The Effects of Fibroblast Growth Factor-2 (FGF-2) on Haematopoietic Cells and the Identification of those Cells Expressing FGF Receptors

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DECLARATION

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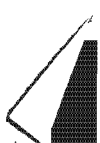

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LIST OF ABBREVIATIONS AND SYNONYMS

7-AAD	7-aminoactinomycin D
AC133	CD133
ac-LDL	acetylated low density lipoprotein
aFGF	acidic fibroblast growth factor (FGF-1)
AGM	aorta-gonad-mesonephros
AML	acute myeloid leukaemia
APC	allophycocyanine
B-CLL	B cell chronic lymphocytic leukaemia
bek	bacterially expressed kinase (FGFR-2)
bFGF	basic fibroblast growth factor (FGF-2)
BFU-E	burst forming unit-erythroid
BL-CFC	blast colony forming cell
BM	bone marrow
BSA	bovine serum albumin
CB	cord blood
CD31	PECAM-1
CFU-E	colony forming unit-erythroid
CFU-GEMM	colony forming unit- granulocyte erythrocyte macrophage megakaryocyte
CFU-GM	colony forming unit-granulocyte macrophage
CFU-M	colony forming unit-macrophage
c-kit	receptor for SCF
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CMML	chronic myelomonocytic leukaemia
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate
DiI-ac-LDL	DiI-acetylated-low density lipoprotein
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethylsulfoxide

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DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease 1
DTT	dithiothreitol
E 9.5	embryonic day 9.5
EB	embryoid body
EB-PE	embryoid derived primitive erythroid
EGF	epidermal growth factor
ES cell	embryonic stem cell
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FGF-1	fibroblast growth factor-1 (aFGF)
FGF-2	fibroblast growth factor-2 (bFGF)
FGF-4	fibroblast growth factor-4 (KGF)
FGFR	fibroblast growth factor receptor
FGFR-/-	fibroblast growth factor receptor deficient
FITC	fluorescein isothiocyanate
flg	fms-like gene (FGFR-1)
Flk-1	fetal liver kinase (VEGFR-2/KDR)
FN	fibronectin
G-CSF	granulocyte-colony-stimulating factor
GM-CSF	granulocyte/magrophage-colony-stimulating factor
HCL	hairy cell leukaemia
HSPG	heparan sulphate proteoglycan
HUVEC	human umbilical vein endothelial cell
IGF-1	insulin growth factor-1
IgG	immunoglobulin
IL-3	interleukin 3
IMDM	Iscove's modified Dulbecco's medium
KDR	kinase domain receptor (VEGFR-2/Flk-1)
KGF	keratinocyte growth factor (FGF-4)
LIF	leukaemia inhibitory factor

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MDS	myelodysplastic syndrome
MEK	mitogen-activated protein kinase/extracellular signal-related kinase
MFI	median fluorescence intensity
mPB	mobilized peripheral blood
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
NEM	N-ethylmaleimide
NOD/SCID	non obese diabetic severe combined immunodeficient
NSC	neural stem cell
PBS	phosphate buffered saline
PCLP1	podcalyxin-like protein 1
PE	phycoerythrin
PECAM-1	platelet-endothelial cell adhesion molecule-1 (CD31)
PE-Cy5	phycoerythrin coupled to cyanine Cy5
pI	isoelectric point
RNA	ribonucleic acid
RPMI medium	Rosewell Park Memorial Institute medium
RT-PCR	reverse transcription-polymerase chain reaction
SCF	stem cell factor (steel factor)
SCGF	stem cell growth factor
SCID	severe combined immunodeficient
SCL	stem cell leukaemia
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tek	tunica interna endothelial kinase (Tie-2)
TGF-ß	transforming growth factor-ß
Tie	tyrosine kinase with IgG loops and epidermal growth factor homology (Tie 1)
TNF-α	tumour necrosis factor-α
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor

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VEGFR-2	vascular endothelial growth factor receptor-2 (KDR/Flk-1)
vWF	von Willebrand factor

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ABSTRACT

Fibroblast growth factor-2 (FGF-2) is a multifunctional growth factor, expressed by a variety of cells, that stimulates the proliferation of stem cells of neuronal, embryonic and haematopoietic origin. Excessive numbers of primitive cells are characteristic of various leukaemias and elevated levels of FGF-2 are found in the haematopoietic cells and serum of individuals with certain types of leukaemias. The first section of my thesis examines the hypothesis that FGF-2 inhibits differentiation, thereby promoting the accumulation of primitive cells. It shows, using cells of the leukaemic cell line, K562, that FGF-2 alone promotes a primitive phenotype in these cells and that it prevents the erythroid differentiation mediated by transforming growth factor-beta. Erythroid differentiation was ascertained by an increase in both haemoglobin content and glycophorin A expression and a decrease in c-kit expression in K562 cells.

The primitive CD34+ haematopoietic population has recently been shown to contain endothelial progenitor cells. As CD34+ haematopoietic cells are known to express receptors for fibroblast growth factors, the hypothesis that the subset of CD34+ cells that expresses fibroblast growth factor receptors contains circulating endothelial progenitor cells was examined. The second part of my thesis describes the isolation and characterization of the fibroblast growth factor receptor-1 expressing subset of CD34+ cells isolated from human bone marrow, cord blood and mobilized peripheral blood, using immunomagnetic selection methods. Four colour flow cytometry was used to identify antigens expressed on these cells. The CD34+FGFR+ cells comprise approximately 5% of the CD34+ population and they express primitive haematopoietic (AC133, Thy-1, c-kit) and endothelial specific (VE-cadherin, P1H12) cell surface antigens, as well as antigens expressed by both haematopoietic and endothelial cells (CD31, TIE, TEK, KDR). CD34+FGFR+ cells mature into endothelial cells when cultured under conditions known to promote endothelial growth, indicating that the CD34+FGFR+ population contains endothelial progenitor cells.

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The experiments in this thesis have been published in two manuscripts (Burger *et al.*, 1994; Burger *et al.*, 2002b) and a third paper has recently been submitted for publication (Burger *et al.*, 2002a). The observation that fibroblast growth factor-2 antagonises the differentiation of haematopoietic cells and maintains their primitive phenotype is novel and may be of significance in understanding the aetiology of leukaemia. The isolation and characterization of the CD34+ population that expresses receptors for fibroblast growth factors, as well as primitive haematopoietic and endothelial antigens and that matures into endothelial cells *in vitro,* is also novel. The results of my work indicate that the circulating CD34+FGFR+ population contains an endothelial progenitor cell population. These cells may be able to repair sites of vascular damage or be genetically engineered to deliver angiostatic or anti-tumour agents to the developing vascular beds of tumours and may therefore have significant clinical potential for the treatment of cardiovascular disease, diabetes and cancer.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

Introduction

Fibroblast growth factor-2 (FGF-2) is a multifunctional growth factor found in a variety of cells that has effects on a wide range of biological processes including embryogenesis, vasculogenesis, angiogenesis and haematopoiesis. It is a member of the FGF family that comprises 23 currently known structurally related polypeptides (Ford-Perriss *et al.*, 2001). The prototypes of this family, acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) were identified as factors present in extracts of bovine brain and pituitary that stimulated the growth of mouse 3T3 fibroblasts (Armelin, 1973; Gospodarowicz *et al.*, 1978). These two growth factors were originally named according to their differing isoelectric points (pI), aFGF having an acidic pI of approximately 5 and bFGF a basic pI of approximately 8 (Klagsbrun and Shing, 1985). They are currently referred to as FGF-1 and FGF-2 respectively.

The human gene encoding FGF-2 is located on chromosome 4 (Mergia *et al.*, 1986). Endogenously produced FGF-2 is synthesised from a common messenger ribonucleic acid (mRNA) as four isoforms with molecular weights of 18 kD, 22 kD, 22.5 kD and 24 kD that are found in different cell compartments: the 18 kD form being localized in the cytosol, while the higher molecular weight forms are associated with the nucleus and ribosomes (Florkiewicz *et al.*, 1991; Renko *et al.*, 1990). All four forms lack a classic signal sequence for secretion, resulting in controversy regarding the manner in which FGF-2 is released from the cell, although a possible mechanism for FGF-2 release may be through exocytosis (Mignatti *et al.*, 1992).

Cellular response to FGF-2 is initiated through two different types of receptors on the cell surface, namely specific high affinity FGF receptors (FGFRs) and low affinity heparan sulphate proteoglycan (HSPG) receptors (Moscatelli, 1987). The specific FGF receptors

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comprise a family of four transmembrane tyrosine kinases that have overlapping affinities with different members of the FGF family (Jaye *et al.*, 1992). The full-length form of these FGFRs contains 3 extracellular immunoglobulin-like loops, a transmembrane domain and an intracellular tyrosine kinase domain (Johnson *et al.*, 1990), although multiple forms of FGFR-1 (*flg*) and FGFR-2 (*bek*) are generated through alternative splicing (Johnson and Williams, 1993). Binding of FGF-2 to FGFR-1 and FGFR-2 has been shown to result in dimerization and autophosphorylation of the receptor and signalling to the cell (Bellot *et al.*, 1991). A fifth member of the receptor family, FGFR-5, has recently been identified (Sleeman *et al.*, 2001). Although FGFR-5 does not have an intracellular tyrosine kinase domain, it demonstrates specific binding to FGF-2. FGF-2 also binds to HSPGs on the cell surface and in the extracellular matrix, though with lower affinity than to FGFR-1 or FGFR-2 (Moscatelli, 1987). Binding of FGF-2 to HSPGs on the cell surface or in the extracellular matrix protects the growth factor from enzymatic or thermal degradation (Gospodarowicz and Cheng, 1986; Saksela *et al.*, 1988), allows the extracellular matrix to act as a reservoir for FGF-2 and allows the growth factor to be localised to a specific site (Flaumenhaft *et al.*, 1990). Interaction between FGF-2 and heparan sulphates or heparin increases the affinity of FGF-2 for its tyrosine kinase receptor (Roghani *et al.*, 1994). FGF-2 has also been shown to act as an attachment factor for suspension cells and may serve as a bridge between cells expressing FGFRs and those possessing HSPGs, thereby promoting cell-cell interactions (Richard *et al.*, 1995).

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FGF-2 is expressed by a wide range of cells, mostly of mesodermal or neuroectodermal origin. Review articles reveal that FGF-2 affects a variety of processes *in vitro* and *in vivo*, including cell proliferation, migration, survival, differentiation, wound healing, angiogenesis and embryogenesis (Burgess and Maciag, 1989; Johnson and Williams, 1993; Mason, 1994; Rifkin and Moscatelli, 1989). FGF-2 stimulates endothelial cell proliferation (Schweigerer *et al.*, 1987) and enhances the survival of endothelial (Fuks *et al.*, 1994), epithelial (Houchen *et al.*, 1999) and haematopoietic (Gallicchio *et al.*, 1991) cells exposed to ionizing radiation-induced injury. It also promotes wound healing in various organs (Hebda *et al.*, 1990; Rieck *et al.*, 1992; Robson *et al.*, 1992; Tsuboi and

Rifkin, 1990), accelerates the healing of duodenal and gastric ulcers (Folkman *et al.*, 1991; Satoh *et al.*, 1997) and is well known as a potent inducer of angiogenesis (Brown *et al.*, 1996; Joseph-Silverstein and Rifkin, 1987; Pepper *et al.*, 1992). FGF-2 also promotes the growth of haematopoietic cells (Gabbianelli *et al.*, 1990; Gallicchio *et al.*, 1991; Han *et al.*, 1992; Oliver *et al.*, 1990; Wilson *et al.*, 1991). Some of the literature implicating FGF-2 in embryogenesis, vasculogenesis, angiogenesis and haematopoiesis will be discussed in more detail below.

Role of FGF-2 in embryogenesis and vasculogenesis

Human embryonic stem (ES) cells are pluripotent cells from the inner cell mass of *in vitro* fertilized human blastocysts and are therefore of a most primitive nature. Experiments show that human ES cells express FGFR-1 (Schuldiner *et al.*, 2000) and that FGF-2 increases the cloning efficiency of these cells and inhibits their differentiation, maintaining their primitive phenotype (Amit *et al.*, 2000). However when FGF-2 is added to embryoid bodies (EBs) derived from ES cells, the morphology changes from a phenotype encompassing many lineages to that of a fibroblastic morphology (Schuldiner *et al.*, 2000). This expression of FGFR-1 on human ES cells, together with evidence of functionality of these receptors, indicates that FGF-2 is a relevant growth factor in early human embryogenesis.

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Studies on the development of Xenopus embryos indicate that FGFs and their receptors are necessary for the induction of mesoderm (Amaya *et al.*, 1991; Amaya *et al.*, 1993; MacNicol *et al.*, 1993; Slack, 1994). Murine embryos expressing dominant negative FGFR-1 die before E 9.5 (embryonic day 9.5) and show severe growth retardation and defective mesoderm structures (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994). Murine chimeras, generated by injecting FGFR-1 deficient (FGFR-1 -/-) ES cells with a high contribution of FGFR-1 -/- cells into wild type blastocysts, die during gastrulation. The majority of embryos with a low contribution of FGFR-1 -/- cells survive, but show limb bud malformation, tail distortions, spina bifida and partial duplication of the neural tube (Deng *et al.*, 1997). FGF-2 is also implicated in the development of most tissues and

organs in the body including the liver (Jung *et al*., 1999), kidney (Dono and Zeller, 1994; Drummond *et al*., 1998), muscle (Joseph-Silverstein *et al*., 1989) and the eye (Consigli *et al*., 1993).

FGF-2 also plays a crucial role in vasculogenesis: a process involving the *de novo* development of vascular structures from endothelial precursor cells or angioblasts that are derived from mesodermal cells, as compared to angiogenesis which entails the formation of new vessels sprouting from existing vasculature. Using dissociated quail blastodiscs, Flamme and Risau showed that treatment with FGF-2 results in the emergence of blood island-like clusters in 100% of epiblast cell cultures, whereas no endothelial or haematopoietic cells are found in cultures without added growth factor (Flamme and Risau, 1992). *In vitro* and *in vivo* quail/chick chimera experiments show that FGF-2 mediates the induction of angioblasts from mesoderm and can induce ectopic vessel formation from lateral mesoderm (Cox and Poole, 2000; Poole *et al*., 2001). Studies using mouse embryos that are injected with antisense FGF-2 ribonucleic acid (RNA) indicate that FGF-2 is crucial for normal vascular assembly in the embryo (Leconte *et al*., 1998). In addition, embryos expressing dominant negative FGFR-1 display disorganised yolk sac development (Lee *et al*., 2000).

There is also evidence that FGF-2 promotes stem cell self-renewal and FGF-2 has been shown to promote a primitive phenotype and inhibit differentiation in a number of systems. For example, FGF-2 promotes myoblast proliferation and inhibits the terminal differentiation of skeletal muscle cells (Clegg *et al*., 1987; Olwin and Rapraeger, 1992). It maintains the primitive phenotype and is a mitogen for stem cells of embryonal (Matsui *et al*., 1992; Resnick *et al*., 1992; Resnick *et al*., 1998) and neuronal (Kalyani *et al*., 1999; Ray *et al*., 1993) origin. Neural stem cells as early as E8.5 proliferate in serum free conditions when in the presence of FGF-2 (Tropepe *et al*., 1999) and FGF-2 plays an important role in neurogenesis (Raballo *et al*., 2000; Vaccarino *et al*., 1999). FGF-2 also promotes the self-renewal of mesenchymal stem cells (Tsutsumi *et al*., 2001). In addition, FGF-2 is a potent mitogen for a primitive erythroid cell line, generated from

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differentiated ES cells (Yuen *et al.*, 1998) and for another ES cell-derived multipotential haematopoietic cell line (Anzai *et al.*, 1999).

Role of FGF-2 in angiogenesis

Angiogenesis is the process whereby new blood vessels are generated from pre-existing vessels. This process involves degradation of extracellular matrix proteins and the proliferation and migration of endothelial cells. Under normal conditions, the endothelial cells lining the inside of blood vessels are quiescent but during angiogenesis capillary endothelial cells undergo rapid proliferation. The angiogenic process is regulated by positive and negative factors (Bussolino *et al.*, 1996; Hanahan and Folkman, 1996; Iruela-Arispe and Dvorak, 1997) and FGF-2 is one of the most potent positive regulators of this process (Basilico and Moscatelli, 1992; Gualandris *et al.*, 1994; Mignatti *et al.*, 1989; Montesano *et al.*, 1986).

Under physiological conditions, angiogenesis occurs in only a few instances such as in the female reproductive system during menses or pregnancy. Maintenance of uterine tissue during the menstrual cycle and the development of the placenta after fertilization are dependent on angiogenesis. There is evidence that FGF-2, FGFR-1 and FGFR-2 are implicated in the growth and re-modelling of endometrial and placental tissue (Arany and Hill, 1998; Moller *et al.*, 2001; Sangha *et al.*, 1997).

In cancer, active angiogenesis is required in order for tumours to acquire new blood vessels that are necessary to support tumour growth. These new vessels also allow the dissemination of malignant cells and therefore metastatic spread is dependent on angiogenesis. Of the 12 currently known angiogenic factors, FGF-2 and vascular endothelial growth factor (VEGF), are the two most commonly found in tumours (Folkman, 1995). There is evidence that FGF-2 induces the expression of VEGF *in vitro* (Mandriota and Pepper, 1997; Seghezzi *et al.*, 1998) and *in vivo* (Claffey *et al.*, 2001) and increases the response of endothelial cells to VEGF by upregulating VEGF receptor-2 (VEGFR-2 /flk1/KDR) (Hata *et al.*, 1999). Recent evidence shows that increased

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angiogenesis also occurs in the bone marrow of patients with some haematopoietic malignancies and that the levels of FGF-2 and/or VEGF are elevated in these malignancies (Aguayo *et al.*, 2000; Bertolini *et al.*, 2000; Hussong *et al.*, 2000; Padro *et al.*, 2000; Pruneri *et al.*, 1999).

FGF-2 is an important tumourigenic factor in prostate cancers and both FGF-2 and FGFR-1 are found expressed in these tumours (Davol and Frackelton, 1999; Ittman and Mansukhani, 1997). Expression of FGF-2 and FGFRs is also found in other tumour types, including breast, gastric and lung carcinomas as well as melanomas (Albino *et al.*, 1991; Berger *et al.*, 1999; Ueki *et al.*, 1995; Yoshimura *et al.*, 1998). In addition, cells derived from malignant melanomas (Reed *et al.*, 1994) and from renal cell carcinomas (Eguchi *et al.*, 1992) show elevated levels of FGF-2, as do urine samples from patients with a variety of carcinomas (Nguyen *et al.*, 1994).

Role of FGF-2 in haematopoiesis

FGF-2 is involved in the regulation of haematopoiesis and both FGF-2 and FGFRs are expressed by haematopoietic and accessory cells. FGF-2 is produced by human bone marrow stromal cells (Brunner *et al.*, 1991; Brunner *et al.*, 1993). It is a potent mitogen for human bone marrow cells and delays the senescence of these cells (Oliver *et al.*, 1990). FGF-2 also increases survival and haematopoietic recovery after whole body irradiation (Ding *et al.*, 1997). Addition of low doses of FGF-2 (0.2-2.0 ng/ml) to human long-term bone marrow results in a significant increase in myelopoiesis (Wilson *et al.*, 1991). In concert with other haematopoietic cytokines such as interleukin 3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF), FGF-2 stimulates the growth of purified haematopoietic progenitor cells (Bruno *et al.*, 1993; Gabbianelli *et al.*, 1990; Gabrilove *et al.*, 1994). FGF-2 has also been shown to stimulate megakaryopoiesis (Bikfalvi *et al.*, 1992; Bruno *et al.*, 1993; Han *et al.*, 1992) as well as the growth of the megakaryocytic cell line, Hel (Bikfalvi *et al.*, 1992).

