.	1.071	1.252	1.066	.	.	0.900
33	T	.	0.943	1.043	1.084	.	T	1.300
34	Q	.	0.043	1.096	1.092	t	T	1.200
35	G	.	0.700	0.950	1.085	t	.	0.950
36	T	.	0.971	1.058	1.058	B	.	0.900
37	F	.	0.271	0.453	1.029	B	.	0.450
38	S	.	-0.114	0.614	1.016	B	.	-0.150
39	N	G	-0.071	0.614	1.012	B	.	-0.150
40	V	.	0.329	1.140	1.017	B	.	0.600
41	S	.	0.129	0.632	1.026	B	.	0.450
42	T	.	0.129	0.526	1.019	B	.	0.450
43	N	G	-0.186	1.111	1.005	B	.	0.000
44	V	.	0.914	1.436	0.991	B	.	0.750
45	S	.	1.300	1.723	0.985	B	.	0.750
46	Y	.	1.300	1.546	0.995	B	T	1.150
47	Q	.	0.900	3.007	1.020	B	T	1.300
48	E	.	1.600	3.238	1.046	.	T	1.300
49	T	.	1.714	3.196	1.063	.	.	0.900

Pos	AA	GlycoS	HyPhil	SurfPr	FlexPr	CF-Pred	GORPred	AI-Ind
50	T	.	1.643	2.473	1.077	.	.	0.900
51	T	.	1.243	2.061	1.082	.	.	0.900
52	P	.	0.200	1.178	1.077	T	.	1.000
53	S	.	0.157	0.807	1.074	T	.	0.850
54	T	.	0.171	0.750	1.066	.	.	0.450
55	L	.	0.171	0.700	1.059	.	.	0.450
56	G	.	0.057	0.700	1.065	.	.	0.450
57	S	.	-0.600	0.400	1.066	t	.	-0.250
58	T	.	-0.243	0.660	1.054	t	.	0.050
59	S	.	0.529	1.031	1.032	t	.	1.100
60	L	.	-0.129	0.571	1.000	.	.	-0.150
61	H	.	-0.129	0.530	0.979	.	.	-0.300
62	P	.	0.271	0.685	0.976	t	.	0.500
63	V	.	0.614	1.131	0.986	t	.	0.950
64	S	.	1.214	0.822	1.003	.	.	0.750
65	Q	.	1.257	0.855	1.019	T	.	1.150
66	H	.	1.529	1.995	1.030	T	.	1.300
67	G	.	1.871	1.504	1.043	t	.	1.100
68	N	.	1.857	1.253	1.052	t	.	1.100
69	E	.	1.457	1.329	1.054	.	.	0.900
70	A	.	1.500	2.160	1.053	b	.	0.900
71	T	.	0.800	0.942	1.047	b	.	0.750
72	T	.	0.400	0.785	1.040	b	.	0.450
73	N	G	0.400	1.345	1.037	b	.	0.600
74	I	.	0.757	1.345	1.040	b	B	0.900
75	T	.	0.757	1.345	1.048	b	B	0.900
76	E	.	0.057	0.621	1.053	b	B	0.450
77	T	.	0.114	1.771	1.053	b	B	0.600
78	T	.	0.357	1.063	1.041	b	B	0.600
79	V	.	0.357	0.886	1.025	b	B	0.450
80	K	.	-0.029	0.822	1.026	b	B	-0.150
81	F	.	-0.029	0.822	1.029	b	.	-0.150
82	T	.	-0.014	1.485	1.049	b	.	0.000
83	S	.	-0.014	0.551	1.062	t	.	0.050
84	T	.	-1.214	0.446	1.045	t	.	-0.250
85	S	.	-0.714	0.446	1.026	.	.	-0.450
86	V	.	-0.700	0.446	1.000	B	B	-0.600
87	I	.	-1.414	0.229	0.981	B	B	-0.600
88	T	.	-1.329	0.268	0.982	B	B	-0.600
89	S	.	-1.386	0.358	0.984	B	B	-0.600
90	V	.	-0.286	0.821	0.990	B	B	-0.300
91	Y	.	0.457	0.821	1.005	B	B	0.450
92	G	.	0.857	0.985	1.030	t	.	0.950
93	N	.	0.857	1.778	1.063	t	.	1.100
94	T	.	1.571	1.521	1.087	T	.	1.300
95	N	G	0.786	1.140	1.097	T	.	1.300
96	S	.	1.229	1.228	1.089	t	.	1.100
97	S	.	0.843	1.140	1.074	t	.	1.100
98	V	.	1.243	1.228	1.070	.	B	0.900
99	Q	.	0.843	1.323	1.077	.	B	0.900
100	S	.	0.843	1.323	1.091	.	B	0.900
101	Q	.	0.129	1.323	1.090	t	B	0.800
102	T	.	0.086	0.535	1.065	t	B	0.650
103	S	.	-0.300	0.535	1.033	.	B	-0.150
104	V	.	-0.314	0.446	0.999	B	B	-0.300
105	I	.	-1.414	0.229	0.981	B	B	-0.600
106	S	.	-1.914	0.148	0.981	B	B	-0.600
107	T	.	-1.929	0.288	0.982	B	B	-0.600
108	V	.	-1.229	0.593	0.985	B	B	-0.600
109	F	.	-0.357	0.685	0.998	B	B	-0.300
110	T	.	-0.729	0.479	1.017	B	B	-0.450
111	T	.	-0.329	1.039	1.029	B	.	0.000
112	P	.	-0.329	0.890	1.034	t	.	0.050
113	A	.	0.186	0.827	1.026	t	.	0.650
114	N	G	0.186	0.827	1.013	t	.	0.650
115	V	.	0.314	0.827	1.014	.	.	0.450
116	S	.	0.586	1.417	1.029	.	.	0.900
117	T	.	0.943	1.272	1.047	.	.	0.900
118	P	.	0.543	2.473	1.062	T	.	1.300
119	E	.	0.600	1.522	1.064	T	B	1.300
120	T	.	1.043	2.109	1.058	.	B	0.900
121	T	.	1.171	2.109	1.049	.	B	0.900
122	L	.	1.057	1.632	1.042	.	B	0.900
123	K	.	0.014	0.932	1.039	.	B	0.450
124	P	.	0.029	0.866	1.036	t	T	1.050
125	S	.	0.157	1.623	1.034	t	T	1.200
126	L	.	0.757	0.803	1.038	.	.	0.750
127	S	.	0.700	0.835	1.044	.	.	0.750
128	P	.	-0.129	0.463	1.048	T	.	0.250