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Receptors for FGF are expressed by normal haematopoietic and leukaemic cells:
(1) The expression of FGFR protein and/or mRNA has been shown on a variety of normal haematopoietic cells, including primitive progenitor and mature cells. Fluorescent activated cell sorter (FACS) analysis has shown the presence of FGFR-1 protein on mononuclear cells from normal bone marrow (Ratajczak *et al*., 1996). Ratajczak et al found that 79% of burst forming units-erythroid (BFU-E) and 64% colony forming units-granulocyte macrophage (CFU-GM) express FGFR-1; 17% of lymphoctyes expressed FGFR-1 strongly, whereas 35% of monocytes displayed weak expression (Ratajczak *et al*., 1996). Receptors for FGFs are also expressed by CD34+ haematopoietic progenitor cells (Berardi *et al*., 1995; Burger *et al*., 1998; Burger *et al*., 1999; Le Bousse-Kerdiles *et al*., 1996; Testa *et al*., 1996). In addition, FGFR-1 and FGFR-2 mRNAs are expressed by megakaryocytes, platelets, macrophages, granulocytes, T cells and B cells (Bikfalvi *et al*., 1992).
(2) FGFRs are also expressed by leukaemic cells and cell lines. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of bone marrow or peripheral blood samples from 15 out of 15 patients with acute myeloid leukaemia (AML) and 19 out of 20 patients with chronic myeloid leukaemia (CML) expressed FGFR-1 (Ratajczak *et al*., 1996). Leukaemic cell lines, such as K562, Molt 3, Jurkat, HL60, TF-1, Hel, Daudi, MO7E, Dami and KG1 have all been shown to express FGFRs (Allouche *et al*., 1995b; Armstrong *et al*., 1992; Liuzzo and Moscatelli, 1996; Ratajczak *et al*., 1996).

Expression of FGF-2 is shown by a variety of normal haematopoietic cells. FGF-2 is found in T cells (Blotnick *et al*., 1994; Peoples *et al*., 1995) megakaryocytes, platelets and granulocytes (Brunner *et al*., 1993). Recent studies show that myeloblasts, erythroblasts, megakaryoblasts and primitive CD34+c-kit+ cells express FGF-2 mRNA (Majka *et al*., 2001). In addition, conditioned media from CD34+ cells and colony forming unit megakaryocyte-derived cells contain detectable amounts of FGF-2 (Majka *et al*., 2001), indicating that FGF-2 is released by these cells in culture.

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Increased FGF-2 production is associated with a number of haematological disorders. Circulating megakaryocytic cells and platelets from patients with idiopathic myelofibrosis express high levels of FGF-2 (Martyre *et al.*, 1997) and increased expression of FGF-2 as well as that of FGFR-1 and FGFR-2 is found on CD34+ cells obtained from the bone marrow of patients suffering from myelofibrosis with myeloid metaplasia as compared to normal donors (Le Bousse-Kerdiles *et al.*, 1996). A variety of types of leukaemic cells produce FGF-2. Lymphocytes from patients with high-risk and intermediate risk chronic lymphocyte leukaemia (CLL) have significantly elevated levels of FGF-2 as compared to the levels found in cells from those individuals with low-risk disease (Menzel *et al.*, 1996). In addition, CLL lymphocytes with high intracellular levels of FGF-2 appear to have increased resistance to fludarabine-mediated apoptosis, indicating that the presence of this growth factor may result in a delay in apoptosis and the prolonged survival of these cells (Menzel *et al.*, 1996). Hairy cell leukaemia (HCL) is a rare form of leukaemia that presents with pancytopenia, bone marrow fibrosis and the appearance of malignant cells co-expressing both the B cell specific antigen, CD19, and the myeloid specific antigen, CD11c. Serum, bone marrow aspirate and conditioned medium from cell culture from patients with HCL show raised levels of FGF-2 compared to samples from healthy individuals (Gruber *et al.*, 1999). Moreover exogenous FGF-2 protects hairy cells from the effects of 2-chlorodeoxyadenosine, a reagent that causes apoptosis of these cells (Gruber *et al.*, 1999). The size of a leukaemic cell population may be regulated by the rate of cell death as well as by the rate of cell proliferation and the protection afforded against apoptosis by FGF-2 in the case of CLL and HCL may indicate that FGF-2 may afford a survival advantage to these leukaemic cells. Aberrant production of FGF-2 is also shown by cells from patients with B cell chronic lymphocytic leukaemia (B-CLL) and those with CML, where plasma levels of FGF-2 are increased by 54% and 44% respectively, compared to samples from normal controls (Krejci *et al.*, 2001). High levels of FGF-2 present in the serum of adult patients with non-Hodgkin's lymphoma before chemotherapy are associated with poor overall survival (Salven *et al.*, 1999). A recent report shows that, compared with normal individuals, FGF-2 plasma levels are increased in patients with AML, CML, CLL, chronic myelomonocytic leukaemia (CMML) and myelodysplastic syndromes (MDS) (Aguayo *et al.*, 2000). There is

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therefore evidence that over-expression of FGF-2 may play a role in the aetiology of leukaemia and myeloproliferative disorders. It is possible that aberrant expression of FGF-2 could lead to excessive proliferation of stem cells or increased survival by preventing apoptosis, resulting in these types of diseases.

Plasticity of stem cells

Within the last four years, the concept of a pluripotential haematopoietic stem cell that is only capable of differentiating linearly along a pathway of increasing limited development potential into mature cells of the peripheral blood, has been seriously challenged. A recent review of the literature shows that the developmental potential of adult haematopoietic stem cells has a greater plasticity than previously thought (Fuchs and Segre, 2000; Goodell *et al.*, 2001; Reya *et al.*, 2001; Weissman, 2000). Purified haematopoietic stem cells are capable of giving rise to liver (Lagasse *et al.*, 2000; Theise *et al.*, 2000a), skeletal muscle (Gussoni *et al.*, 1999), cardiac muscle and vascular endothelium (Jackson *et al.*, 2001) as well as epithelial cells of lung, gastrointestinal tract, and skin (Krause *et al.*, 2001). Bone marrow derived cells have been shown to give rise to skeletal muscle (Ferrari *et al.*, 1998), brain (Brazelton *et al.*, 2000; Mezey *et al.*, 2000), and liver (Petersen *et al.*, 1999; Theise *et al.*, 2000b), while cells able to regenerate bone have been found in the blood (Kuznetsov *et al.*, 2001). Donor-derived epithelial cells have recently been detected in biopsy specimens of skin, liver and gastrointestinal tract of recipients that received sex-mismatched transplants of stem cells obtained from mobilized peripheral blood (Korbling *et al.*, 2002), indicating that adult human circulating stem cells may have a similar plasticity to that noted for bone marrow cells *in vivo*. Conversely, stem cells from adult mouse brain injected into sublethally irradiated mice, give rise to multipotent haematopoietic stem cells in the host animal (Bjornson *et al.*, 1999) and murine muscle stem cells are able to reconstitute haematopoiesis (Jackson *et al.*, 1999). Human neural stem cells, derived from foetal brain, have been shown to be capable of long-term haematopoietic reconstitution in severe combined immunodeficient (SCID) mice (Shih *et al.*, 2001). These neural stem cells express nestin, FGFR-1 and receptors for epidermal growth factor (EGF) and form

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neurospheres in the presence of FGF-2 and/or EGF. The ability of these cells, which are multipotent for neurons, astrocytes and oligodendrocytes, to show haematopoietic potential, suggests the existence of a human neurohaematopoietic stem cell. In addition, haematopoietic stem cells from adult mouse bone marrow injected into mouse blastocysts are able to generate chimeric embryos that express foetal globin genes (Geiger *et al.*, 1998). These experiments demonstrate that cells once described as haematopoietic progenitor cells may have a broader potential than previously recognised and that haematopoietic stem cells may have the capacity to de-differentiate in culture to a more primitive type of stem cell.

During early vertebrate ontogeny, haematopoiesis and the development of the vascular system are closely associated. In fact, a common precursor for haematopoietic and endothelial cells, the haemangioblast, has long been hypothesized (Murray, 1932; Sabin, 1920; Wagner, 1980). Recent research has revived an interest in the haemangioblast (Lacaud *et al.*, 2001; Ogawa *et al.*, 2001). A number of studies using embryos and/or ES cells have shown that cells regarded as endothelial progenitors are able to give rise to haematopoietic cells (Jaffredo *et al.*, 1998; Nishikawa *et al.*, 1998b; Ogawa *et al.*, 1999; Pardanaud and Dieterlen-Lievre, 1999). Conversely, cells regarded as haematopoietic progenitors are able to give rise to endothelial cells (Choi *et al.*, 1998). Blast colony forming cells (BL-CFCs), derived from ES cells, are capable of generating multilineage haematopoietic cells, primitive erythroid, definitive erythroid, and myeloid cells (Kennedy *et al.*, 1997). Recently, BL-CFCs have been shown to give rise to both haematopoietic and endothelial cells (Choi *et al.*, 1998) and are considered to represent the *in vitro* equivalent of the haemangioblast. Furthermore, experiments on BL-CFCs show that FGF-2 supports haemangioblast proliferation and promotes the haematopoietic development of these cells (Faloon *et al.*, 2000).

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There is also evidence that the CD34+ haematopoietic progenitor cell population contains endothelial stem/progenitor cells (Asahara *et al.*, 1997; Kocher *et al.*, 2001; Nieda *et al.*, 1997; Peichev *et al.*, 2000; Schatteman *et al.*, 2000; Shi *et al.*, 1998; Takahashi *et al.*, 1999): (1) CD34+ cells isolated from human peripheral blood are incorporated into the

endothelium of ischaemic blood vessels of recipient animals (Asahara *et al.*, 1997; Takahashi *et al.*, 1999). (2) Human CD133+ (AC133+) cells isolated from mobilized peripheral blood are able to differentiate into endothelial cells *in vitro* and to form new blood vessels *in vivo* when injected into immunodeficient mice (Gehling *et al.*, 2000). (3) Endothelial cells of donor origin have been cultured from the peripheral blood of human subjects who had previously received sex-mismatched bone marrow transplants, confirming the existence of circulating progenitor endothelial cells (Ikpeazu *et al.*, 2000; Lin *et al.*, 2000).

FGFR-1 and FGF-2 knockout mice

The absence of high affinity receptors for FGF has significant effects: FGFR-1 knockout mice die by E9.5 (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994), and depending on the extent of the deletion, FGFR-2 mutation is either lethal (Arman *et al.*, 1998) or results in defects in limb and lung development (Arman *et al.*, 1999), while FGFR-3 mutants show skeletal and inner ear abnormalities (Colvin *et al.*, 1996). Surprisingly however, studies on FGF-2 knockout mice show that these animals are viable and fertile, displaying only mild defects such as delayed wound healing, impaired vascular tone and abnormalities in cerebral cortex development (Dono *et al.*, 1998; Ortega *et al.*, 1998; Vaccarino *et al.*, 1999; Zhou *et al.*, 1998) as well as a decrease in the ability of the bone marrow stromal layer to support haematopoiesis (Miller *et al.*, 2000). Recently, FGF-1 knockout and FGF-1/FGF-2 double knockout mice have been generated and show either no abnormalities or mild defects similar to those shown by the FGF-2 null mice (Miller *et al.*, 2000), indicating that FGF-1 is not compensating for FGF-2 in the FGF-2 null mouse. It is possible, however, that the lack of gross phenotypic defects in the FGF-2 null mouse may be due to compensation by other FGFs.

Murine studies, using microangiography, demonstrate that when embryos are injected with adenovirus encoding antisense FGF-2 RNA, vascular development is disrupted, whereas co-injection of the virus carrying FGF-2 cDNA in the sense direction prevents abnormal development (Leconte *et al.*, 1998). The authors suggest that, compared to FGF-2 null mice (that show no gross vascular abnormalities), the inhibition of FGF-2

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expression, caused by the injection of antisense FGF-2 RNA, occurs too rapidly to allow time for the upregulation and compensation of FGF-2 by other FGFs (Leconte *et al.*, 1998). These experiments support the view that overlap by other members of the FGF family may account for the lack of difference between the wild type and FGF-2 null mouse.

Summary

This review of the literature has cited evidence that FGF-2 is a relevant cytokine in embryogenesis, vasculogenesis, angiogenesis and haematopoiesis (Figure 1A). Cells that express FGFRs and that respond to FGF-2 are found amongst cells of a very primitive nature, such as ES cells, haemangioblasts, stem cells of the haematopoietic, neuronal, mesenchymal and myogenic lineages. FGFR-expressing and FGF-2-responsive cells are also found amongst haematopoietic cells, including primitive CD34+, mature and leukaemic cells, and endothelial progenitor cells and mature endothelial cells (Figure 1B). As FGF-2 is a haematopoietic cytokine produced by both leukaemic and normal haematopoietic cells, I determined the effects of this cytokine on the cell growth and differentiation of a leukaemic cell line (Figure 1C (i)). In addition, I isolated and characterized a subset of normal CD34+ progenitor cells that expresses receptors for FGF and that contains endothelial precursor cells (Figure 1C (ii)).

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A	B	C
Processes in which FGF-2 is relevant	**Cells that express FGFRs and that respond to FGF-2**	**Aims of study**
Embryogenesis Vasculogenesis Angiogenesis Haematopoiesis	ES cells Mesodermal cells Haemangioblasts Stem cells Leukaemic cells * CD34+ haematopoietic cells ** Mature haematopoietic cells Endothelial stem/progenitor cells** Mature endothelial cells	*(i) Effect of FGF-2 on the erythroid differentiation of the K562 leukaemic cell line. **(ii) Isolation and characterization of a subset of CD34+ cells that expresses FGFR-1 and that contains endothelial stem/progenitor cells.

Figure 1. Schematic representation of the processes in which FGF-2 is a relevant cytokine (A), the cell types that express FGFRs and respond to FGF-2 (B) and the aims of this study (C).

Aims of this study

As discussed in the literature review, FGF-2 has a relevant role in haematopoiesis and both FGF-2 and FGFRs are expressed by normal and leukaemic cells. Aberrant production of FGF-2 is associated with a number of cancers and high levels of FGF-2 are found in cells from individuals with various leukaemias and myeloproliferative diseases. The presence of excessive numbers of primitive cells that occur in these disorders could be explained by a number of processes, such as the increased proliferation of primitive cells and the survival of increased numbers of primitive cells due to diminished apoptosis or the inhibition of differentiation. An accumulation of primitive cells could result from any of these processes, either singly or in combination and it is possible that FGF-2 may be involved in these events.

Aim 1. The first aim of this study was to investigate whether FGF-2 was able to inhibit the differentiation and maintain the primitive phenotype of haematopoietic progenitor cells. In order to accomplish this, the leukaemic cell line, K562, was used as a model for haematopoietic progenitor cells and the effect of FGF-2 on the growth and erythroid differentiation of this cell line was determined (Figure 1C (i))

On completion of the work for Aim 1 and having shown functional effects of FGF-2 on cells of the leukaemic cell line K562, I decided to examine normal haematopoietic cells for the expression of FGFR-1. Human CD34+ haematopoietic progenitor cells express FGFRs (Berardi *et al.*, 1995; Burger *et al.*, 1998; Burger *et al.*, 1999; Le Bousse-Kerdiles *et al.*, 1996; Testa *et al.*, 1996) and the CD34+ haematopoietic progenitor cell population contains endothelial stem/progenitor cells (Asahara *et al.*, 1997; Kocher *et al.*, 2001; Nieda *et al.*, 1997; Peichev *et al.*, 2000; Schatteman *et al.*, 2000; Shi *et al.*, 1998; Takahashi *et al.*, 1999). As cells that are considered to represent haemangioblasts (i.e. cells capable of giving rise to both haematopoietic and endothelial cells) express FGFR-1 and respond to FGF-2 (Faloon *et al.*, 2000), and as a subset of CD34+ haematopoietic cells express FGFRs, I thought that it would be interesting to determine whether the CD34+ cells that express FGFR-1 are able to give rise to endothelial cells.

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Aim 2. The second aim of this study was to isolate and characterize the subset of primitive CD34+ haematopoietic cells that express FGFR-1. These CD34+FGFR+ cells were isolated from bone marrow, umbilical cord blood and mobilized peripheral blood samples. The antigenic phenotype of the CD34+FGFR+ cells was determined and studies on the growth characteristics of these cells were performed in order to ascertain whether the CD34+FGFR+ population contains endothelial stem/progenitor cells (Figure 1C (ii)).

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CHAPTER 2

EFFECTS OF FGF-2 ON ERYTHROID DIFFERENTIATION OF K562 CELLS

Introduction

Haematopoietic cell differentiation and proliferation is regulated by a complex network of signals, including those produced by cell-cell interactions and growth factors or cytokines. FGF-2 is a multifunctional growth factor involved in embryogenesis and vasculogenesis (Cox and Poole, 2000; Flamme and Risau, 1992; Leconte *et al.*, 1998; Pardanaud and Dieterlen-Lievre, 1999; Poole *et al.*, 2001), angiogenesis (Brown *et al.*, 1996; Joseph-Silverstein and Rifkin, 1987; Pepper *et al.*, 1992) and haematopoiesis (Allouche, 1995a; Gabbianelli *et al.*, 1990; Gallicchio *et al.*, 1991; Han *et al.*, 1992; Oliver *et al.*, 1990; Wilson *et al.*, 1991).

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FGF-2 is a mitogen for a variety of stem cells and, in many cases, FGF-2 also inhibits stem cell differentiation, thereby maintaining the cells in a primitive state: 1) FGF-2 stimulates the growth of a primitive erythroid cell line and inhibits its ability to undergo erythroid differentiation (Yuen *et al.*, 1998). 2) It supports the self-renewal of a multipotent haematopoietic progenitor cell line isolated from embryonic stem cells (Anzai *et al.*, 1999) and enhances the proliferation of primitive blast colony forming cells from embryoid bodies (Faloon *et al.*, 2000). 3) FGF-2 also promotes the self-renewal of mesenchymal stem cells (Tsutsumi *et al.*, 2001). 4) It maintains the primitive phenotype and is a mitogen for stem cells of neuronal (Kalyani *et al.*, 1999; Ray *et al.*, 1993; Reimers *et al.*, 2001) and embryonal (Matsui *et al.*, 1992; Resnick *et al.*, 1992; Resnick *et al.*, 1998) origin. 5) FGF-2 also promotes myeloblast proliferation and inhibits the terminal differentiation of skeletal muscle cells (Clegg *et al.*, 1987; Olwin and Rapraeger, 1992). As elevated levels of FGF-2 are associated with leukaemias and myeloproliferative disorders (Aguayo *et al.*, 2000; Gruber *et al.*, 1999; Krejci *et al.*, 2001; Le Bousse-Kerdiles *et al.*, 1996; Martyre *et al.*, 1997; Menzel *et al.*, 1996), it is

possible that the accumulation of primitive cells that occurs in these diseases is due to inhibition of differentiation and that aberrant production of FGF-2 may be involved in the aetiology of these haematological disorders.

The work for this thesis originally arose from a wish to determine if FGF-2 could inhibit the differentiation of haematopoietic progenitor cells, thereby promoting a primitive phenotype and perhaps enhancing the proliferation of cells of an immature nature. In order to initiate this study, I chose the human erythroleukaemic cell line, K562, as a model for the following reasons: (1) The K562 line is a continuous cell line comprising human haematopoietic cells that are easily grown in culture. (2) K562 cells have receptors for FGF-2 on their surface (Allouche *et al.*, 1995b; Liuzzo and Moscatelli, 1996). Both low affinity HSPGs and specific high affinity FGFRs bind FGF-2 (Liuzzo and Moscatelli, 1996) and K562 cells have been shown to express functional FGFRs (Allouche *et al.*, 1995b; Burger *et al.*, 1994). (3) K562 cells can be induced to differentiate along a number of lineage pathways (Leary *et al.*, 1987; Sutherland *et al.*, 1986), allowing examination of the effects of the addition of FGF-2 on the differentiation of these cells along a particular lineage pathway.

The K562 cell line, established in culture in 1971 by Lozzio and Lozzio from a patient with chronic myelogenous leukaemia in blast crisis, was originally thought to be granulocytic in nature (Lozzio and Lozzio, 1975). However the expression of the erythroid specific markers, glycophorin A (Gahmberg *et al.*, 1979) and spectrin (Marie *et al.*, 1981), indicated that this line was erythroleukaemic. Later, the ability of K562 cells to undergo megakaryocytic differentiation (Tetteroo *et al.*, 1984) and to express antigens associated with erythroid, granulocytic, monocytic and megakaryocytic cells in response to different inducing agents, resulted in the K562 line being recognized as a multipotent cell line (Leary *et al.*, 1987; Sutherland *et al.*, 1986).

K562 cells can be induced to undergo erythroid differentiation by the use of a variety of chemical agents, for example, daunorubicin and thymidine-hypoxanthine (Leary *et al.*, 1987; Rowley *et al.*, 1992), haemin (Dean *et al.*, 1981; Rutherford *et al.*, 1979;

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Sutherland *et al.*, 1986), herbimycin A (Honma *et al.*, 1989), sodium butyrate (Sutherland *et al.*, 1986), ethidium bromide and acridine orange (Sasaki *et al.*, 1991). Erythroid differentiation, resulting in the haemoglobinization of K562 cells, can also be induced by physiologically relevant agents such as activin (Yu *et al.*, 1987) and transforming growth factor-ß (TGF-ß) (Burger *et al.*, 1994; Chen *et al.*, 1989a). As I considered the use of a physiological inducer of differentiation to be more appropriate than that of a chemical inducer, I decided to examine the effects of FGF-2 on the TGF-ß-mediated induction of erythroid differentiation in K562 cells.

TGF-ß is an inhibitory haematopoietic cytokine (Ishibashi *et al.*, 1987; Keller *et al.*, 1990; Ohta *et al.*, 1987; Sing *et al.*, 1988) that suppresses human haematopoietic progenitor colony formation (Ottmann and Pelus, 1988; Sargiacomo *et al.*, 1991; Sing *et al.*, 1988). The inhibitory effects of TGF-ß on colony formation can be negated by the addition of FGF-2 (Gabrilove *et al.*, 1993). Furthermore, the negation by FGF-2 of the suppressive effects of TGF-ß on these haematopoietic progenitor cells is dose dependent (Gabrilove *et al.*, 1993). In long term cultures prepared to support growth of myeloid cells (Dexter cultures), TGF-ß causes arrest of haematopoiesis (Hayashi *et al.*, 1989), however addition of neutralizing antibodies to TGF-ß results in a significant increase in progenitor cell proliferation (Waegell *et al.*, 1994), indicating that the inhibitory effects of TGF-ß are specific but are also reversible. FGF-2 is a mitogen for human marrow stromal cells and it enhances myelopoiesis in human long-term bone marrow culture (Wilson *et al.*, 1991). A possible mechanism for the stimulatory effects of FGF-2 in long-term bone marrow culture may be the ability of FGF-2 to negate the inhibitory effects of TGF-ß. There is therefore evidence that FGF-2 and TGF-ß have opposing effects on haematopoietic progenitor cells. Considering that FGF-2 is relevant for stem cell renewal (Anzai *et al.*, 1999; Kalyani *et al.*, 1999; Matsui *et al.*, 1992; Ray *et al.*, 1993; Reimers *et al.*, 2001; Resnick *et al.*, 1992; Resnick *et al.*, 1998; Tsutsumi *et al.*, 2001) and inhibits differentiation in a variety of cells (Amit *et al.*, 2000; Clegg *et al.*, 1987; Olwin and Rapraeger, 1992; Yuen *et al.*, 1998), it is possible that FGF-2 and TGF-ß may have opposing effects on haematopoietic differentiation. I therefore determined whether FGF-2 could inhibit TGF-ß-mediated erythroid differentiation in K562 cells.

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In order to study the effect of FGF-2 on erythroid differentiation, K562 cells were cultured in the presence or absence of FGF-2 and/or TGF-ß. The effects of FGF-2 on TGF-ß-induced erythroid differentiation were determined by measuring the haemoglobin content of these cells. As the expression of glycophorin A increases (Burger *et al.*, 1994; Burger *et al.*, 2002b; Leary *et al.*, 1987; Rowley *et al.*, 1992) and that of c-kit, the receptor for stem cell factor (SCF), decreases as cells mature along the erythroid lineage (Dai *et al.*, 1994; Ogawa *et al.*, 1994; Uoshima *et al.*, 1995), I also determined the expression of these two cell surface antigens on K562 cells after treatment with FGF-2 alone and together with TGF-ß. I found that FGF-2 inhibits erythroid differentiation in K562 cells in a dose-related, time-dependent manner and that the effects are reversible. I also ascertained the effect of FGF-2 on K562 cell growth and found that although FGF-2 causes a slight inhibition of growth as determined by measurement of DNA synthesis and total cell count, the presence of FGF-2 results in an increase in both the numbers and sizes of colonies in soft agar.

The observation that FGF-2 inhibits erythroid differentiation of K562 cells is novel. It is possible that FGF-2 may similarly inhibit differentiation of haematopoietic progenitor cells. The use of antibodies against FGF-2 or of inhibitors capable of blocking either the receptor or subsequent signal transduction pathways, might reduce proliferation of primitive cells or act synergistically with agents known to stimulate differentiation. Such treatments could be of therapeutic relevance in some leukaemias, where elevated levels of FGF-2 may be responsible for the accumulation of immature cells. In addition, FGF-2 may prove useful in the *in vitro* amplification of stem cells, where it could be used to antagonise the differentiation that usually accompanies the proliferation of cells, thereby allowing an increase in stem cell numbers.

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Materials and Methods

Reagents

The following reagents were obtained from the indicated sources: benzidine (Fluka AG, Buchs, Switzerland); RPMI-1640 medium, haemin, 0.4% Trypan Blue (Sigma, St Louis, MO, USA); agar (Difco Laboratories, Detroit, MI, USA); 0.5% agarose (Sea Plaque Agarose, FMC Bioproducts, Rocklands, M.E., USA);{6-^{3}H} thymidine (Amersham International, Buckinghamshire, UK); paraformaldehyde (BDH Chemicals Ltd., Poole, UK); recombinant human TGF-ß1 (Berlex Biosciences, South San Francisco, CA, USA); recombinant human FGF-2 (Synergen, Boulder, CO, USA or Amgen, Thousand Oaks, CA, USA). Recombinant human FGF-1 was a gift from Dr. Y. Schlessinger (NYU Medical Center, NY, USA) and FGF-4 was a gift from Dr. C. Basilico (NYU Medical Center, NY, USA). Mouse anti-human glycophorin A, mouse $IgG1_K$ and RPE-labelled goat anti-mouse $F(ab')_2$ IgG were obtained from Dako A/S (Glostrup, Denmark); CD41a-FITC from Pharmingen (San Diego, CA, USA) and fluorescein isothiocyanate (FITC)-labelled goat anti-mouse $F(ab')_2$ IgG from Cappel (West Chester, PA, USA). Mouse anti-human c-kit (ICI-H7) was a gift from Dr H-J Bühring (Tübingen University, Germany).

Maintenance of K562 cells in culture

K562 cells were cultured in RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% heat inactivated foetal calf serum (FCS) (State Vaccine Institute, Cape Town, South Africa), penicillin (50 units/ml) and streptomycin sulphate (20 µg/ml) at 37° C in a humidified atmosphere with 5% CO_2. Cultures were fed every three to four days by centrifuging the cells and re-seeding the cells at 5×10^4 cells/ml in fresh medium.

Benzidine-peroxide assay for the detection of haemoglobin

The haemoglobin content of K562 cells was assayed using a benzidine-peroxide reagent prepared according to a published method (Lo *et al.*, 1981). Briefly, a solution containing 3 mg benzidine (in laboratories where use of benzidine is considered an unacceptable health hazard, O-dianisidine may be substituted), 2 ml double distilled water and 100 µl

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glacial acetic acid was stirred gently at 37 °C until dissolved. Immediately before use, 50 μl 30% hydrogen peroxide was added. K562 cells (5 x 10^4 cells/ml or 1x10^5 cells/ml) were incubated in medium alone or in the presence of various concentrations of FGF-2 alone or in combination with various concentrations of TGF-ß or haemin. After incubation for 3 or 4 days, the cells were resuspended and washed twice in 1 ml PBS pH 7.4. Cells were resuspended in 100 μl PBS and 20μl benzidene-peroxide reagent was added to each sample 5 minutes before scoring cells with a blue colour under the microscope. A minimum of 400 cells per sample was counted and each sample was counted in duplicate. Cells with a blue colour were scored as benzidine positive.

Haemin solution

A 4 mM stock solution of haemin was prepared according to a published method (Zuhrie and Wickramasinghe, 1991). Briefly, 13 mg haemin (Sigma Chemical Co., St Louis, MO, USA) was dissolved in 0.2 ml of 0.5M NaOH. The solution was buffered by the addition of 0.25ml of 1M Tris-HCl (pH 8.0) and adjusted to 5 ml.

Immunofluorescent staining and flow cytometry

K562 cells, seeded at 1 x 10^5 cells/ml in RPMI containing 10% FCS and antibiotics were cultured in tissue culture dishes, in the absence or presence of FGF-2 and/or TGF-ß at the indicated concentrations. After 3 days, the cells were recovered from the dishes, counted on a haemocytometer and washed with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin and 0.01% NaN_3 (staining buffer). Glycophorin A expression by K562 cells was determined as follows: aliquots of cells (5 x 10^5) in 100 μl staining buffer, were incubated with mouse anti-human glycophorin A (8 μg IgG/ml) for 30 minutes at 4 °C. Cells were subsequently washed twice with 3ml ice-cold PBS to remove unbound antibody, resuspended in FITC-labelled goat anti-mouse IgG (15 μg/ml), and incubated for 30 minutes at 4°C in the dark. The cells were then washed twice with 3ml ice-cold PBS and resuspended in 300 μl 1% paraformaldehyde in PBS, pH 7.4. c-Kit expression was determined in a similar manner, using 20 μl of mouse anti-human c-kit (ICI-H7) as primary antibody and RPE-labelled goat anti-mouse $F(ab')_2$ IgG, according

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to the manufacturer's recommendation, as secondary antibody. Mouse IgG1 was used as an isotype-matched negative control for each sample. Samples were analysed on a flow cytometer (EPICS Profile II; Coulter Corp, Hialeah, FL, USA). Ten thousand events, occurring within the bitmap (forward scatter versus log side scatter), were analysed for each sample, voltages and scale factors being kept constant within each experiment. Histograms were smoothed (a weighted 3-point smooth was used) and overlaid using EPICS CytoLogic Software (Coulter Electronics).

1% Paraformaldehyde in PBS

Paraformaldehyde (10g) was dissolved in 100 ml 1N NaOH and then diluted with approximately 700 ml PBS pH 7.4. The solution was adjusted to pH 7.2 ± 0.1 using concentrated HCL and subsequently diluted to 1000 ml with PBS. The solution was stored in the dark at 4° C.

DNA synthesis

K562 cells were seeded at 1 x 10^5 cells/ml in RPMI-1640 containing 10% FCS, either in medium alone or medium containing TGF-ß (1 ng/ml) and/or FGF-2 at the indicated concentrations. For each condition, 200µl aliquots of cell suspension in replicates of six in 96 well tissue culture plates, were incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2. ^{3}H-thymidine (0.2 µCi/well) was added to each well for the last 18 hours of culture. The cells were harvested on paper filters, dried and counted in a liquid scintillation counter.

Viable cell count

K562 cells were collected from culture dishes, centrifuged and washed once in medium without FCS. The cells were resuspended in a FCS-free medium and an equal volume of 0.4% Trypan Blue was added to suitably diluted aliquots of cells. After 3 minutes, the number of blue cells and the total number of cells present were counted using phase microscopy and a Neubauer haemocytometer. Trypan Blue is a blue dye that, if the cell membrane is not patent, diffuses into the cell and therefore stains dead cells. As the dye

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may react with serum in the medium, it is recommended that FCS should be removed before staining with Trypan Blue.

Colony formation in soft agar or agarose

K562 cells were seeded in RPMI medium containing 10% FCS and agar (0.33%) or agarose (0.5%) at 5 x 10^3 cells/ 35 mm dish. Cells were seeded in quadruplicate or triplicate dishes in the absence and presence of FGF-2 (10 ng/ml or 100 ng/ml) and incubated at 37°C in a humidified incubator with 5% CO_2. After the indicated number of days, clusters of cells greater than 20 cells were scored as "colonies", while clusters of cells comprising more than 100 cells were considered to be "large colonies".

Statistical analysis of results

Data are expressed as the mean ± standard deviation (SD). When comparing the data from two populations, paired Student *t* tests or, where necessary, analysis of variance or the application of the linear model were used to determine levels of significance. A p value of < 0.05 was considered statistically significant.

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Results

FGF-2 antagonises the TGF-ß-mediated increase in haemoglobin production

In order to determine the effect of FGF-2 on TGF-ß-induced erythroid differentiation, K562 cells were incubated in the presence of TGF-ß (1 ng/ml) and FGF-2 (10 ng/ml). After 3 or 4 days in culture, the cells were examined for the presence of haemoglobin by staining with a benzidine-peroxide reagent.

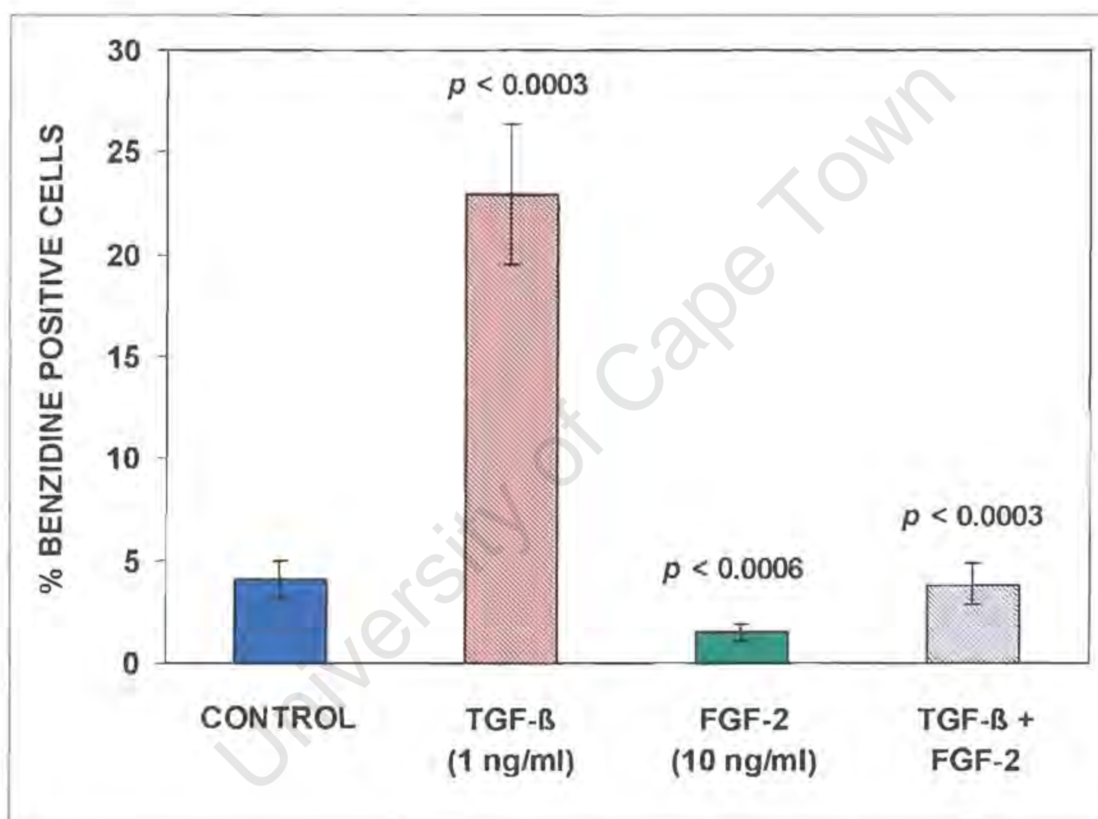

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Figure 2. FGF-2 antagonises the TGF-ß-mediated induction of haemoglobin in K562 cells. K562 cells (5×10^4 cells/ml) were cultured in medium alone or in the presence of FGF-2 (10 ng/ml) or TGF-ß (1 ng/ml), alone or in combination. After 3 or 4 days, the presence of haemoglobin in the cells was determined by scoring the percentage of cells that stained blue with a benzidine-peroxide stain. The graph represents the combined results from 5 experiments.

Data from a set of 5 experiments show that 4.1 ± 0.9% of untreated K562 cells contained endogenously produced haemoglobin (Figures 2 and 3A). In the presence of TGF-ß (1 ng/ml), 22.9 ± 3.5% of K562 cells were benzidine positive, indicating that addition of TGF-ß resulted in a 5.5 fold increase in the number of cells producing haemoglobin, compared to untreated cells ($p < 0.0003$) (Figures 2 and 3B). The simultaneous addition of FGF-2 (10 ng/ml) antagonised the TGF-ß-mediated induction of haemoglobin, such that only 3.9 ± 1.0% of the cells showed positive staining with benzidine-peroxide, indicating that the presence of FGF-2 abrogated the TGF-ß-induced erythroid differentiation ($p < 0.0003$) (Figure 2 and 3C). Interestingly, the percentage of cells containing haemoglobin decreased from 4.1% ± 0.9% in untreated cells to 1.5 ± 0.5% when FGF-2 was added alone, showing that FGF-2 also reduced the endogenous expression of haemoglobin by K562 cells ($p < 0.0006$) (Figure 2 and 3D).

The results therefore confirm that treatment of K562 cells with TGF-ß induces erythroid differentiation in these cells (Burger *et al.*, 1994; Chen *et al.*, 1989a). More importantly they show, for the first time, that FGF-2 antagonises the TGF-ß-mediated induction of erythroid differentiation, as well as inhibiting endogenous haemoglobin production (Burger *et al.*, 1994).