Pos	AA	GlycoS	HyPhil	SurfPr	FlexPr	CF-Pred	GORPred	AI-Ind
129	G	.	-0.129	0.752	1.052	T	.	0.250
130	N	G	0.914	0.937	1.046	t	.	0.950
131	V	.	0.257	0.500	1.036	t	B	0.650
132	S	.	0.143	0.677	1.034	t	B	0.650
133	D	.	0.186	0.607	1.034	.	B	0.450
134	L	.	-0.214	1.181	1.036	.	B	0.000
135	S	.	0.500	1.181	1.053	.	B	0.900
136	T	.	0.486	1.021	1.068	t	.	0.800
137	T	.	0.100	1.658	1.080	t	.	0.800
138	S	.	0.100	1.021	1.085	.	.	0.600
139	T	.	-0.271	0.714	1.064	b	.	-0.150
140	S	.	-0.271	0.714	1.041	b	.	-0.150
141	L	.	-0.257	0.714	1.026	b	.	-0.150
142	A	.	-0.143	0.765	1.024	b	.	-0.150
143	T	.	-0.143	0.824	1.044	b	.	-0.150
144	S	.	0.300	1.999	1.068	.	.	0.600
145	P	.	1.071	3.060	1.079	T	T	1.700
146	T	.	1.514	3.322	1.079	T	T	1.700
147	K	.	1.514	3.577	1.069	.	.	0.900
148	P	.	1.514	3.100	1.061	t	T	1.500
149	Y	.	1.400	2.879	1.064	t	T	1.500
150	T	.	1.414	1.929	1.085	t	T	1.500
151	S	.	1.086	1.929	1.110	T	T	1.700
152	S	.	0.214	0.863	1.113	T	.	0.850
153	S	.	-0.514	0.493	1.098	t	.	-0.250
154	P	.	-0.500	0.493	1.062	h	.	-0.450
155	I	.	-0.114	0.615	1.025	h	.	-0.150
156	L	.	-0.871	0.321	1.011	h	H	-0.450
157	S	.	-0.429	0.416	1.017	h	H	-0.450
158	D	.	-0.914	0.599	1.030	h	H	-0.450
159	I	.	0.229	1.258	1.032	h	H	0.600
160	K	.	0.129	0.658	1.028	h	H	0.450
161	A	.	0.571	0.788	1.018	h	H	0.750
162	E	.	-0.286	0.603	1.003	h	H	-0.150
163	I	.	0.471	0.404	1.005	h	H	0.450
164	K	.	-0.029	0.396	1.010	h	H	-0.150
165	C	.	-0.414	0.160	1.011	h	T	-0.050
166	S	.	-0.271	0.447	1.022	T	T	0.650
167	G	.	0.871	0.387	1.024	T	T	1.550
168	I	.	-0.286	0.537	1.021	h	.	-0.150
169	R	.	0.629	0.801	1.024	h	.	0.750
170	E	.	-0.029	0.667	1.019	h	T	0.250
171	V	.	0.014	1.374	1.016	h	T	1.000
172	K	.	1.157	1.215	1.028	h	T	1.300
173	L	.	0.571	0.694	1.035	h	T	1.150
174	T	.	-0.571	0.656	1.042	h	T	-0.050
175	Q	.	-0.329	0.176	1.040	h	T	0.250
176	G	.	-1.429	0.176	1.014	.	B	-0.450
177	I	.	-0.386	0.211	0.988	h	B	-0.300
178	C	.	0.014	0.211	0.984	h	B	0.300
179	L	.	0.014	0.343	1.000	h	B	0.450
180	E	.	0.514	0.977	1.037	h	B	0.750
181	Q	.	1.257	2.632	1.077	h	T	1.300
182	N	G	1.729	4.276	1.099	h	T	1.300
183	K	.	2.386	3.309	1.106	h	T	1.300
184	T	.	1.529	1.024	1.091	h	T	1.300
185	S	.	0.771	0.643	1.065	T	.	1.150
186	S	.	0.771	0.557	1.025	T	H	1.150
187	C	.	-0.186	0.334	0.981	t	H	-0.100
188	A	.	0.271	0.499	0.964	H	H	0.300
189	E	.	0.714	0.745	0.964	H	H	0.600
190	F	.	1.100	2.320	0.985	H	H	0.750
191	K	.	2.100	4.497	1.020	H	H	0.900
192	K	.	2.414	2.570	1.046	H	H	0.900
193	D	.	2.414	5.140	1.062	H	H	0.900
194	R	.	2.871	2.543	1.073	H	H	0.900
195	G	.	1.771	1.049	1.073	.	H	0.900
196	E	.	0.957	0.634	1.057	t	H	0.950
197	G	.	1.100	0.634	1.028	t	H	0.950
198	L	.	-0.143	0.476	0.992	B	H	-0.300
199	A	.	-0.743	0.227	0.958	B	H	-0.600
200	R	.	-1.600	0.123	0.932	B	H	-0.600
201	V	.	-1.600	0.147	0.927	B	H	-0.600
202	L	.	-0.557	0.252	0.939	B	H	-0.600
203	C	.	0.200	0.223	0.965	B	H	0.300
204	G	.	0.057	0.521	1.004	.	H	0.450
205	E	.	0.400	0.638	1.035	H	H	0.450
206	E	.	1.443	1.988	1.045	H	H	0.900
207	Q	.	1.543	2.029	1.041	H	H	0.900