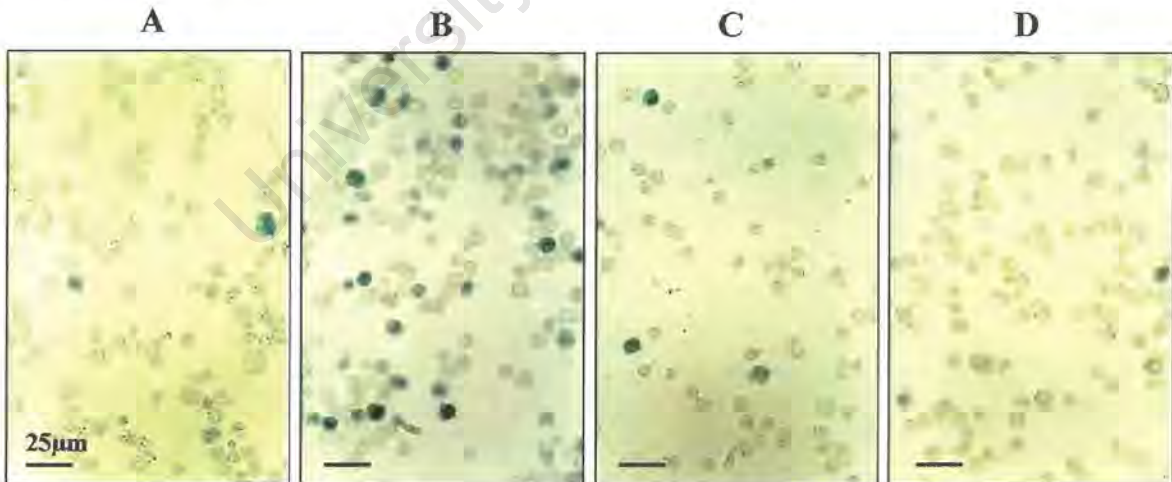

Figure 3. The effect of FGF-2 on the haemoglobin content of K562 cells. K562 cells were incubated in the absence or presence of FGF-2 (10 ng/ml) and/or TGF-ß (1 ng/ml) for 3 days, washed and stained for the presence of haemoglobin. (A) Untreated cells; (B) TGF-ß (1 ng/ml); (C) FGF-2 plus TGF-ß; (D) FGF-2 (10 ng/ml). Cells containing haemoglobin stain blue with benzidine-peroxide. Scale bars = 25µm.

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FGF-2 antagonises the TGF-ß-mediated induction of haemoglobin in a dose dependent manner

Experiments were performed to determine the effects of adding increasing amounts of FGF-2 to K562 cells, in the presence and absence of TGF-ß (1 ng/ml) and to ascertain the minimal dose of FGF-2 required to abrogate TGF-ß-induced haemoglobin production in these cells. I found that the TGF-ß-mediated induction of haemoglobin was antagonised by FGF-2 in a dose-dependent manner, with 0.1 ng/ml and 1 ng/ml FGF-2 inhibiting haemoglobin induction by 41% ($p<0.007$) and 67% ($p<0.013$) respectively, while 10 or 100 ng/ml completely abrogated the increase in haemoglobin synthesis ($p<0.003$, $p<0.007$) (Figure 4). FGF-2 alone at 1, 10 or 100 ng/ml diminished the number of cells that endogenously produced haemoglobin (Figure 4). Similar results were found in 6 separate experiments, confirming that FGF-2 antagonises the TGF-ß-mediated induction of haemoglobin in a dose related manner.

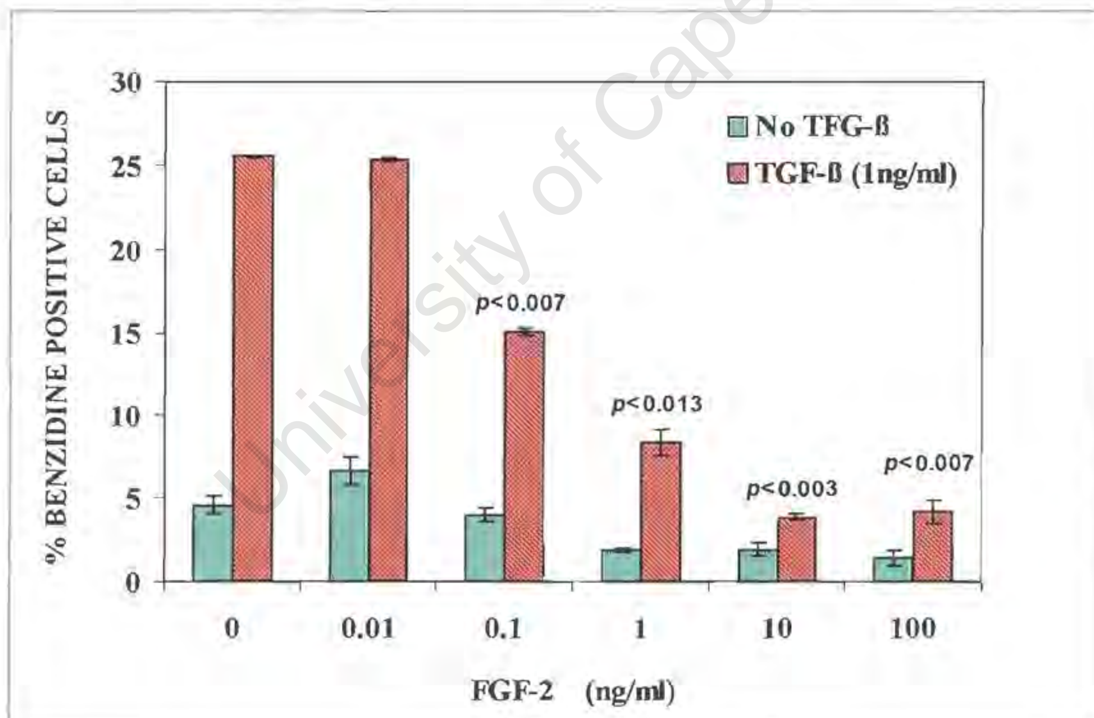

Figure 4. FGF-2 antagonises the TGF-ß-mediated induction of haemoglobin in a dose dependent manner. Increasing concentrations of FGF-2 (0.01-100 ng/ml) were added to K562 cells (5 x 10^4 cells/ml) either alone or in combination with TGF-ß (1 ng/ml) and cultured for 4 days. The presence of haemoglobin in the cells was determined by scoring the percentage of cells that stained blue with a benzidine-peroxide stain.

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Effect of duration of incubation on the inhibition of TGF-ß-induced haemoglobin production by FGF-2 in K562 cells

To determine whether the length of time that FGF-2 was present in culture had an effect on the inhibition of TGF-ß-induced erythroid differentiation in K562 cells, replicate dishes of cells were incubated for 96 hours with TGF-ß (1 ng/ml), while FGF-2 (10 ng/ml) was added either at time zero or after 24, 48 or 72 hours. After 96 hours the cells were assayed for the presence of haemoglobin by staining with benzidine-peroxide.

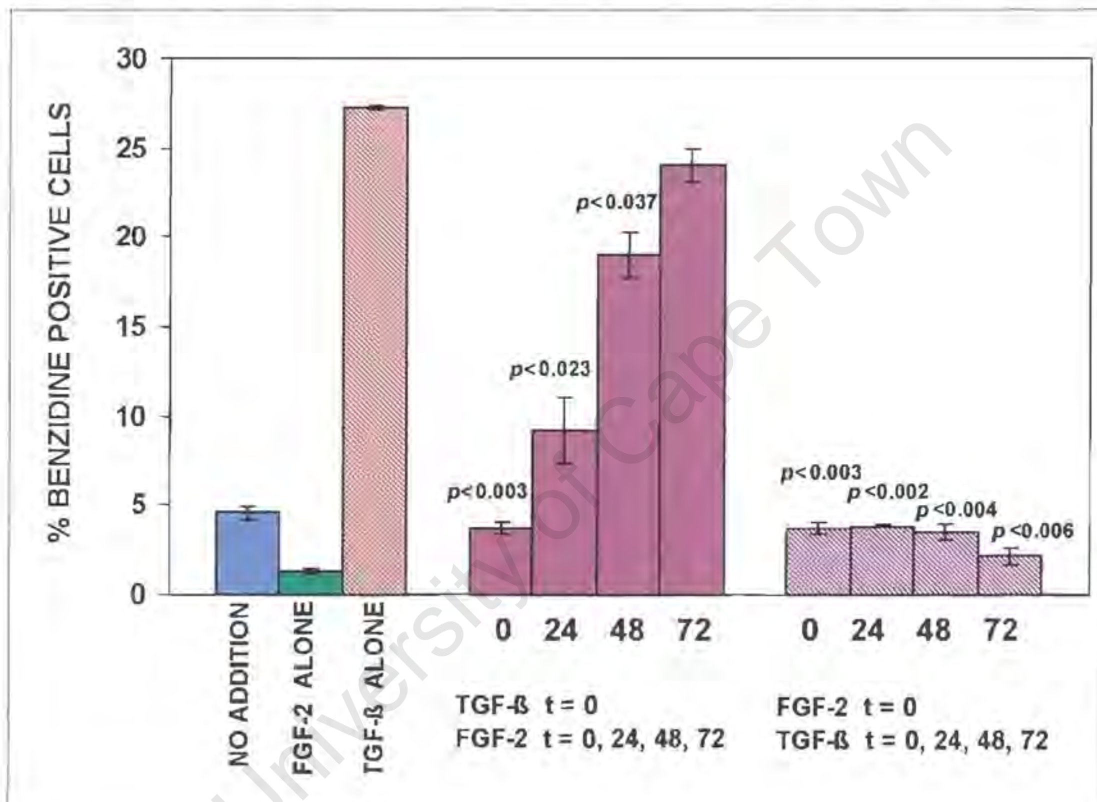

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Figure 5. Effect of duration of incubation on the inhibition of TGF-ß-mediated haemoglobin production by FGF-2 in K562 cells. K562 cells (5 x 10^4 cells/ml) were incubated with either no additions, with FGF-2 (10 ng/ml) or with TGF-ß (1 ng/ml) for 4 days and benzidine positive cells were scored. Four duplicate sets of cultures were prepared in which TGF-ß was added at time 0 and FGF-2 was added at time 0 and after 24, 48 and 72 hours. In addition, 4 sets of cultures were prepared in which FGF-2 was added at time 0 and TGF-ß was added at time 0 and after 24, 48, and 72 hours. All cells were examined for evidence of benzidine staining 4 days after inception of the experiment.

FGF-2 was most effective at antagonising the TGF-ß-mediated induction of haemoglobin if it and TGF-ß were added simultaneously to K562 cells (Figure 5). Delayed addition of FGF-2 to TGF-ß-treated cells, partially abrogated the induction of haemoglobin in K562 cells. If FGF-2 was added 24, 48, or 72 hours after TGF-ß addition, the inhibition of haemoglobinization was 69% (p<0.023), 30% (p<0.037) and 12% respectively, indicating that substantial inhibition was still noted when FGF-2 was added to cells 24 hours after TGF-ß addition, and that partial inhibition was obtained when FGF-2 was added 48 hours after the addition of TGF-ß (Figure 5). Even after 72 hours of treatment with TGF-ß, the presence of FGF-2 for the remaining 24 hours in culture, resulted in a small reduction in the number of benzidine positive cells, however this reduction was not statistically significant (p<0.069) (Figure 5). Addition of FGF-2 from time zero resulted in inhibition of haemoglobin production for all time points of TGF-ß addition (p<0.006) (Figure 5). Similar results were obtained in a total of 4 experiments.

The results show that in order to completely abrogate the effect of TGF-ß-mediated haemoglobin production, FGF-2 is required to be present in culture for the same length of time as TGF-ß. If FGF-2 is present for 75% of the culture time i.e. added after 24 hours, haemoglobin production is reduced by 69%, while if it is present for 50% of culture time i.e. added after 48 hours, haemoglobin production is reduced by 30%. Therefore the extent to which FGF-2 opposes the effects of TGF-ß on haemoglobin production depends on the length of time that FGF-2 is present in culture.

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The effect of FGF-2 on the TGF-ß-mediated haemoglobinization of K562 cells is reversible

To ascertain whether the inhibitory action of FGF-2 on the erythroid differentiation of K562 cells was reversible, the ability of TGF-ß to induce haemoglobin production in K562 was determined before and after removal of FGF-2 from culture. K562 cells were cultured in medium alone or with either TGF-ß (1 ng/ml) or FGF-2 (10 ng/ml) alone, or in combination for 3 days and aliquots of cells were removed in order to determine the number of cells expressing haemoglobin (Figure 6A). The remaining cells were washed with fresh medium to remove reagents and re-incubated in either medium alone (Figure 6B) or in medium containing TGF-ß at 1 ng/ml (Figure 6C) for a further 3 days, after which the numbers of benzidine-positive cells were determined.

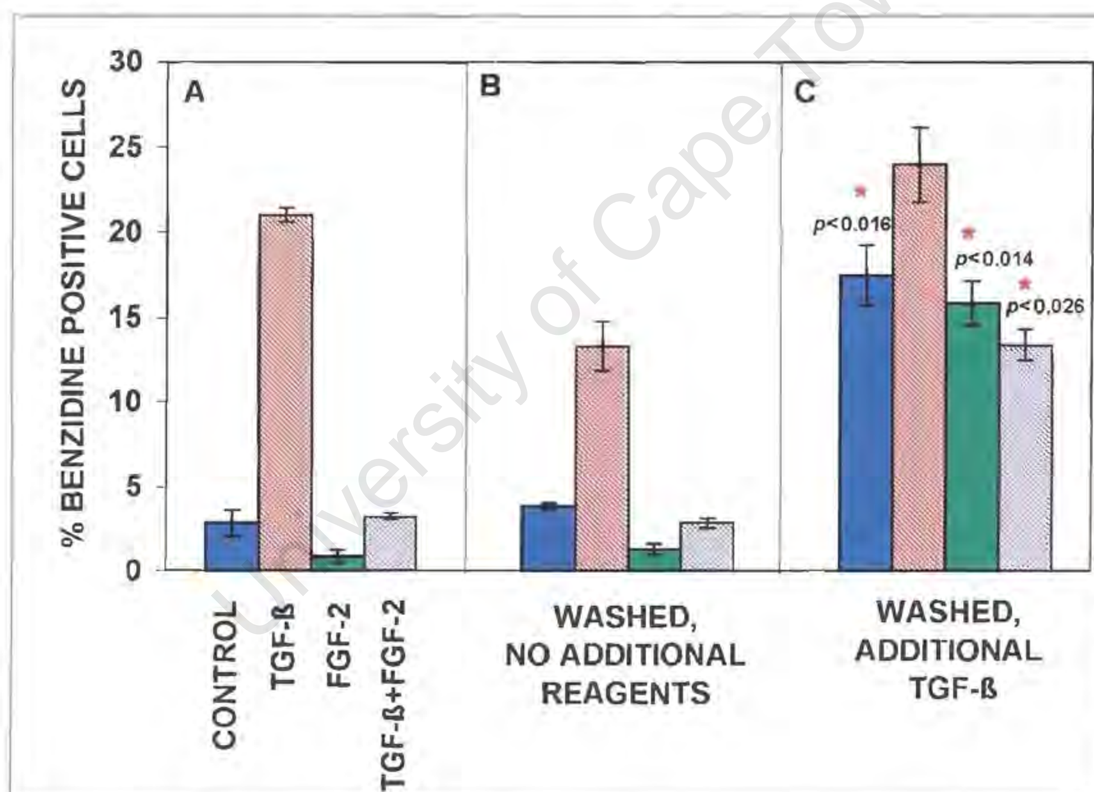

Figure 6. The effects of FGF-2 are reversible. K562 cells (5×10^4 cells/ml) were incubated with either no additions (control), with TGF-ß (1 ng/ml), with FGF-2 (10 ng/ml) or with TGF-ß + FGF-2 for 3 days (A). Cultures were washed free of reagents and then incubated with fresh medium for an additional 3 days in the absence (B) or the presence (C) of additional TGF-ß (1 ng/ml). Benzidine positive cells were determined.
* These values were obtained by comparing the percentages of benzidine positive cells before washing, with those after washing and subsequent TGF-ß treatment.

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Approximately 1% of K562 cells that had been cultured for 3 days with FGF-2 (10 ng/ml) contained haemoglobin (Figure 6A, column 3). When these cells were washed free of FGF-2 and subsequently incubated with TGF-ß (1 ng/ml) alone for an additional 3 days, 16% of the cells in these cultures contained haemoglobin ($p<0.014$ * this value is obtained by comparing the percentages of benzidine positive cells before washing with those after washing and subsequent TGF-ß treatment) (Figure 6C, column 3), compared to 18% of cells that had been treated with TGF-ß alone for 3 days ($p<0.016$ * see above) (Figure 6C, column 1). When TGF-ß and FGF-2 were added simultaneously to cells for 3 days, 3% of the population contained haemoglobin (Figure 6A, column 4). Removal of both reagents and the subsequent addition of TGF-ß alone for a further 3 days (an additional test of the reversibility of the effects of FGF-2) resulted in haemoglobin expression in 13% of the cells ($p<0.026$ * see above) (Figure 6C, column 4). Similar results were obtained in 5 separate experiments.

These results show that prior treatment with FGF-2 for 3 days did not alter the ability of the cells to respond subsequently to the differentiation-inducing effects of TGF-ß. This indicates that the effect of FGF-2 on erythroid differentiation of K562 cells is reversible.

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FGF-2 inhibits the haemin-induced increase in haemoglobin production in K562 cells

As haemin has also been shown to induce haemoglobin production in K562 cells (Cioe *et al*., 1981; Rutherford *et al*., 1979), I determined whether addition of FGF-2 is able to inhibit haemin-induced haemoglobin production in these cells. Concentrations of haemin that induced similar numbers of benzidine positive K562 cells as that induced by TGF-ß (1 ng/ml) were used. Interestingly, FGF-2 was much less effective at inhibiting haemin-induced haemoglobin production than TGF-ß-induced haemoglobin synthesis. Haemoglobin production induced by 10, 20 and 40 μM haemin was inhibited by 54% ($p<0.034$), 51% ($p<0.007$) and 14%, respectively by FGF-2 (10 ng/ml) (Figure 7). The haemin-mediated induction of haemoglobin synthesis could therefore be inhibited by FGF-2 by a maximum of approximately 50% when lower concentrations of haemin (10-20μM) were used, as compared with the ability of FGF-2 to completely abrogate the TGF-ß-mediated induction of haemoglobin synthesis. Similar results were obtained in 2 separate experiments. This indicates that the mechanisms by which TGF-ß and haemin influence haemoglobin synthesis may differ.

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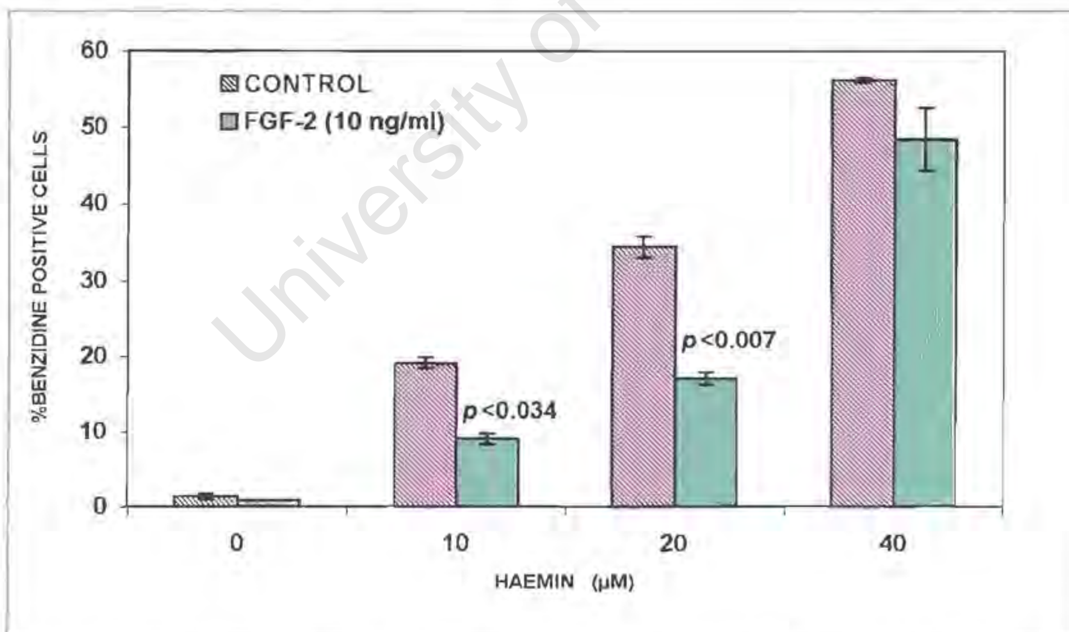

Figure 7. FGF-2 inhibits the haemin-induced increase in haemoglobin production in K562 cells. K562 cells (1 x 10^5 cells/ml) were incubated with or without FGF-2 (10 ng/ml) and varying concentrations of haemin as indicated. Cells were stained with benzidine for haemoglobin determination after 4 days.