Pos	AA	GlycoS	HyPhil	SurfPr	FlexPr	CF-Pred	GORPred	AI-Ind
208	A	.	1.986	1.957	1.021	H	H	0.900
209	D	.	1.229	1.142	1.002	H	H	0.900
210	A	.	0.786	0.652	0.992	H	H	0.600
211	D	.	0.029	0.652	0.985	H	H	0.300
212	A	.	0.786	0.676	0.982	H	H	0.600
213	G	.	-0.314	0.497	0.971	H	H	-0.300
214	A	.	-0.414	0.160	0.958	H	H	-0.600
215	Q	.	-0.800	0.212	0.938	H	H	-0.600
216	V	.	-1.086	0.176	0.926	H	H	-0.600
217	C	.	-1.686	0.144	0.920	H	H	-0.600
218	S	.	-1.971	0.069	0.919	H	H	-0.600
219	L	.	-2.729	0.093	0.928	H	H	-0.600
220	L	.	-1.629	0.301	0.936	H	.	-0.600
221	L	.	-1.157	0.301	0.960	H	.	-0.600
222	A	.	-0.771	0.633	0.988	H	.	-0.600
223	Q	.	-0.829	0.570	1.017	H	T	-0.050
224	S	.	0.357	1.353	1.042	H	T	1.000
225	E	.	1.129	2.071	1.044	H	T	1.300
226	V	.	1.886	2.071	1.039	H	T	1.300
227	R	.	1.029	0.828	1.025	H	.	0.750
228	P	.	0.371	0.395	1.004	.	T	0.850
229	Q	.	-0.671	0.438	0.982	B	T	-0.200
230	C	.	-0.614	0.185	0.954	B	B	-0.600
231	L	.	-1.857	0.089	0.934	B	B	-0.600
232	L	.	-2.629	0.042	0.921	B	B	-0.600
233	L	.	-3.386	0.080	0.918	B	B	-0.600
234	V	.	-2.529	0.155	0.927	B	B	-0.600
235	L	.	-1.343	0.368	0.942	B	H	-0.600
236	A	.	-0.700	0.644	0.966	B	H	-0.600
237	N	G	0.343	1.503	0.995	B	H	0.450
238	R	.	0.300	1.278	1.017	.	H	0.600
239	T	.	0.957	1.695	1.033	.	H	0.900
240	E	.	1.329	1.413	1.045	.	H	0.900
241	I	.	1.386	1.442	1.054	.	H	0.900
242	S	.	0.200	0.824	1.061	.	H	0.450
243	S	.	0.600	0.824	1.058	t	H	0.950
244	K	.	-0.443	0.970	1.035	t	H	-0.250
245	L	.	-0.071	0.716	0.996	H	H	-0.300
246	Q	.	0.371	1.069	0.966	H	H	0.450
247	L	.	0.814	1.069	0.959	H	H	0.750
248	M	.	0.714	1.763	0.966	H	H	0.750
249	K	.	1.757	1.763	0.997	H	H	0.750
250	K	.	1.371	2.865	1.027	H	H	0.900
251	H	.	2.414	4.835	1.044	H	H	0.900
252	Q	.	2.143	1.994	1.060	t	H	1.100
253	S	.	2.143	1.994	1.068	t	H	1.100
254	D	.	2.143	2.930	1.066	t	H	1.100
255	L	.	1.143	1.395	1.055	h	H	0.900
256	K	.	0.700	1.030	1.042	h	H	0.900
257	K	.	-0.057	0.433	1.015	h	H	-0.150
258	L	.	-1.100	0.433	0.985	h	B	-0.600
259	G	.	-0.057	0.361	0.961	.	B	-0.300
260	I	.	-1.014	0.156	0.939	h	B	-0.600
261	L	.	-1.471	0.274	0.940	h	B	-0.600
262	D	.	-0.429	0.479	0.956	h	B	-0.600
263	F	.	0.014	1.183	0.990	h	H	0.450
264	T	.	1.157	2.396	1.033	h	H	0.900
265	E	.	1.100	1.065	1.058	h	H	0.900
266	Q	.	0.343	1.242	1.065	h	H	0.600
267	D	.	0.857	1.154	1.043	h	H	0.900
268	V	.	1.214	0.907	1.004	h	H	0.750
269	A	.	1.214	0.907	0.979	h	H	0.600
270	S	.	0.829	0.727	0.970	h	H	0.600
271	H	.	0.514	1.536	0.979	h	.	0.750
272	Q	.	1.229	2.037	0.998	T	.	1.150
273	S	.	1.986	2.633	1.019	T	.	1.300
274	Y	.	2.429	3.869	1.037	t	T	1.500
275	S	.	2.071	3.224	1.058	t	T	1.500
276	Q	.	1.029	1.984	1.070	.	T	1.300
277	K	.	0.271	0.888	1.054	.	B	0.450
278	T	.	-0.171	0.669	1.024	B	B	-0.150
279	L	.	-0.829	0.319	0.979	B	B	-0.600
280	I	.	-1.929	0.118	0.938	B	B	-0.600
281	A	.	-2.386	0.118	0.927	B	B	-0.600
282	L	.	-2.371	0.192	0.945	B	B	-0.600
283	V	.	-1.771	0.271	0.979	B	B	-0.600
284	T	.	-1.386	0.271	1.016	B	B	-0.450
285	S	.	-1.671	0.271	1.034	t	.	-0.250
286	G	.	-1.671	0.301	1.019	t	.	-0.250