Measurement of glycophorin A and c-kit expression

I wished to examine, in a sensitive and quantitative manner, the direct effect of FGF-2 on K562 cells, to ascertain whether this cytokine could influence the expression of differentiation antigens by these cells. As less than 5% of untreated K562 cells contain haemoglobin (Figures 2-7), it was not feasible to use the benzidine assay for haemoglobin content to study direct effects of FGF-2. In contrast, changes in the expression of cell surface molecules can readily be quantitated in thousands of cells by flow cytometry. As the expression of the red cell lineage membrane protein, glycophorin A, increases during erythroid differentiation of K562 cells (Burger *et al.*, 1994; Leary *et al.*, 1987; Rowley *et al.*, 1992) and as expression of c-kit, the receptor for SCF decreases as cells mature along the erythroid lineage (Dai *et al.*, 1994; Ogawa *et al.*, 1994; Uoshima *et al.*, 1995), I determined the effect of FGF-2 on the expression of glycophorin A and c-kit, using monoclonal antibodies to glycophorin A and c-kit, fluorescent labelled secondary antibodies and flow cytometry.

FGF-2 decreases glycophorin A

Glycophorin A was expressed by approximately 60-90% of untreated K562 cells. The addition of FGF-2 significantly decreased glycophorin A levels on K562 cells (upper panel of Table 1, Figure 8). Low concentrations of FGF-2 (0.1 ng/ml) reduced both the number of cells expressing this antigen and the amount of the antigen expressed per cell (fluorescence intensity) (Table 1). FGF-2 at 0.1, 1, 10 and 100 ng/ml reduced the numbers of glycophorin A expressing cells by 25, 42, 49 and 53% respectively and the fluorescence intensity of the cells also decreased (Table 1). Therefore, both the numbers of K562 cells that expressed glycophorin A were significantly diminished and the amount of glycophorin A expressed per cell was also decreased (as evidenced by the reduced fluorescence intensity expressed as median fluorescence intensity (MFI) in Table 1). Similar results were obtained in 9 separate experiments, confirming that the addition of FGF-2 alone to K562 cells resulted in a dose dependent decrease in glycophorin A, indicating that FGF-2 inhibits erythroid differentiation.

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TABLE 1. FGF-2 REDUCES GLYCOPHORIN A AND ANTAGONISES THE EFFECTS OF TGF-ß ON THE EXPRESSION OF GLYCOPHORIN A ON K562 CELLS

FGF-2 (ng/ml)	TGF-ß (ng/ml)	Glycophorin A Expressing Cells (%)	Log Median Fluorescence Intensity
0.0	0.0	70.8	5.2
0.1	0.0	53.4	4.6
1.0	0.0	41.2	3.6
10.0	0.0	36.2	3.6
100.0	0.0	33.1	3.7
0.0	0.0	70.8	5.2
0.0	0.1	82.7	7.9
0.0	0.5	88.4	11.7
0.0	2.0	89.2	11.1
0.0	0.1	82.7	7.9
0.1	0.1	70.5	5.2
1.0	0.1	56.1	4.2
10.0	0.1	45.3	4.2
0.0	0.5	88.4	11.7
0.1	0.5	79.1	8.0
1.0	0.5	66.2	5.9
10.0	0.5	63.8	5.1
0.0	2.0	89.2	11.1
0.1	2.0	79.9	8.5
1.0	2.0	67.6	6.5
10.0	2.0	62.1	5.9

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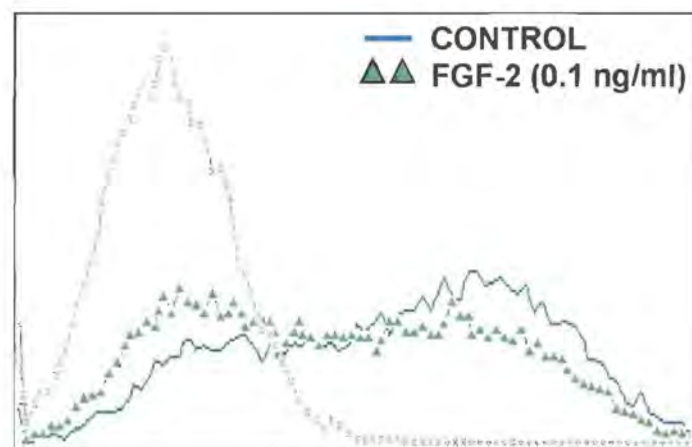

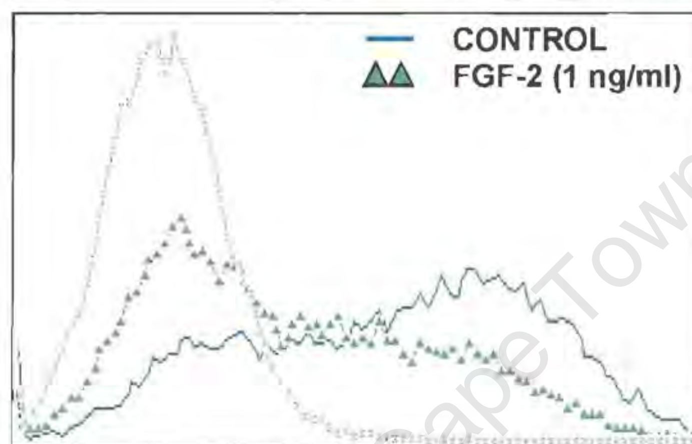

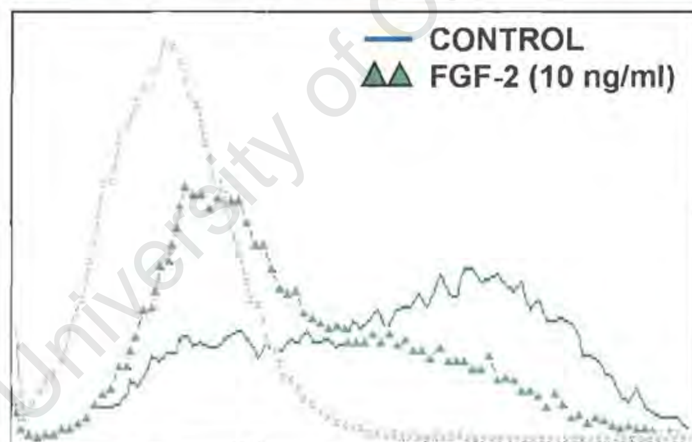

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Figure 8. FGF-2 reduces glycophorin A expression on K562 cells. K562 cells were incubated for 3 days in the absence or presence of FGF-2 at 0.1, 1 or 10 ng/ml. Untreated cells (——); cells treated with the indicated concentrations of FGF-2 (▲▲); isotype matched negative control (xxxx).

A study of the time course of glycophorin A expression after addition of FGF-2 (10 ng/ml) for various periods of time, showed that at 24 hours glycophorin A expression was reduced by half that amount noted at 71 hours, and at 48 hours, the expression was diminished to values that were only 15% above those found at 71 hours (Figure 9). Continued incubation with FGF-2 for longer than 71 hours did not result in increased loss of glycophorin A from the surface of K562 cells (results not shown). These results indicate that the inhibition of glycophorin A expression is a time dependent process showing half maximal effects after 24 hours and requiring 3 days in culture for maximal inhibition. Similar results were obtained in a total of 4 experiments.

In order to determine whether the decrease in glycophorin A expression, induced by FGF-2, was reversible, cells previously treated with FGF-2 were washed and the expression of glycophorin A was assayed after culture in the medium alone. K562 cells (1×10^5 cells/ml) were cultured for 3 days in the presence of FGF-2 (10 ng/ml). The cells were washed twice with fresh medium in order to remove FGF-2 and the expression of glycophorin A was measured on an aliquot of cells (Figure 10, 0 hours) to determine its level at the start of the experiment. The remaining cells were re-seeded at 1×10^5 cells/ml and cultured for either 21 or 46 hours in medium alone, after which the expression of glycophorin A was determined (Figure 10, 21 hours and 46 hours). The results of this experiment show that the phenotype of K562 cells was not permanently altered by FGF-2, as its effects on glycophorin A expression were fully reversible by 46 hours, and were reversed by 68% within 24 hours of removal of FGF-2 from the cells (Figure 10). The reversibility of the effects of FGF-2 was confirmed in an additional 2 experiments.

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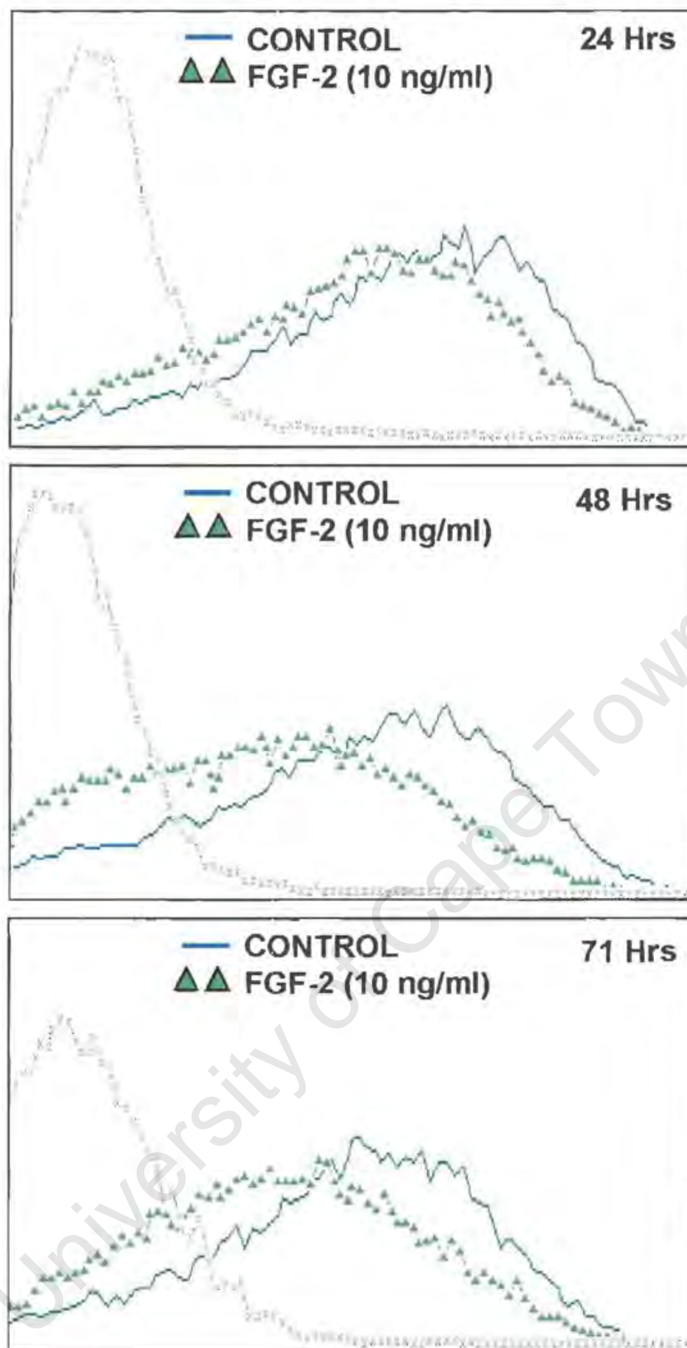

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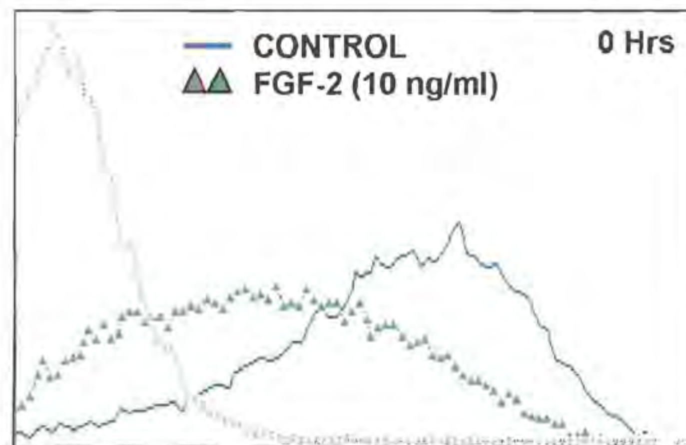
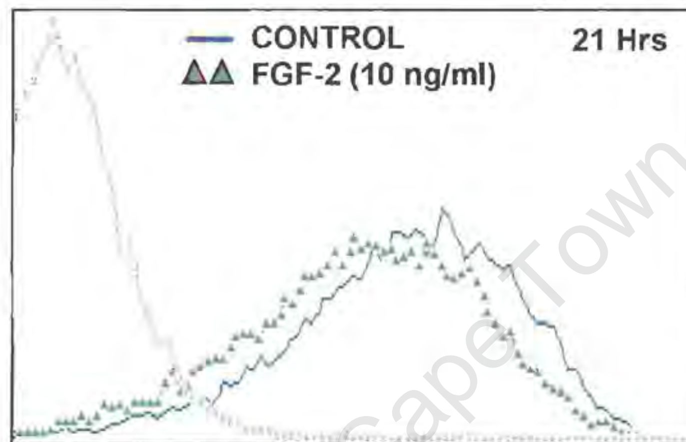
Figure 9. Time course of glycophorin A expression on K562 cells treated with FGF-2. K562 cells were incubated for 24, 48 or 71 hours in the absence or presence of FGF-2 (10 ng/ml). Untreated cells (—); cells treated with FGF-2 (▲▲); isotype matched negative control (xxxx).





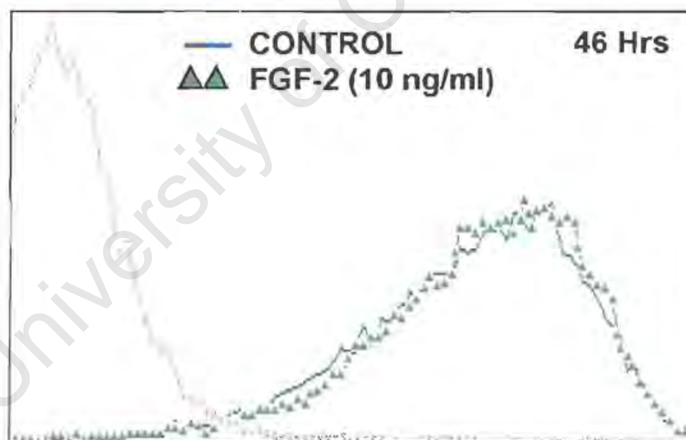


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Figure 10. The effect of FGF-2 (10ng/ml) on the expression of glycophorin A on K562 cells is reversible. K562 cells were incubated for 3 days in the absence or presence of FGF-2, washed and cultured in fresh medium without FGF-2 for 21 and 46 hours. Untreated cells (——); cells previously treated with FGF-2 (10 ng/ml) prior to its removal at the start of this experiment(▲▲); isotype matched negative control (xxxx).

Two other members of the FGF family, FGF-1 and FGF-4, were examined for evidence of direct effects on glycophorin A expression. In the absence of heparin, treatment of K562 cells with FGF-1 or FGF-4 (100 ng/ml) resulted in a reduction in the expression of glycophorin A of approximately 6%, whereas treatment with FGF-2 (100 ng/ml) diminished glycophorin A expression by 48% (data not shown), indicating that FGF-2 was far more potent than FGF-1 or FGF-4. Heparin is not required for the binding of FGF-2 to its tyrosine kinase receptors, although it increases the binding affinity of FGF-2 for these receptors (Roghani *et al.*, 1994). As heparin has a minimal effect on FGF-2 activity but potentiates the activity of FGF-1 considerably (Damon *et al.*, 1989; Mueller *et al.*, 1989), I determined whether either FGF-1 or FGF-4 would inhibit glycophorin A expression in the presence of heparin. K562 cells were cultured for 3 days in the presence of FGF-1, FGF-2 or FGF-4 (1, 10 and 100 ng/ml) together with heparin (500 ng/ml) and the cells were analysed for the expression of glycophorin A. In the presence of heparin and low concentrations of the relevant FGF (1 ng/ml), glycophorin A expression was inhibited by 34%, 4% and 4% by FGF-2, FGF-1 and FGF-4 respectively (Figure 11), indicating that FGF-2 was considerably more effective than either FGF-1 or FGF-4 in inhibiting erythroid differentiation in either the absence (see above) or presence of heparin. Higher concentrations (10 ng/ml) of FGF-2, FGF-1 or FGF-4 caused reductions of glycophorin A expression of 43%, 12% and 23% respectively (Figure 11). When FGF-1 or FGF-4 was added at 100 ng/ml, glycophorin A expression was reduced by 24% and 32% respectively compared to 42% inhibition by FGF-2 (100 ng/ml) (Figure 11). The results indicate that FGF-2 at 1 ng/ml was more effective than either FGF-1 or FGF-4 at 100 ng/ml in reducing glycophorin A expression. Although heparin was able to potentiate the effects of FGF-1 and FGF-4, even in its presence both of these cytokines were considerably less effective than FGF-2 at inhibiting the erythroid differentiation of K562 cells. The data were statistically analysed by application of the linear model (Graybill, 1976) and the results confirm that FGF-2 is more effective than either FGF-1 or FGF-4 in reducing glycophorin A expression by K562 cells, both in the absence ($p<0.003$) and in the presence ($p<0.01$) of heparin.

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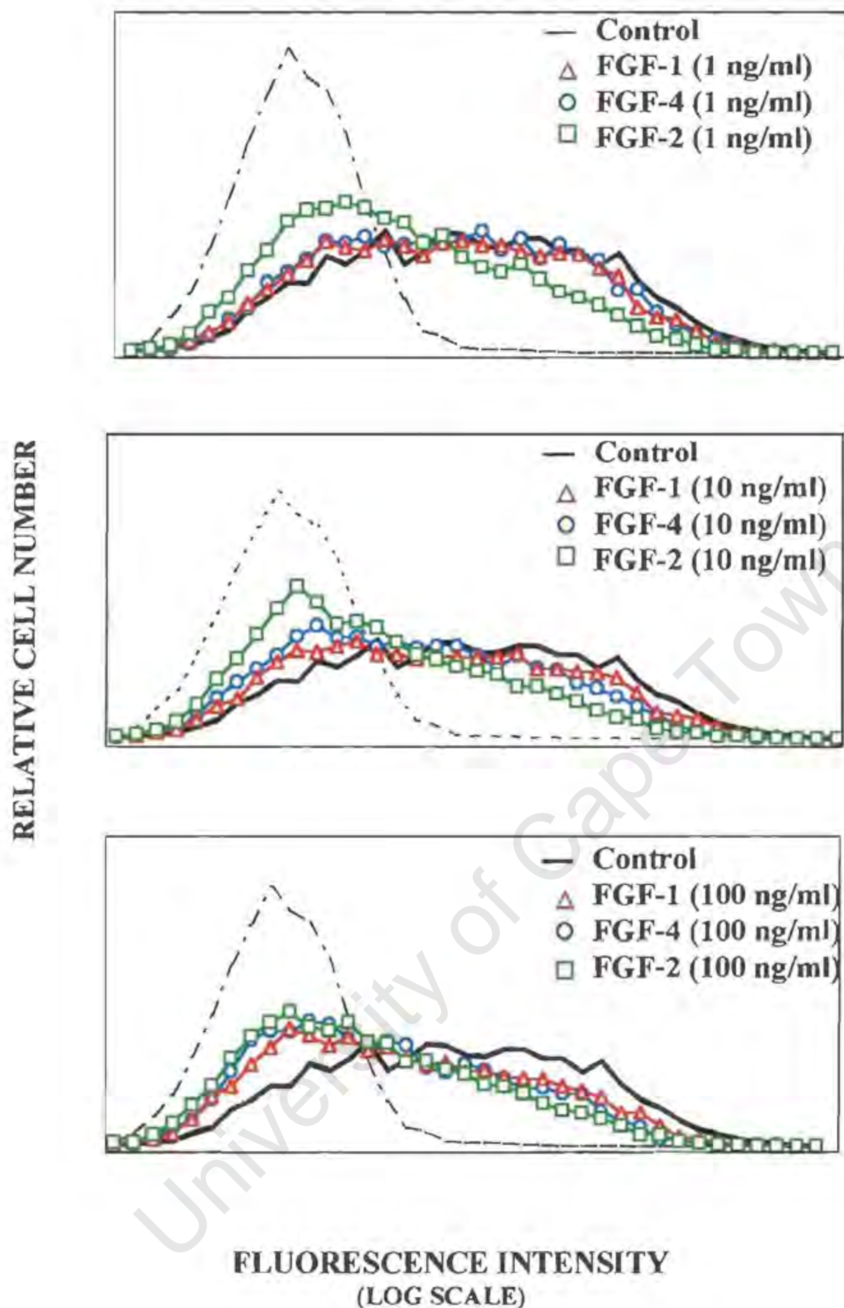

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Figure 11. FGF-1 and FGF-4 are less effective than FGF-2 in reducing glycophorin A expression in K562 cells. K562 cells were incubated in the absence or presence of FGF-1, FGF-2 or FGF-4 at 1, 10, or 100 ng/ml for 3 days. Heparin (500 ng/ml) was present in all cultures. Untreated cells (——); FGF-1 (△); FGF-2 (□); FGF-4 (○); isotype matched negative control (------)

The results obtained by determining the expression of glycophorin A (Table 1 and Figures 8-10) indicate that FGF-2 alone inhibits the erythroid differentiation of K562 cells and that the effect of this cytokine is more potent than that of either FGF-1 or FGF-4 (Figure 11). They also support my results indicating that the numbers of haemoglobin expressing cells were diminished by FGF-2 and confirm that the inhibitory effect of FGF-2 on erythroid differentiation of K562 cells is reversible and both dose and time dependent.

FGF-2 increases c-kit expression

The expression of c-kit, the receptor for SCF, diminishes during erythroid differentiation (Dai *et al.*, 1994; Ogawa *et al.*, 1994; Uoshima *et al.*, 1995). To confirm my results showing that FGF-2 inhibits erythroid differentiation (using haemoglobin production and glycophorin A expression), with an additional parameter, I determined the effect of FGF-2 on the expression of c-kit. As FGF-2 inhibits erythroid differentiation, I expected to find that c-kit expression would either be unaffected or increased by FGF-2.

I found that approximately 60-90% of K562 cells expressed c-kit and that treatment with FGF-2 resulted in an increase in c-kit expression (upper panel of Table 2A). Although cells treated with 0.1-100 ng/ml FGF-2 showed only a slight increase (1 to 7%) in the numbers of cells expressing c-kit when compared to untreated cells, there was a definite increase (17-50%) in c-kit expression per cell, as measured by the median fluorescence intensity (MFI) (upper panel of Table 2A). As the measurement of MFI is in logarithmic units, this increase reflects a considerable enhancement in the number of c-kit molecules expressed per cell e.g., the addition of FGF-2 (100 ng/ml) increased c-kit MFI from 1.2 to 1.8 (upper panel of Table 2A) which, on a logarithmic scale, is a 4 fold increase.

In order to further investigate the FGF-induced modulation of the expression of both glycophorin A and c-kit, I wished to determine the effects of FGF-2 on the expression of both these antigens on the same cells. At the time that I was performing these experiments, I was unable to obtain antibodies to glycophorin A or c-kit that were directly fluorochrome labelled. I therefore analysed the expression of glycophorin A and c-kit (using indirect staining and single colour flow cytometry) on cell aliquots from the

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TABLE 2A. FGF-2 INCREASES C-KIT AND ANTAGONISES THE EFFECTS OF TGF-ß ON THE EXPRESSION OF C-KIT ON K562 CELLS

FGF-2 (ng/ml)	TGF-ß (ng/ml)	c-Kit Expressing Cells (%)	Log Median Fluorescence Intensity
0.0	0.0	59.7	1.2
0.1	0.0	60.2	1.4
1.0	0.0	62.0	1.7
10.0	0.0	64.6	1.7
100.0	0.0	63.8	1.8
0.0	0.0	59.7	1.2
0.0	0.2	50.1	0.8
0.0	2.0	46.5	0.7
0.0	0.2	50.1	0.8
0.1	0.2	56.2	1.1
1.0	0.2	59.9	1.4
10.0	0.2	62.7	1.5
100.0	0.2	63.2	1.6
0.0	2.0	46.5	0.7
0.1	2.0	55.3	1.2
1.0	2.0	62.9	1.9
10.0	2.0	66.3	2.4
100.0	2.0	67.7	2.6

This table represents the results of one experiment, from a set of two separate experiments, performed using the indicated concentrations of FGF-2 and TGF-ß.

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same cell cultures, that had been incubated in the absence or presence of FGF-2. As the effects of FGF-2 on the numbers of cells expressing c-kit were slight, whereas FGF-2 caused a definite increase in the number of molecules of c-kit expressed per cell (upper panel of Table 2A), I used the MFI indices for c-kit and glycophorin A to compare the effects of FGF-2 on both these parameters. To compare the results obtained from 8 separate experiments, the MFI indices for glycophorin A and c-kit expression after treatment with FGF-2 were 'normalised' by setting the MFI values of untreated cells at 100% and calculating the appropriate value as a % of control for the treated cells. When compared to untreated K562 cells, the addition of FGF-2 (100 ng/ml) reduced the number

of molecules of glycophorin A expressed per cell by 63.2 ± 18.8%, n=8 and increased that of c-kit by 23.2 ± 14.8%, n=8) (Table 2B and Figure 12). These results therefore indicate that FGF-2 acts on K562 cells to promote a primitive cell surface phenotype, as judged by diminished expression of the erythroid lineage specific marker, glycophorin A and the increased expression of c-kit.

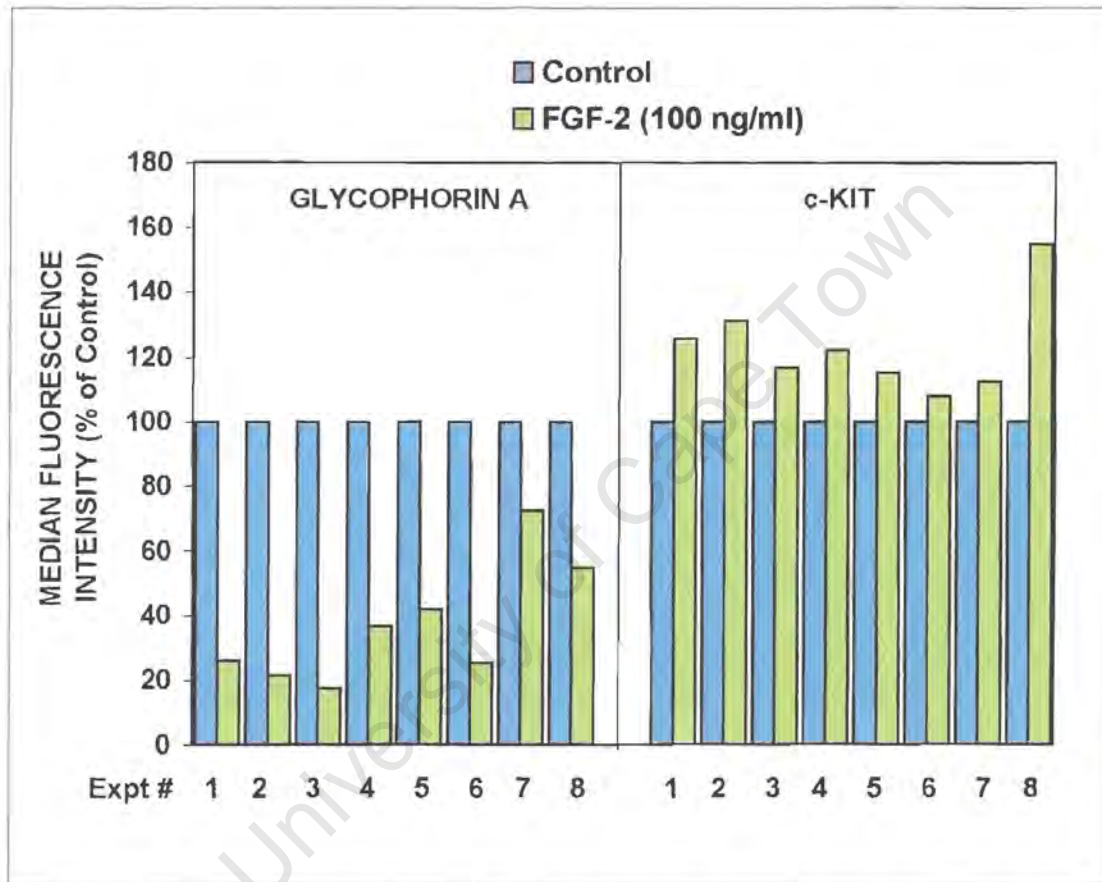

Figure 12. FGF-2 decreases glycophorin A and increases c-kit expression on K562 cells. In eight separate experiments, K562 cells were incubated in the absence or presence of FGF-2 (100 ng/ml) and the expression of glycophorin A and c-kit was determined. The median fluorescence intensity of each antigen was calculated after setting the MFI of glycophorin A or c-kit expression by untreated cells to 100%.

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TGF-ß increases glycophorin A and reduces c-kit expression

As TGF-ß has been shown to induce haemoglobin production in K562 cells (Burger *et al.*, 1994; Chen *et al.*, 1989a) and decrease c-kit expression in normal and leukaemic cells (de Vos *et al.*, 1993; Dubois *et al.*, 1994; Heinrich *et al.*, 1995; Sansilvestri *et al.*, 1995) and, since a reduction of c-kit expression is noted as cells mature along the erythroid pathway (Dai *et al.*, 1994; Ogawa *et al.*, 1994; Uoshima *et al.*, 1995), I determined the effects of TGF-ß on glycophorin A and c-kit expression in K562 cells. I found that TGF-ß at 0.1, 0.5 and 2.0 ng/ml increased the numbers of glycophorin A expressing cells by 17, 25 and 26% respectively, and increased the MFI by 52, 125 and 113%, respectively (middle panel of Table 1, Figure 13). The expression of c-kit was reduced by TGF-ß (middle panel of Table 2A, middle panel of Figure 14). The numbers of cells expressing c-kit were decreased by 16 and 22% and a decrease in the MFI of 33 and 42% was noted in K562 cells treated with 0.2 and 2.0 ng/ml TGF-ß, respectively (middle panel of Table 2A).

Within eight experiments, addition of TGF-ß (2 ng/ml) increased the number of molecules of glycophorin A and reduced that of c-kit (Table 2B). These results demonstrate that TGF-ß promotes erythroid differentiation in K562 cells as determined by changes in glycophorin A and c-kit expression.

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TABLE 2B. EFFECTS OF FGF-2 AND TGF-ß ON THE EXPRESSION OF GLYCOPHORIN A AND c-KIT BY K562 CELLS.

Expt	GLYCOPHORIN A MFI (Log)				c-KIT MFI (Log)				GPA (% Con)	c-KIT (% Con)
#	**Con**	**FGF**	**TGF**	**T+F**	**Con**	**FGF**	**TGF**	**T+F**	**FGF**	**FGF**
1	12.93	3.33	35.81	13.33	1.48	1.86	1.69	2.12	25.8	125.7
2	8.29	1.77	19.31	5.50	1.95	2.56	0.74	2.04	21.4	131.3
3	9.14	1.58	21.59	6.60	2.17	2.53	0.73	1.88	17.3	116.6
4	1.70	0.62	3.93	0.85	0.54	0.66	0.51	0.69	36.5	122.2
5	4.51	1.87	38.98	11.39	2.46	2.83	1.31	2.40	41.5	115.0
6	13.40	3.35	33.71	12.92	2.16	2.33	0.90	2.29	25.0	107.9
7	3.66	2.65	8.80	6.34	2.46	2.76	1.51	3.12	72.4	112.2
8	4.90	2.67	12.84	6.05	1.24	1.92	0.89	2.41	54.5	154.8
Mean	**7.32**	**2.23**	**21.87**	**7.87**	**1.81**	**2.18**	**1.04**	**2.12**	**36.8**	**123.2**
SD	4.33	0.94	13.14	4.30	0.67	0.71	0.42	0.69	18.8	14.8

MFI = Median Fluorescence Intensity (Log)
Expt # = Experiment number
Con = Control (untreated cells)
FGF = FGF-2 (100 ng/ml)
TGF = TGF-ß (2 ng/ml)
F+T = FGF-2 (100 ng/ml) + TGF-ß (2 ng/ml)
GPA (% Con) = Glycophorin A "normalised" MFI (% Control)
c-Kit (% Con) = c-kit "normalised" MFI (% Control)

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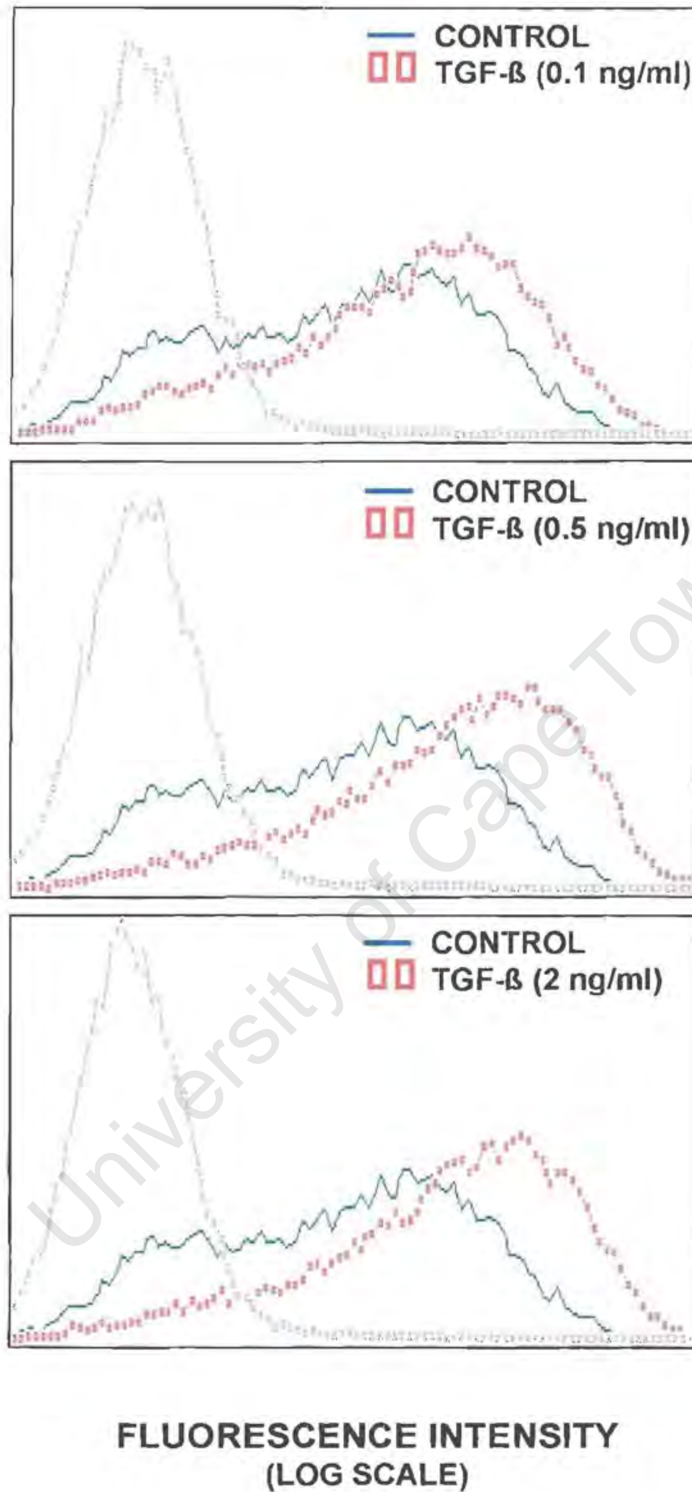

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Figure 13. TGF-ß increases the expression of glycophorin A on K562 cells. K562 cells were incubated for 3 days in the absence or presence of TGF-ß at 0.1, 0.5 or 2 ng/ml. Untreated cells (—); cells treated with TGF-ß (▯▯); isotype matched negative control (xxxx).

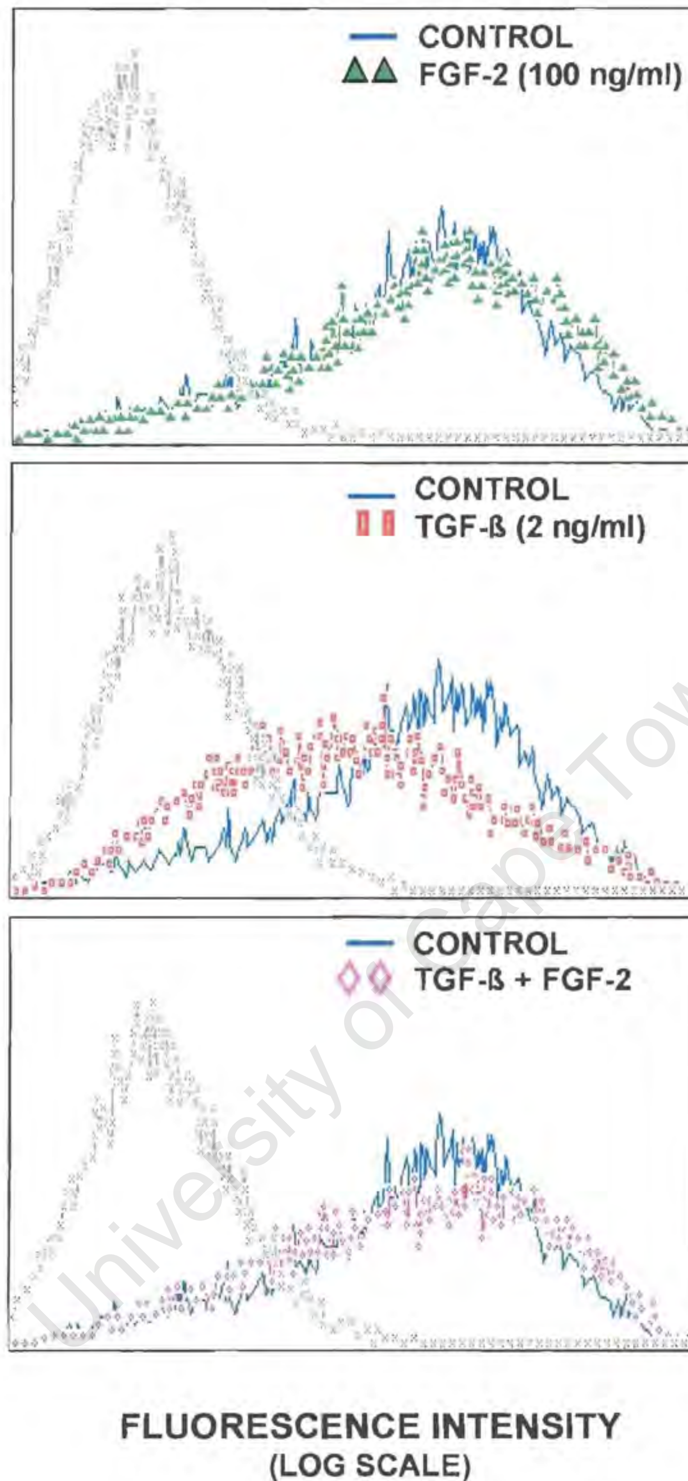

Figure 14. FGF-2 increases c-kit expression and antagonises the TGF-ß-mediated decrease in c-kit expression on K562 cells. K562 cells were incubated for 3 days in the absence or presence of FGF-2 (100 ng/ml); TGF-ß (2 ng/ml) or with the addition of both cytokines. Untreated cells (—); cells treated with FGF-2 (▲▲);TGF-ß (▮▮); TGF-ß + FGF-2 (◇◇); isotype control (xxxx).