Pos	AA	GlycoS	HyPhil	SurfPr	FlexPr	CF-Pred	GORPred	AI-Ind
287	A	.	-1.329	0.211	0.987	H	B	-0.600
288	L	.	-2.029	0.117	0.952	H	B	-0.600
289	L	.	-2.686	0.097	0.928	H	B	-0.600
290	A	.	-2.686	0.095	0.919	H	B	-0.600
291	V	.	-3.071	0.081	0.926	H	B	-0.600
292	L	.	-2.429	0.142	0.944	H	B	-0.600
293	G	.	-1.829	0.139	0.965	.	B	-0.600
294	I	.	-1.386	0.293	0.986	.	B	-0.600
295	T	.	-1.186	0.308	0.995	.	B	-0.600
296	G	.	-1.186	0.257	0.987	.	B	-0.600
297	Y	.	-1.514	0.363	0.962	B	B	-0.600
298	F	.	-0.371	0.404	0.942	B	B	-0.300
299	L	.	0.171	0.800	0.935	B	B	0.300
300	M	.	0.757	0.999	0.944	B	B	0.600
301	N	.	0.686	1.547	0.975	B	T	1.150
302	R	.	1.214	1.972	0.997	T	T	1.550
303	R	.	1.871	2.670	1.011	T	T	1.700
304	S	.	2.371	2.568	1.020	T	T	1.700
305	W	.	1.971	1.892	1.018	.	.	0.900
306	S	.	1.386	0.956	1.035	.	.	0.750
307	P	.	1.243	1.235	1.054	T	.	1.300
308	T	.	1.771	2.301	1.070	T	.	1.300
309	G	.	1.100	1.416	1.078	t	.	1.100
310	E	.	1.043	0.906	1.066	.	.	0.750
311	R	.	1.314	1.088	1.052	.	.	0.900
312	L	.	1.714	1.835	1.039	.	.	0.900
313	G	.	1.886	1.639	1.041	.	.	0.900
314	E	.	1.571	1.311	1.041	.	.	0.900
315	D	.	1.114	2.491	1.032	.	.	0.900
316	P	.	1.757	3.632	1.023	T	.	1.300
317	Y	.	2.200	3.632	1.008	T	.	1.300
318	Y	.	2.200	3.498	1.011	.	.	0.900
319	T	.	1.757	2.239	1.037	.	.	0.900
320	E	.	1.586	1.414	1.069	t	T	1.500
321	N	.	1.457	0.893	1.102	t	T	1.350
322	G	.	1.771	1.072	1.127	T	T	1.700
323	G	.	1.729	0.612	1.132	T	T	1.550
324	G	.	1.414	0.597	1.121	T	T	1.550
325	Q	.	1.029	0.808	1.100	T	.	1.150
326	G	.	1.086	1.094	1.077	T	.	1.300
327	Y	.	1.086	1.094	1.067	t	T	1.500
328	S	.	1.257	0.977	1.073	T	T	1.550
329	S	.	0.814	0.977	1.091	T	.	1.150
330	G	.	0.857	0.900	1.103	T	.	1.150
331	P	.	0.786	0.900	1.111	T	.	1.150
332	G	.	0.900	1.038	1.112	T	.	1.300
333	T	.	1.286	1.817	1.101	t	.	1.100
334	S	.	0.971	1.187	1.094	t	.	1.100
335	P	.	1.243	2.077	1.077	T	.	1.300
336	E	.	1.243	1.424	1.065	T	.	1.300
337	A	.	1.700	2.126	1.063	h	.	0.900
338	Q	.	1.329	1.389	1.062	h	.	0.900
339	G	.	1.214	1.075	1.064	h	.	0.900
340	K	.	0.114	0.790	1.050	h	.	0.450
341	A	.	0.871	0.733	1.029	h	.	0.750
342	S	.	1.014	1.451	1.007	h	.	0.900
343	V	.	1.014	0.718	1.000	h	.	0.600
344	N	.	0.200	0.718	1.006	t	.	0.650
345	R	.	0.957	0.928	1.018	T	.	1.150
346	G	.	1.400	2.500	1.034	T	.	1.300
347	A	.	2.500	2.500	1.047	.	.	0.900
348	Q	.	2.057	1.263	1.063	.	.	0.900
349	K	.	1.514	1.842	1.081	T	.	1.300
350	N	G	1.514	1.805	1.097	T	.	1.300
351	G	.	2.271	1.805	1.104	t	.	1.100
352	T	.	1.514	0.912	1.098	T	.	1.150
353	G	.	1.057	0.818	1.088	T	.	1.150
354	Q	.	0.671	1.108	1.076	.	.	0.900
355	A	.	1.257	1.503	1.071	.	.	0.900
356	T	.	1.657	2.443	1.077	.	.	0.900
357	S	.	1.657	1.396	1.083	t	.	1.100
358	R	.	1.614	1.880	1.076	T	T	1.700
359	N	.	1.986	1.746	1.062	T	T	1.700
360	G	.	1.629	1.316	1.037	t	T	1.500
361	H	.	2.157	1.316	1.013	t	.	1.100
362	S	.	2.014	1.418	1.003	h	.	0.900
363	A	.	1.971	1.949	1.002	h	.	0.900
364	R	.	1.314	1.063	0.998	h	B	0.750
365	Q	.	0.257	0.589	0.987	h	B	0.300

366	H	.	-0.114	0.589	0.969	h	B	-0.300
367	V	.	0.643	0.502	0.957	h	B	0.600
368	V	.	0.100	0.418	0.963	h	B	0.300
369	A	.	0.100	0.532	0.984	h	B	0.300
370	D	.	-0.900	0.592	1.000	h	B	-0.450
371	T	.	-0.350	1.019	1.000	h	B	0.000
372	E	.	0.420	1.289	1.000	h	.	0.600
373	L	.	0.975	0.987	1.000	h	.	0.750

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SHORT COMMUNICATION

The cloning and sequencing of cDNAs encoding two isoforms of feline stem cell factor

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cDNA clones encoding two isoforms of feline stem cell factor (fSCF) have been isolated using RT-PCR and their sequences determined. The cDNAs encode a predicted full length fSCF protein of 274 amino-acids and a shorter isoform of 246 amino acids. Feline SCF shows a high degree of homology to the SCFs of other species at both the nucleic acid and protein level.

KEY WORDS: cDNA, feline, kit-ligand, mast cell growth factor, polymerase chain reaction, stem cell factor

Stem cell factor (SCF), also known as steel factor (SLF), *kit* ligand (KL), and mast cell growth factor (MGF) is a pleiotropic growth factor having important roles in melanogenesis, gametogenesis and haematopoiesis both during development and in adult life (for review see Galli *et al.*, 1994). SCF is allelic to the steel locus (*Sl*) in the mouse (Huang *et al.*, 1990; Zsebo *et al.*, 1990; Copeland *et al.*, 1990) and is the ligand for the *c-kit* proto-oncogene product, a transmembrane tyrosine kinase which maps to the mouse dominant white spotting (*W*) locus (Chabot *et al.*, 1988; Geissler *et al.*, 1988). The haematological effects of SCF have suggested potential therapeutic uses including treatment of bone marrow failure syndromes, bone marrow support during chemotherapy and mobilisation of peripheral blood progenitor cells with marrow repopulating ability (for review see Morstyn *et al.*, 1994).

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cDNAs have been cloned for human, rat (Martin *et al.*, 1990), murine (Anderson *et al.*, 1990), porcine (Zhang and Anthony, 1994), bovine (Zhou *et al.*, 1994), canine (Shull *et al.*, 1992) and chicken (Zhou *et al.*, 1993) SCF. In all species (except the rat for which only a partial cDNA is described) the longer isoform has been cloned, with a shorter isoform also described in the case of human (Anderson *et al.*, 1991), murine (Anderson *et al.*, 1990; Flanagan *et al.*, 1991), canine (Shull *et al.*, 1992) and bovine SCF (Zhou *et al.*, 1994). Both isoforms exist as transmembrane proteins, the shorter of which is generated by alternate mRNA splicing and, lacking exon 6, this results in an SCF isoform 28 amino-acids shorter than the full length SCF. A soluble form of SCF is produced by proteolytic cleavage of the transmembrane protein, largely of the longer isoform. The shorter isoform lacks the proteolytic cleavage site which lies within exon 6; this isoform of mouse but not human SCF is cleaved with lower efficiency at an alternate site (Huang *et al.*, 1992; Majumdar *et al.*, 1994). The respective roles of soluble and membrane bound forms of SCF have yet to be fully elucidated. We here report the cloning and sequencing of two isoforms of feline stem cell factor (fSCF).

Messenger RNA (mRNA) was isolated from an FeLV-A transformed feline fibroblast cell line, using a QuickPrep mRNA purification kit (Pharmacia P-L Biochemicals). Complementary

DNA (cDNA) was synthesised from the mRNA, primed with a Not-dT primer, using a First strand cDNA synthesis Kit (Pharmacia P-L Biochemicals) and used as a template for PCR amplification. SCF cDNA was specifically amplified using Taq polymerase (Perkin Elmer Cetus, Norwalk, C.T.) with a PCR cycle consisting of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min, repeated for 30 cycles. Primers were designed from conserved areas within the 5′ and 3′ non-coding regions of published SCF sequences as follows: 5′ primer CCAGAACAGC-TAAACGGAGT, 3′ primer ATGAAGCAAACAT-GAACTGT. The PCR product was cloned into a pCR™II vector (Invitrogen, San Diego, U.S.A.) and sequenced by the dideoxy chain termination method using a Sequenase Kit (United States Biochemical, Cleveland, Ohio, USA). Three separate clones, taken from different PCR reactions were sequenced on both DNA strands; these were identical except for base changes at nucleotides 16 (A to G) and 261 (A to G) in clone 3. A further clone was sequenced and found to be identical to

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   1                  CCAGAACAGCTAAACGGAGTCGCCACACCGCTGCCTGGACTGGATCACAGCGCTGCCTTTCCTT  64

  65 ATG AAG AAG ACA CAA ACT TGG ATT GTC ACT TGC ATT TAT CTT CAG CTG CTC CTA TTT AAC 124
   1  M   K   K   T   Q   T   W   I   V   T   C   I   Y   L   Q   L   L   L   F   N  20

 125 CCT CTG GTC AAA ACT AAA GGG CTC TGC AGG AAC CGT GTG ACT GAT GAT GTG AAA GAC GTT 184
  21  P   L   V   K   T   K   G   L   C   R   N   R   V   T   D   D   V   K   D   V  40

 185 ACA AAA TTG GTG GCA AAT CTT CCA AAA GAC TAT AAG ATA GCC CTC AAA TAT GTC CCC GGG 244
  41  T   K   L   V   A   N   L   P   K   D   Y   K   I   A   L   K   Y   V   P   G  60

 245 ATG GAT GTT TTG CCT AGT CAT TGT TGG ATA AGC GTG ATG GTG GAA CAG TTG TCA GTC AGT 304
  61  M   D   V   L   P   S   H   C   W   I   S   V   M   V   E   Q   L   S   V   S  80

 305 TTG ACT GAT CTT CTG GAC AAG TTT TCG AAT ATT TCT GAA GGC TTG AGT AAT TAT TCT ATC 364
  81  L   T   D   L   L   D   K   F   S   N   I   S   E   G   L   S   N   Y   S   I 100

 365 ATA GAC AAA CTT GTG AAA ATA GTG GAT GAC CTT GTG GAG TGC GTG GAA GGA CAC TCA TCT 424
 101  I   D   K   L   V   K   I   V   D   D   L   V   E   C   V   E   G   H   S   S 120

 425 GAG AAT GTA AAA AAA TCA TCT AAG AGC CCA GAA CCC AGG CTT TTT ACT CCT GAA GAA TTC 484
 121  E   N   V   K   K   S   S   K   S   P   E   P   R   L   F   T   P   E   E   F 140

 485 TTT AGA ATT TTT AAT AGA TCC ATT GAT GCC TTC AAG GAC TTG GAG ATG GTG GCA TCT AAA 544
 141  F   R   I   F   N   R   S   I   D   A   F   K   D   L   E   M   V   A   S   K 160

 545 ACT AGT GAA TGT GTG GTT TCT TCA ACA TTA AGT CCT GAA AAA GAT TCC AGG GTC AGT GTC 604
 161  T   S   E   C   V   V   S   S   T   L   S   P   E   K   D   S   R   V   S   V 180

 605 ACA AAA CCA TTT ATG TTA CCC CCT GTT GCA GCC AGC TCC CTT AGG AAT GAC AGC AGT AGC 664
 181  T   K   P   F   M   L   P   P   V   A   A   S   S   L   R   N   D   S   S   S 200

 665 AGT AAT AGG AAG GCC ACA AAT CCC ATA GAA GAC TCC AGC ATA CAA TGG GCA GTC ATG GCA 744
 201  S   N   R   K   A   T   N   P   I   E   D   S   S   I   Q   W   A   V   M   A 220

 725 TTA CCA GCG TGC TTT TCT CTT GTA ATC GGA TTT GCT TTT GGA GCC TTC TAC TGG AAG AAG 804
 221  L   P   A   C   F   S   L   V   I   G   F   A   F   G   A   F   K   W   K   K 240

 785 AAA CAA CCG AAT CTC ACA AGG ACA GTT GAA AAT ATA CAG ATT AAC GAA GAG GAT AAT GAG 864
 241  K   Q   P   N   L   T   R   T   V   E   N   I   Q   I   N   E   E   D   N   E 260

 845 ATA AGT ATG CTG CAA GAA AAA GAG AGA GAG TTT CAA GAG GTG TAA TTGTGGCTTATATCAACAC 908
 261  I   S   M   L   Q   E   K   E   R   E   F   Q   E   V   *                     274

 909 TGTTACTTTTGTGCCTTGGCGGGTAACAGTTCATGTTTGCTTCAT                                  953
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Figure 1 Nucleotide sequence and deduced amino-acid sequence of feline stem cell factor cDNA. The nucleotides depicted in bold type and overlined are those deleted in the shorter isoform of fSCF, which results in a removal of amino-acids 150–178 and the insertion of a glycine residue at this site. Primers used in the PCR are underlined.