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FGF-2 opposes the TGF-ß-induced modulation of glycophorin A and c-kit expression

As FGF-2 and TGF-ß have been shown to have opposing effects in a number of systems (Pepper *et al.*, 1990; Saksela *et al.*, 1987; Saksela and Rifkin, 1990), and as I had noted opposing effects of these cytokines on haemoglobin production in K562 cells, I determined the effects of FGF-2 on the TGF-ß-mediated increase in glycophorin A and decrease in c-kit expression by K562 cells. TGF-ß alone (0.1, 0.5 and 2 ng/ml), or TGF-ß together with FGF-2 (0.1, 1.0 and 10 ng/ml), was added to K562 cells for 72 hours, and the amount of cell surface glycophorin A was measured (Table 1). FGF-2 alone at 0.1, 1.0 and 10 ng/ml was also added to K562 cells for the duration of these experiments (Table 1). FGF-2 effectively antagonised the TGF-ß mediated induction of glycophorin A (bottom panel of Table 1, Figure 15A, B, C and Figure 16). Eight separate experiments showed that the simultaneous addition of FGF-2 (100 ng/ml) and TGF-ß (2 ng/ml) prevented the increase in glycophorin A expression induced by TGF-ß, indicating that FGF-2 inhibits the erythroid differentiation of K562 cells (Table 2B).

FGF-2 was also able to prevent the TGF-ß-mediated decrease in c-kit expression. As shown in Figure 14, the ability of TGF-ß (2 ng/ml) to decrease c-kit was abrogated by the simultaneous addition of FGF-2 (100 ng/ml). TGF-ß at 0.2 and 2.0 ng/ml was equally effective in decreasing the number of molecules of c-kit expressed per cell (MFI decreased from 1.2 to 0.8 and 0.7 respectively) and low concentrations of FGF-2 (0.1 ng/ml) effectively negated the effects of TGF-ß on c-kit expression (bottom panel of Table 2A). Similar results indicating that FGF-2 (100 ng/ml) maintained c-kit expression in the presence of TGF-ß (2 ng/ml) were noted in 8 experiments, indicating that FGF-2 antagonises the effect of TGF-ß and inhibits erythroid differentiation of K562 cells (Table 2B).

The ability of FGF-2 to overcome the differentiation-inducing effects of TGF-ß was dependent on the relative doses of TGF-ß and FGF-2. Low doses of FGF-2 (0.1 ng/ml) effectively antagonised the effects of approximately equivalent amounts of TGF-ß (0.1-2.0 ng/ml) on the expression of both glycophorin A (bottom panel of Table 1, Figure

15A, B, C) and c-kit (bottom panel of Table 2A). As the molecular weights of FGF-2 and TGF-ß1 are approximately equivalent, this implies that these two cytokines counteract each other approximately on a molar basis. Thus, small excesses of either cytokine, relative to one another, may either promote or prevent differentiation along the erythroid lineage in these cells. Even when higher doses of TGF-ß were used (2.0 ng/ml), its effects were readily negated by FGF-2 when added at approximately equivalent amounts (1.0 ng/ml) as shown for both glycophorin A (Table 1, Figure 15C) and c-kit (Table 2A) expression. In two out of four experiments, this higher dose of TGF-ß (2 ng/ml) resulted in the cells being more responsive to FGF-2 (10-100 ng/ml), as noted by an increase in the MFI of c-kit to levels above those seen with FGF-2 alone (Table 2A and Table 2B). The reason for the "overshoot" in c-kit expression is unknown.

Analysis of the data in Table 2B, using the Kruskal-Wallis method (non parametric ANOVA), shows that when K562 cells are treated with FGF-2 (100 ng/ml), TGF-ß (2 ng/ml) or a combination of both cytokines, significant differences in the MFI indices of glycophorin A ($p<0.005$) and c-kit ($p<0.02$) occur. The Mann Whitney test confirms that the addition of FGF-2 alone reduces glycophorin A expression ($p<0.007$) and inhibits the TGF-ß induced increase in glycophorin A expression by K562 cells ($p<0.04$). Although the difference in c-kit expression between untreated cells and those treated with FGF-2 alone is not significant ($p>0.05$), FGF-2 inhibition of the effect of TGF-ß on c-kit expression in these cells is statistically significant ($p<0.009$).

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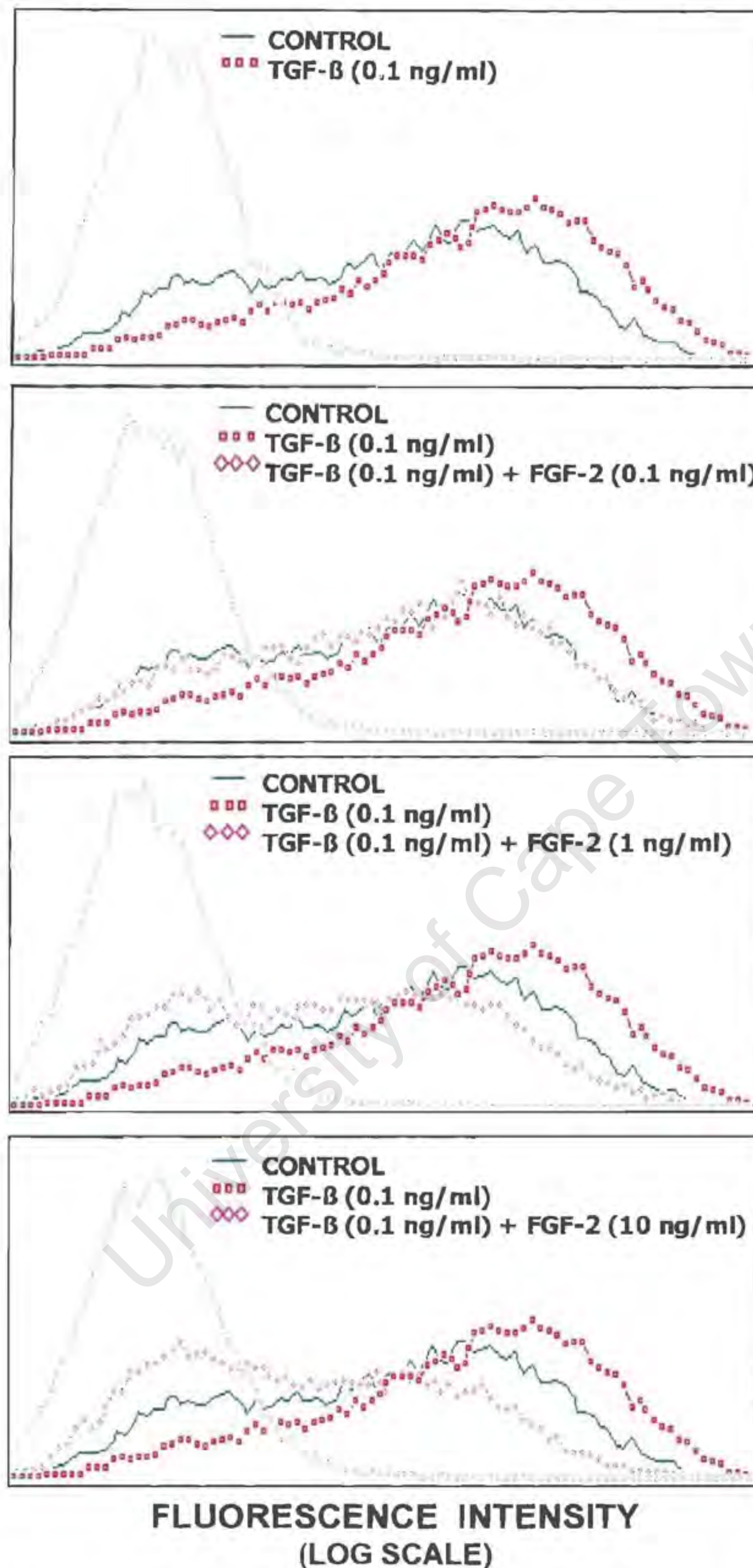

Figure 15A. FGF-2 antagonises the TGF-ß (0.1 ng/ml)-mediated increase in glycophorin A expression. K562 cells were incubated for 3 days in the absence or presence of TGF-ß at 0.1 ng/ml with the simultaneous addition of FGF-2 at 0.1, 1.0 or 10 ng/ml. Untreated cells (—); TGF-ß (▫▫▫); TGF-ß + FGF-2 (◇◇◇).

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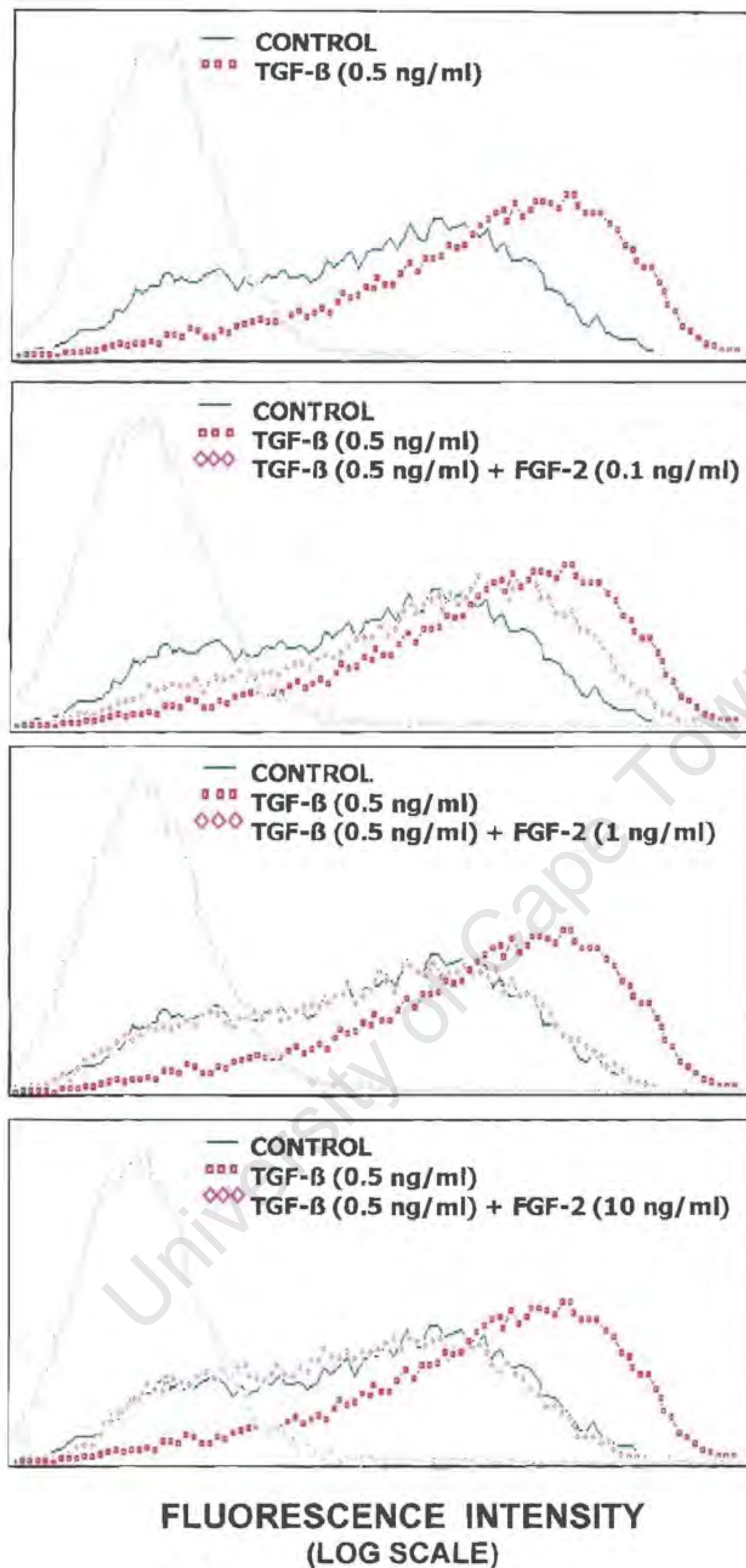

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Figure 15B. FGF-2 antagonises the TGF-ß (0.5 ng/ml)-mediated increase in glycophorin A expression. K562 cells were incubated for 3 days in the absence or presence of TGF-ß at 0.5 ng/ml with the simultaneous addition of FGF-2 at 0.1, 1.0 or 10 ng/ml. Untreated cells (—); TGF-ß (▫▫▫); TGF-ß + FGF-2 (◇◇◇).

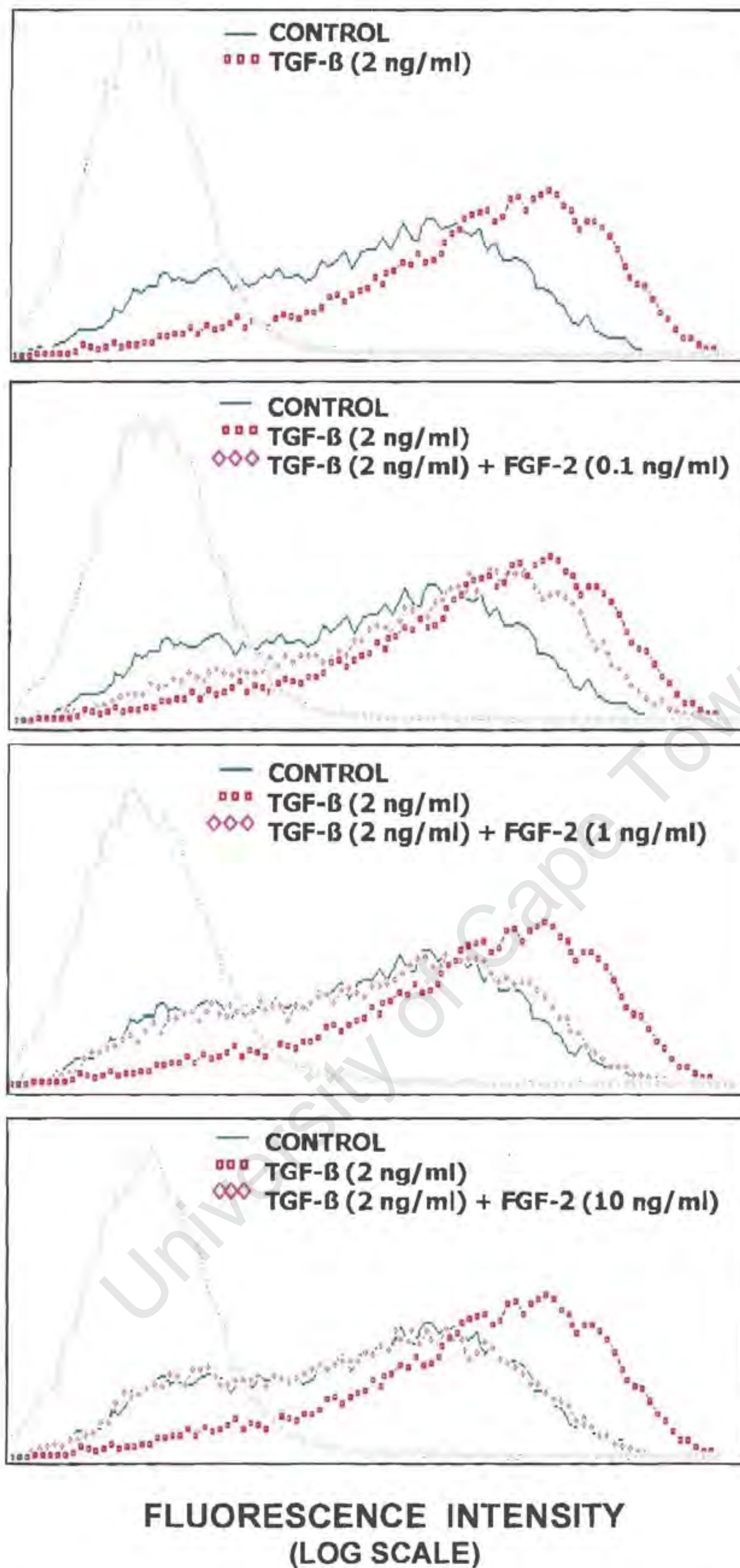

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Figure 15C. FGF-2 antagonises the TGF-ß (2 ng/ml)-mediated increase in glycophorin A expression. K562 cells were incubated for 3 days in the absence or presence of TGF-ß at 2 ng/ml with the simultaneous addition of FGF-2 at 0.1, 1.0 or 10 ng/ml. Untreated cells (—); TGF-ß (▫▫▫); TGF-ß + FGF-2 (◇◇◇).

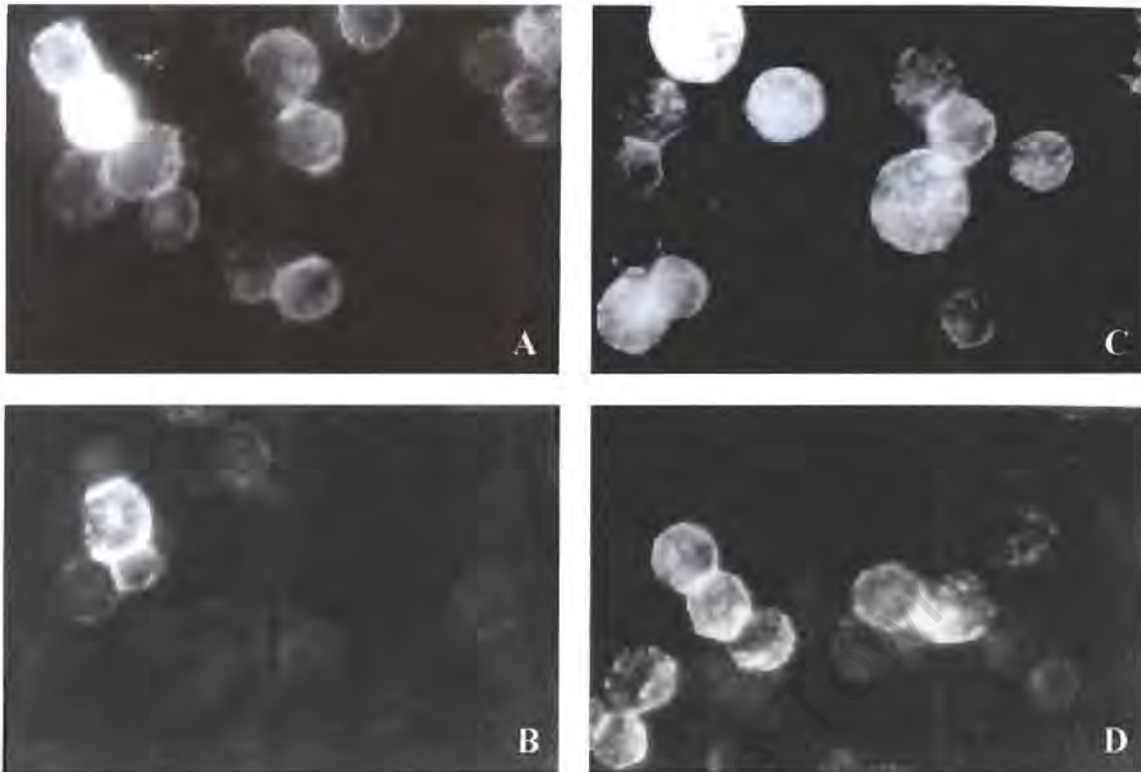

Figure 16. Indirect immunofluorescence staining for glycophorin A on K562 cells. Cells were incubated for 3 days in the absence or presence of FGF-2 and/or TGF-ß and then stained with anti-glycophorin A monoclonal antibody (8 μg/ml) followed by R-phycoerythrin (RPE)-conjugated $F(ab')_2$ goat anti-mouse immunoglobin (50 μg/ml). (A) Untreated cells; (B) FGF-2 (100 ng/ml); (C) TGF-ß (2 ng/ml); (D) FGF-2 (100 ng/ml) + TGF-ß (2 ng/ml). Magnification 475-fold.