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                _____S-P_____♦ •
-25 MKKTQTWIVT CIYLQLLLFN PLVKTKGLCR NRVTDDVKDV TKLVANLPKD YKIALKYVPG
-25 --------I- ---------- ---R-Q-I-- ---------- ---------- ---T------
-25 --------I- ---------- -------I-G K--------- ---------- ----------
-25 --------I- ---------- ---H-Q-I-S ---------- ---------- -M-T------
-25 --------L- ---------- -----E-I-- ----NN---- ---------- -M-T------
-25 --------I- ---------- ------EI-G -P---N---I --------N- -M-T-N--A-
-25 ---A----I- -FC-----L- ----AQSS-G -P-----N-I A---G---N- -L-T-----K

        •                         *         *                   •
36  MDVLPSHCWI SVMVEQLSVS LTDLLDKFSN I...SEGLSN YSIIDKLVKI VDDLVECVE.
36  ---------- -E-------- ---------- ---------- ---------- -------M--
36  ---------- ---------- ---------- ---------- ---------- -------T--
36  ---------- -E-------- ---------- ---------- -C-------- -------M--
36  ---------- -E--V---D- ---------- ---------- --------N- --------K-
36  ---------L RD--I---L- --T------- ---------- -------G-- -----L-M--
36  --S--N---L HL--PEF-R- -HN--Q---D -SDM-DV--- ----NN-TR- IN--MA-LAF

                                      *                   •          →
92  GHSSENVKKS SKSPEPRLFT PEEFFRIFNR SIDAFKDLEM VASKTSECVV SSTL.SPEKD
92  E--F------ ---------- --K--G---- ---------- --P------I -----T----
92  -Y-F-----A P----L---- ---------- ---------T ----S----- -------D--
92  E--------- -------Q-- --K--G---K ----------I ----M----I ---S------
92  EN--KDL--- F--------- ---------- -------F.V ---E--D--- ----------
92  ENAPK-I-E- P-R--T-S-- -----S---- -------F.- ---D--D--L -----G----
96  DKNKDFI-EN GHLY-EDR-I --N---L--S T-EVY-EFAD SLD-.ND-IM P--VET--N-

                    ↓↓    *           ←                  _____T-M_____
151 SRVSVTKPFM LPPVAASSLR ND......SS SSNRKATNPI EDSSIQWAVM ALPACFSLVI
151 ---------- ---------- ---------- ------SDS- ----L---AV ----F-----
151 ---------- ---------- ---------- ------S-S- G--NL---A- ----F-----
151 ---------- ---------- ---------- ------S-S- ----L---AV ----F-----
150 ---------- ---------- ---------- ------K--P G---LH--A- ----L---I-
150 ---------- ---------- ---------- ------AKAP ---GL--TA- ----LI----
155 ---A---TIS F--------- --SIGSNT-- N--KE-LGF- SS--L-GISI --TSLL--L-

    ___________
205 GFAFGAFYWK KKQPN.LTRT VENIQIN..E EDNEISMLQE KEREFQEV 249 FELINE
205 ------L--- ---------- ---------- ---------- -------- 249 PORCINE
205 ------L--- ---------- ---------- ---------- -------- 249 CANINE
205 ---------- ---------- ---R------ ---------- -------- 249 BOVINE
204 ------L--- -R--S----A ---------- ---------- -------- 248 HUMAN
204 ------L--- ---SS----A ---------- ---------Q -------- 248 MURINE
215 --IL--I--- -TH-KSRPES N-T--CHGCQ -E-------Q --K-HLQ- 262 CHICKEN
```

Figure 2 Alignment of the amino-acid sequences of feline, porcine, canine, bovine, human, murine and chicken stem cell factor polypeptides. Identical amino-acids are indicated by dashes (-) and dots indicate gaps introduced to maintain optimal sequence alignment. The likely signal peptide and transmembrane domains are indicated by _**S-P**_ and _**T-M**_ respectively. The predicted amino terminus is marked "♦". Conserved cysteine residues are depicted by "•" and potential N-glycosylation sites by "*". The amino acids shown in bold type between the horizontal arrows (→ ←) are those deleted in the shorter isoforms of feline, canine, human, murine and bovine SCF; in each case this deletion results in the replacement of the 29 deleted amino-acids by a glycine residue. The major proteolytic cleavage sites, responsible for generation of soluble SCF are indicated by "↓".

Table 1 Features of interest within the predicted full length feline SCF protein. These are discussed further within the text.

Feature:	*Position in Predicted Peptide Sequence*	*Comments*
Signal Peptide	–25--1	Predicted from similarity to other species SCF
Extracellular Domain	1–190	as above
Transmembrane Domain	191–213	as above
Intracellular Domain	214–249	as above
Potential N-glycosylation sites	65, 72, 120 and 171.	as above
Conserved cysteine residues	4, 43, 89 and 139.	Cysteine residues 4 and 89 and residues 43 and 139 likely form disulphide bonds.
Proteolytic cleavage site(s)	between Ala^{165} and Ala^{166} and/or Ala^{166} and Ser^{167}	Cleavage results in release of soluble SCF.

clone three except for a deletion of 84 nucleotides (nt. 588–671 inclusive). In order to confirm the likely identity of nucleotide 261 (the above change would lead to an amino-acid change from Asn to Ser), RT-PCR was repeated, as described above except for cycling conditions consisting of an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C 1 min, 50°C 1 min, 72°C 1 min, and a final extension step of 72°C for 10 mins. The primers used were TGGCAAATCTTCCAAAAGACTATAAGA (5′ primer) and AGATGAGTGTCCTTCCACGCACTC (3′ primer) resulting in the amplification of a 230 bp fragment. Two clones from each of two independent PCR reactions were cloned and sequenced on both DNA strands using a Sequitherm long-read cycle-sequencing kit (Epicentre Technologies, Madison, Wisconsin) and M13 universal IRD41 labelled primers (Licor, Lincoln, N.E.); the reaction products were run on a Licor Model 4000 automated sequencer. All four clones showed the nucleotide G at position 261. The full length consensus nucleotide sequence of 953bp is shown in Figure 1. The sequence contains an open reading frame of 822 nucleotides that encodes a deduced polypeptide of 274 amino-acids. The shorter isoform has a deletion of 84 nucleotides (see figure 1) that corresponds to the deletions reported for human, murine, canine and bovine SCFs.

Comparison of the full-length fSCF cDNA to published sequences in other species shows homology of 95%, 93%, 93%, 92%, 87% and 71% to canine, bovine, porcine, human, murine and chicken SCFs respectively. The derived protein shows identity of 92%, 92%, 91%, 88%, 80% and 53% to canine, porcine, bovine, human, murine and chicken homologues. The high sequence homology to the SCFs of other species allows the identification of a signal peptide (aa –25 to –1) that is cleaved to produce the mature protein, an extracellular domain (aa 1 to 190), a transmembrane domain (aa 191 to 213) and an intracellular domain (aa 214 to 249) within the longer fSCF isoform. In common with bovine, canine and porcine SCFs, fSCF has an extra amino-acid (Glu^{130}) when compared to human and rodent SCF sequences. Four cysteine residues (aa 4, 43, 89, 139), implicated as important in forming intramolecular disulphide bridges in rat SCF (Lu *et al.*, 1991), are conserved in fSCF. Potential N-glycosylation sites within the extracellular domain of fSCF are present at amino-acids 65, 72, 120 and 171; the site corresponding to Asn^{93} of human SCF is absent in fSCF. A proteolytic cleavage site has been identified in rat SCF between Ala^{164} and Ala^{165} and/or Ala^{165} and Ser^{166} (Martin *et al.*, 1990; Lu *et al.*, 1991), the amino-acid sequence in this region is well conserved in fSCF suggesting the existence of an analogous site in fSCF, and thus a similar soluble form of fSCF.

Work is in progress to express the fSCF protein which will then enable characterisation of its bioactivity both in-vitro and in-vivo.

The nucleotide sequence data reported in this paper will appear in GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number D50833.

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REFERENCES

Anderson, D.M., Lyman, S.D., Baird, A., Wignall, J.M., Eisenman, J., Rauch, C., March, C.J., Boswell, H.S., Gimpel, S.D., Cosman, D. and Williams, D.E. (1990). Molecular Cloning of Mast Cell Growth Factor, a Hematopoietin That Is Active in Both Membrane Bound and Soluble Forms. *Cell*, **63**, 235–243.

Anderson, D.M., Williams, D.E., Tushinski, R., Gimpel, S.,

Eisenman, J., Cannizzaro, L.A., Aronson, M., Croce, C.M., Huebner, K., Cosman, D. and Lyman, S.D. (1991). Alternate Splicing of mRNAs Encoding Human Mast Cell Growth Factor and Localisation of the Gene to Chromosome 12q22–q24. *Cell Growth and Differentiation*, **2**, 373–378.

Chabot, B., Stephenson, D.A., Chapman, V.M., Besmer, P. and Bernstein, A. (1988). The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. *Nature*, **335**, 88–89.

Copeland, N.G., Gilbert, D.J., Cho, B.C., Donovan, P.J., Jenkins, N.A., Cosman, D., Anderson, D., Lyman, S.D. and Williams, D.E. (1990). Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. *Cell*, **63**, 175–183.

Flanagan, J.G., Chan, D.C. and Leder, P. (1991). Transmembrane Form of the kit Ligand Growth Factor is determined by Alternative Splicing and Is Missing in the Sl^{d} Mutant. *Cell*, **64**, 1025–1035.

Galli, S.J., Zsebo, K.M. and Geissler, E.N. (1994). The kit ligand, stem cell factor. *Adv. Immunol.*, **55**, 1–96.

Geissler, E.N., Ryan, M.A. and Housman, D.E. (1988). The Dominant-White Spotting (W) Locus of the Mouse Encodes the c-kit Proto-Oncogene. *Cell*, **55**, 185–192.

Huang, E., Nocka, K., Beier, D.R., Chu, T., Buck, J., Lahm, H.W., Wellner, D., Leder, P. and Besmer, P. (1990). The Hematopoietic Growth Factor KL is Encoded by the Sl Locus and Is the Ligand of the c-kit Receptor, the Gene Product of the W Locus. *Cell*, **63**, 225–233.

Huang, E.J., Nocka, K.H., Buck, J. and Besmer, P. (1992). Differential Expression and Processing of Two Cell Associated Forms of the Kit-Ligand: KL-1 and KL-2. *Mol. Biol. Cell*, **3**, 349–362.

Lu, H.S., Clogston, C.L., Wypych, J., Fausset, P.R., Lauren, S., Mendiaz, E.A., Zsebo, K.M. and Langley, K.E. (1991). Amino acid sequence and post-translational modification of stem cell factor isolated from buffalo rat liver cell-conditioned medium. *J. Biol. Chem.*, **266**, 8102–8107.

Majumdar, M.K., Feng, L., Medlock, E., Toksoz, D. and Williams, D.A. (1994). Identification and mutation of primary and secondary proteolytic cleavage sites in murine stem cell factor cDNA yields biologically active, cell-associated protein. *J. Biol. Chem.*, **269**, 1237–1242.

Martin, F.H., Suggs, S.V., Langley, K.E., Lu, H.S., Ting, J., Okino, K.H., Morris, C.F., McNiece, I.K., Jacobsen, F.W., Mendiaz, E.A., Birkett, D.C., Smith, K.A., Johnson, M.J., Parker, V.P., Flores, J.C., Patel, A.C., Fisher, E.F., Erjavec, H.O., Herrera, C.J., Wypych, J., Sachdev, R.K., Pope, J.A., Leslie, I., Wen, D., Lin, C., Cupples, R.L. and Zsebo, K.M. (1990). Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* **63**, 203–211.

Morstyn, G., Brown, S., Gordon, M., Crawford, J., Demetri, G., Rich, W., McGuire, B., Foote, M. and McNiece, I. (1994). Stem cell factor is a potent synergistic factor in hematopoiesis. *Oncology* **51**, 205–214.

Shull, R.M., Suggs, S.V., Langley, K.E., Okino, K.H., Jacobsen, F.W. and Martin, F.H. (1992). Canine stem cell factor (c-kit ligand) supports the survival of hematopoietic progenitors in long-term canine marrow culture. *Exp. Hematol.*, **20**, 1118–1124.

Zhang, Z. and Anthony, R.V. (1994). Porcine stem cell factor/c-kit ligand: its molecular cloning and localization within the uterus. *Biol. Reprod.* **50**, 95–102.

Zhou, J.H., Ohtaki, M. and Sakurai, M. (1993). Sequence of a cDNA encoding chicken stem cell factor. *Gene* **127**, 269–270.

Zhou, J.H., Hikono, H., Ohtaki, M., Kubota, T. and Sakurai, M. (1994). Cloning and characterization of cDNAs encoding two normal isoforms of bovine stem cell factor. *Biochim. Biophys. Acta* **1223**, 148–150.

Zsebo, K.M., Williams, D.A., Geissler, E.N., Broudy, V.C., Martin, F.H., Atkins, H.L., Hsu, R.Y., Birkett, N.C., Okino, K.H., Murdock, D.C., Jacobsen, F.W., Langley, K.E., Smith, K.A., Takeishi, T., Cattenach, B.M., Galli, S.J. and Suggs, S.V. (1990). Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* **63**, 213–224.

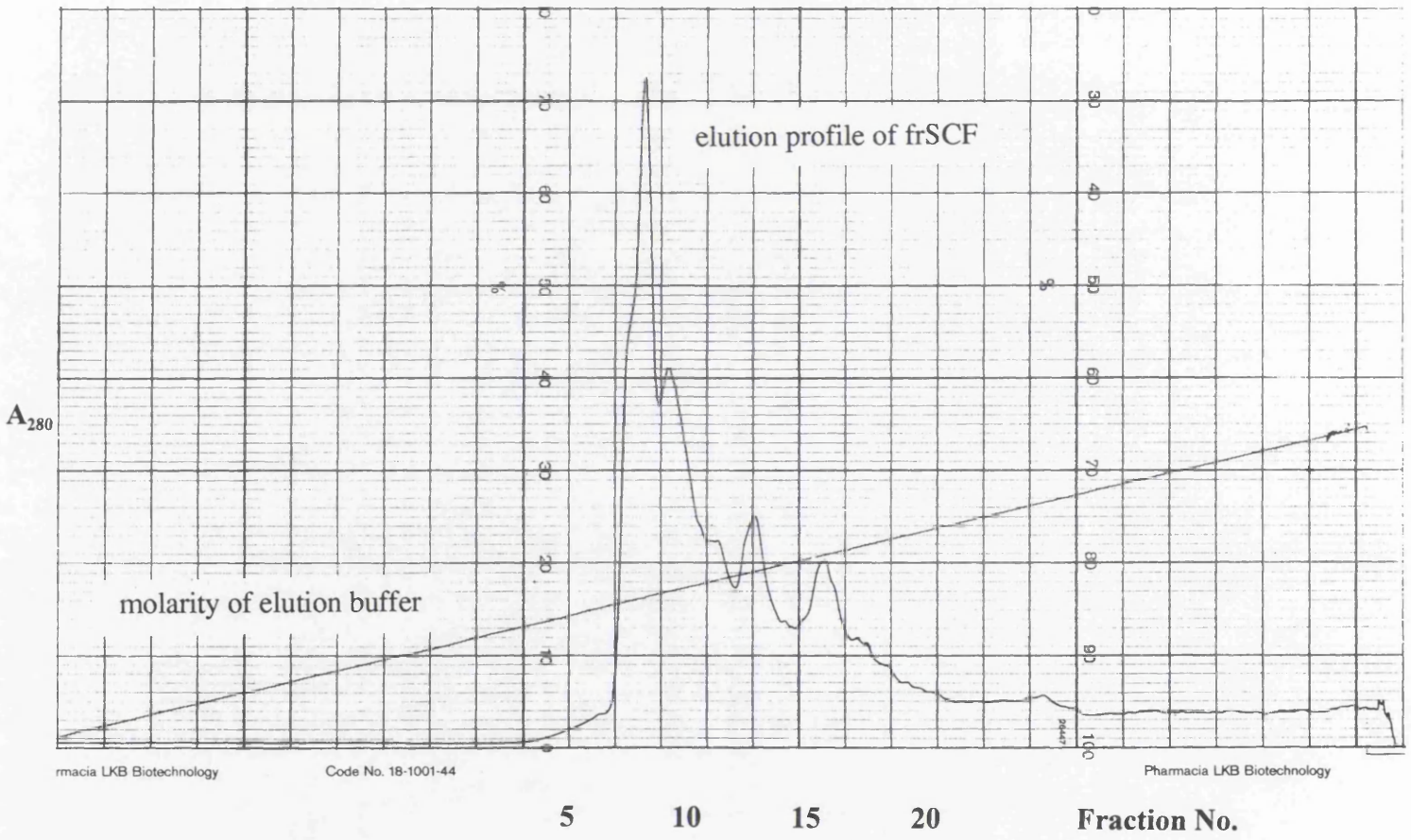

Figure 5.2: Elution profile of fSCF seen with anion exchange chromatography. The curved trace depicts the A_{280} of the sample (AUFS = 2.0) whilst the straight line shows the increasing molarity of NaCl in the elution buffer (10% = 100 mM). Feline SCF corresponds to the first major peak, eluted between 160 - 170 mM mM NaCl, in this example. Flow rate = 1.0 ml/min; chart speed = 1.0 cm/min.