These results show that FGF-2 antagonises the TGF-ß-mediated erythroid differentiation in K562 cells as indicated by its ability to prevent the decrease in c-kit and the increase in glycophorin A expression that accompany erythroid differentiation in this cell line. This demonstrates that FGF-2 acts to maintain a more primitive phenotype and to antagonise the effects of cytokines that enhance differentiation and indicates that increased levels of this cytokine may be relevant in promoting the excessive blast accumulation that accompanies a number of myeloproliferative and leukaemic disorders.

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Effects of FGF-2 and TGF-ß on cell growth

As FGF-2 promotes myelopoiesis in long-term bone marrow cultures (Wilson *et al.*, 1991) and stimulates the growth of primitive haematopoietic cells in synergy with other cytokines (Gabbianelli *et al.*, 1990), I determined whether FGF-2 stimulates the proliferation of K562 cells. In addition, as TGF-ß inhibits the growth of human erythroid progenitor cells (Ottmann and Pelus, 1988; Sing *et al.*, 1988) and K562 cells (Chen *et al.*, 1989a), I wished to ascertain whether FGF-2 could abrogate the inhibitory effects of TGF-ß on the growth of K562 cells. I therefore determined the effects of FGF-2 and TGF-ß on K562 cell proliferation by measuring (a) DNA synthesis (b) cell number and (c) colony growth in soft agar.

(a) **DNA synthesis**

Deoxyribonucleic acid (DNA) synthesis, as measured by the incorporation of radiolabelled thymidine, is an indicator of cell proliferation. K562 cells were cultured for 72 hours in replicates of 6, with and without TGF-ß (1 ng/ml) and in the presence of increasing concentrations of FGF-2 (0.01-100 ng/ml). Tritiated thymidine was added for the last 18 hours of culture. The cells were harvested and counted in a liquid scintillation counter to determine the incorporation of radioactive thymidine into the cells.

Surprisingly, addition of FGF-2 did not result in an increase in DNA synthesis. In fact, exposure of K562 cells to 0.01 or 0.1 ng/ml FGF-2 for 3 days had no effect on cell proliferation, while addition of 1,10 and 100 ng/ml FGF-2 resulted in a decrease in DNA synthesis of 10% ($p < 0.006$), 15% ($p < 0.003$) and 13% ($p <0$ 002) respectively, relative to that of untreated cells (Figure 17). Addition of TGF-ß alone (1 ng/ml) inhibited DNA synthesis by 13% ($p < 0.002$) (Figure 17). This inhibitory effect of TGF-ß on thymidine incorporation by K562 cells is in agreement with results noted previously (Chen *et al.*, 1989a). Interestingly, addition of FGF-2 (1, 10 or 100 ng/ml) did not abrogate the inhibitory action of TGF-ß (1 ng/ml) when these factors were added together but conversely resulted in a further decrease in DNA synthesis of 33% ($p < 0.0002$), 41% ($p < 0.000003$), 36% ($p < 0.000005$) respectively, compared to that noted in untreated cells (Figure 17). The decrease in DNA synthesis that occurred when TGF-ß and FGF-2 were

added together was greater than that expected if this were due to an additive effect: DNA synthesis was inhibited by 13% in the presence of TGF-ß alone and by a mean of 13 ± 3% in the presence of FGF-2 (1,10 or 100 ng/ml), therefore if the effects were additive one would expect a decrease of approximately 26%. However when TGF-ß and FGF-2 (1-100 ng/ml) were added together, DNA synthesis was inhibited by a mean of 37 ± 4%, indicating that TGF-ß and FGF-2 inhibit K562 cell growth in a synergistic manner.

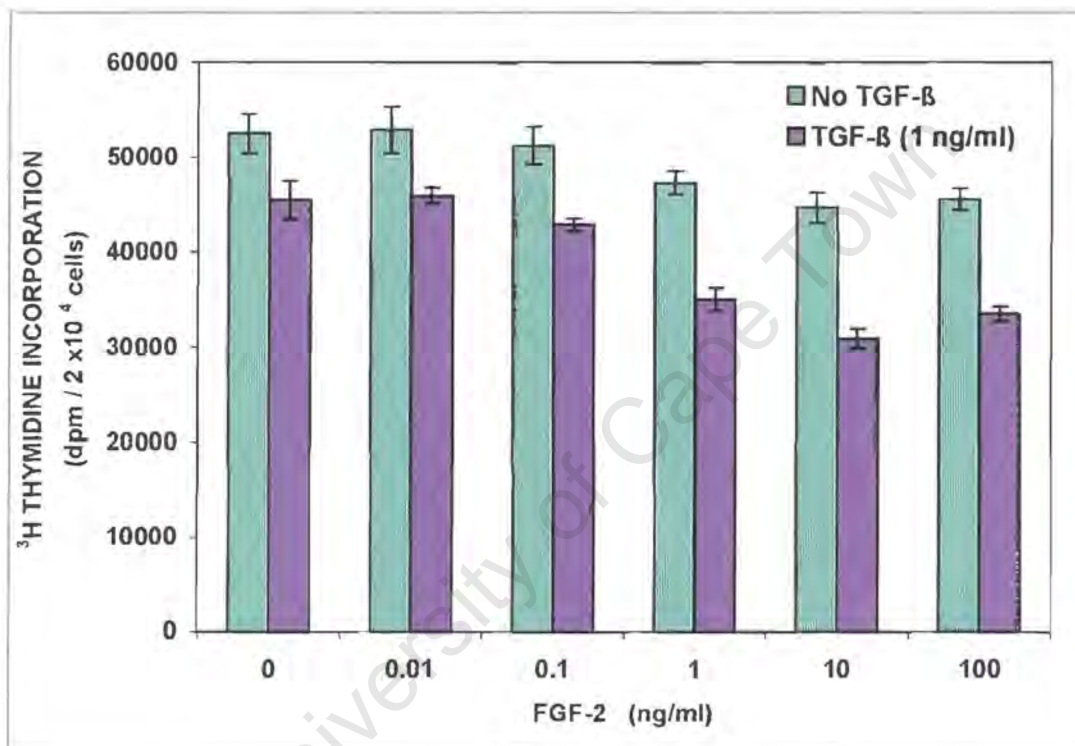

Figure 17. Effect of FGF-2 and TGF-ß on DNA synthesis in K562 cells. Two hundred microlitres of K562 cells (10^5 cells/ml) were incubated with or without TGF-ß (1 ng/ml) and varying concentrations of FGF-2 as indicated for 72 hours. Tritiated thymidine (0.2 µCi/well) was added for the last 18 hours of the culture period. The columns and error bars for each condition represent the means and standard deviations from six replicates.

(b) **Cell number**

In order to confirm the results obtained from DNA synthesis studies, I determined the effects of FGF-2 and TGF-ß on K562 cell proliferation by counting the number of viable cells in culture over a period of 8 days.

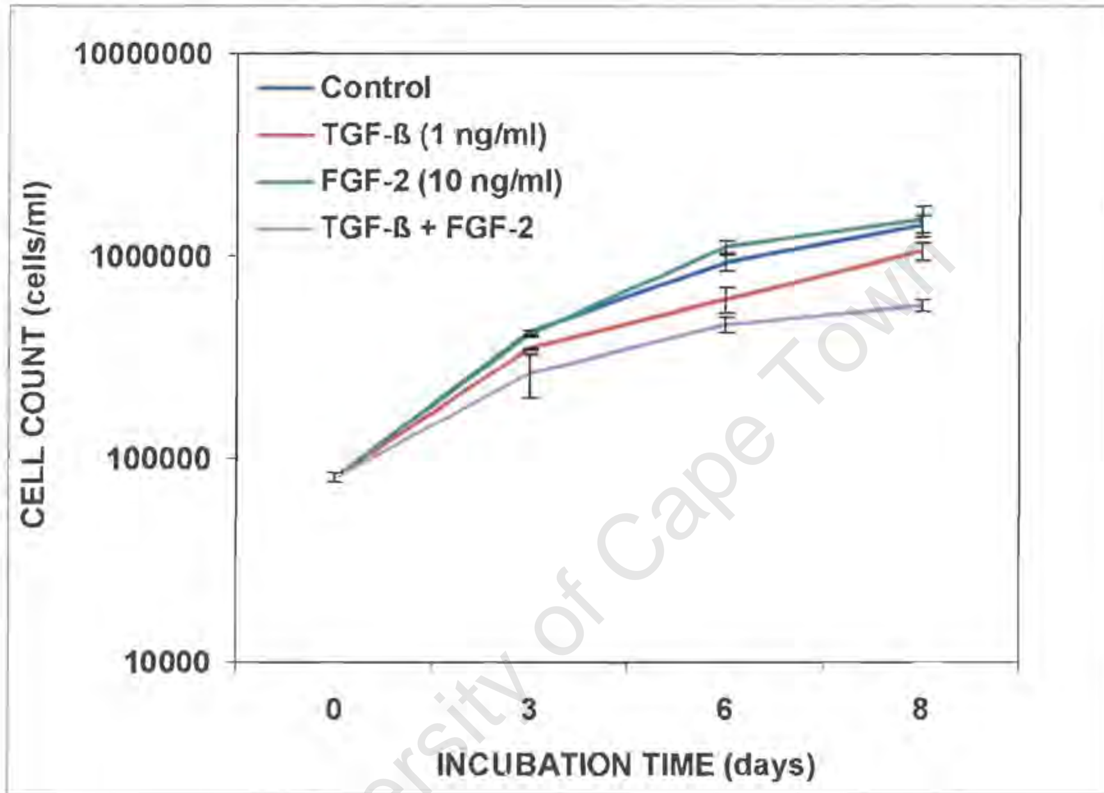

Figure 18. Effect of FGF-2 and TGF-ß on cell proliferation of K562 cells. K562 cells (8×10^4 cells/ml) were incubated with or without FGF-2 (10 ng/ml) and/or TGF-ß (1 ng/ml) for 8 days. Cell aliquots were removed at the indicated times and counted. At day 3 and day 6 medium was replaced with fresh medium and factors.

Exposure of K562 cells to FGF-2 (10 ng/ml) alone for 3 days resulted in a 3% decrease in cell number compared to untreated cells ($p < 0.05$) (Figure 18). After 6 or 8 days in the presence of FGF-2 (10 ng/ml), the number of viable cells in culture increased by 19% and 6% respectively, compared to untreated cells, although these increases were not statistically significant (NS) (Figure 18). Cells cultured in the presence of TGF-ß alone (1 ng/ml) showed an inhibition of cell growth of 17% ($p < 0.04$), 35% ($p < 0.007$) and 26%

($p < 0.04$) after 3, 6 and 8 days respectively compared to that of untreated cells, (Figure 18). Culture of K562 cells with FGF-2 (10 ng/ml) and TGF-ß (1 ng/ml) together, showed a greater decline in cell growth compared to untreated cells, inhibiting cell proliferation by 37% (NS), 51% ($p < 0.04$) and 60% ($p < 0.05$) after 3, 6 and 8 days respectively (Figure 18). The synergistic inhibition of proliferation, noted when FGF-2 and TGF-ß were added together, is similar to that noted in the experiments measuring DNA synthesis (see (a) above, Figure 17).

These results show that addition of FGF-2 alone to K562 cells had a slightly inhibitory effect on cell proliferation after 3 days in culture. This in agreement with published results (Allouche *et al.*, 1995b) and with my experiments measuring DNA synthesis 3 days after FGF-2 addition (see (a) above, Figure 17). Although there was a slight increase in cell count after 6 or 8 days culture with FGF-2, the results were not statistically significant. The inhibitory effect of TGF-ß is in agreement with the results obtained from measurement of DNA synthesis (Figure 17) and has been noted previously in K562 cells (Chen *et al.*, 1989a). Consistent with the results of my DNA synthesis studies, addition of FGF-2 and TGF-ß together resulted in a greater inhibition of growth than that observed when cells were exposed to either cytokine alone (Figure 18). In fact, after 3 days in culture with FGF-2 (10 ng/ml) and TGF-ß (1 ng/ml) the inhibition of cell proliferation was 37% whether measured by DNA synthesis or by viable cell counts, confirming the synergistic inhibition of FGF-2 and TGF-ß on cell growth in liquid culture.

(c) **Colony growth in soft agar**

To further characterize the effects of FGF-2 on the growth of K562 cells, the ability of this cytokine to influence colony formation in soft agar or agarose was determined. K562 cells were seeded in either agar or agarose in the absence or presence of FGF-2 (10 or 100 ng/ml) and the numbers and sizes of colonies were measured (Table 3, Figure 19). Results from two representative experiments show that the number of colonies per dish increased by 2.4 fold ($p < 0.01$) or 1.7 fold ($p < 0.02$) after the addition of FGF-2, as compared to control dishes (Table 3). In addition, there was a significant increase (7 fold or 3.5 fold) in the number of large colonies in those dishes cultured in the presence of

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FGF-2 as compared to those with no added growth factor ($p < 0.02$ and $p < 0.03$ respectively) (Table 3).

TABLE 3. THE EFFECT OF FGF-2 ON COLONY FORMATION BY K562 CELLS

Matrix	n	Culture time (days)	Conditions	Total colonies (mean ± SD)	Increase in total colonies (fold)	***Large colonies (mean ± SD)	Increase in ***large colonies (fold)
Agarose	4	16	Control	110 ± 52	-	5 ± 9	-
	4	16	FGF-2 *	266 ± 35	2.4 ($p < 0.01$)	35 ± 13	7 ($p < 0.02$)
Agar	3	6	Control	1322 ± 152	-	19 ±11	-
	3	6	FGF-2 **	2200 ± 274	1.7 ($p < 0.02$)	67 ± 23	3.5 ($p < 0.03$)

n = number of replicate dishes
* FGF-2 (10 ng/ml)
** FGF-2 (100 ng/ml)
***Large colonies refers to colonies comprising more than 100 cells (agarose experiment) and to more than 50 cells (agar experiment)

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These results show that the presence of FGF-2 promotes an increase in the number of colonies formed when K562 cells are cultured in agar or agarose. This in agreement with studies that show that FGF-2 stimulates colony formation by K562 cells (Komatsu *et al.*, 1995). I also found that there was a significant increase in the number of large colonies formed when FGF-2 is present, indicating that FGF-2 stimulates cell division or has an anti-apoptotic effect under these conditions. FGF-2 stimulates the size of BFU-E, CFU-E and CFU-GM colonies (Ratajczak *et al.*, 1996). It also promotes an increase in the numbers and sizes of murine megakaryocyte colonies (Han *et al.*, 1992). The stimulatory effects of FGF-2 on colony formation is in agreement with a number of previous studies (Han *et al.*, 1992; Komatsu *et al.*, 1995; Ratajczak *et al.*, 1996) but conflicts with its inhibitory effects on cell growth as determined by my results measuring DNA synthesis (Figure 17) and cell numbers (Figure 18). However, the results are consistent with the premise that FGF-2 inhibits differentiation. It is possible that in liquid culture FGF-2

maintains the cells in a primitive state, inhibiting the generation of later progenitors that have a faster proliferation rate, whereas in semi-solid media the effect of the inhibition of differentiation would be to increase colony number and size.

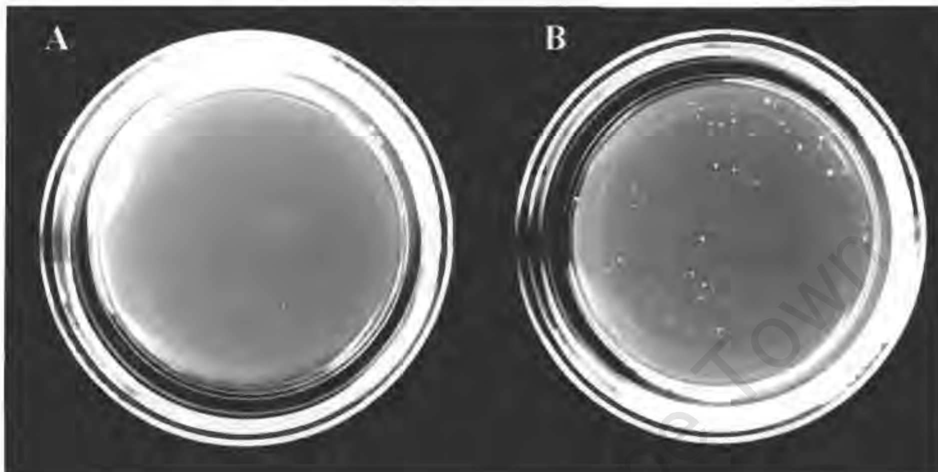

Figure 19. The effect of FGF-2 on colony formation by K562 cells. K562 cells (5×10^3 cells/35 mm dish) were seeded in RPMI medium containing 10% FCS and 0.5% agarose in the absence (A) and presence (B) of FGF-2 (10 ng/ml). After 16 days incubation, the numbers and sizes of the colonies that formed were scored. The results of one set of quadruple dishes is shown. There was a 2.4 fold increase in the number of colonies formed and a 7 fold increase in the number of colonies comprising more than 100 cells in the presence of FGF-2 (B) compared to untreated cells (A). Magnification 2 fold.

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Discussion

My results show that FGF-2 inhibits the erythroid differentiation of the erythroleukaemic cell line, K562. Two different methods were used to assess erythroid differentiation, namely staining with benzidine-peroxide for the determination of the numbers of cells containing haemoglobin and flow cytometric analysis to determine the amounts of the cell surface antigens, glycophorin A and c-kit, expressed by the cells. The results obtained with both techniques are similar and show that FGF-2 antagonises the TGF-ß-mediated induction of erythroid differentiation of K562 cells (Table 1, Table 2A and 2B; Figures 2-6, 14-16). Flow cytometry ascertained that FGF-2 alone decreased the expression of the erythroid lineage antigen, glycophorin A and increased c-kit expression (Table 1, Table 2A and 2B; Figures 8, 12 and 14). As glycophorin A expression increases, while that of c-kit decreases as cells mature along the erythroid lineage (Dai *et al.*, 1994; Ogawa *et al.*, 1994; Uoshima *et al.*, 1995), my results indicate that FGF-2 promotes a primitive cell surface phenotype in K562 cells. The inhibitory effects of FGF-2 on erythroid differentiation were dose dependent (Tables 1 and 2A, Figures 4 and 8) and time related (Figures 5 and 9). In addition, they were reversible, indicating that the phenotype of K562 cells was not permanently altered by FGF-2 (Figures 6 and 10). Other members of the FGF family, FGF-1 and FGF-4 were also able to reduce the expression of glycophorin A but did so only in the presence of heparin and even then were far less potent than FGF-2 (Figure 11). Interestingly, FGF-2 inhibited the TGF-ß-mediated erythroid differentiation of K562 more efficiently than that induced by haemin (Figure 7). As treatment of K562 cells with antisense oligonucleotides to the retinoblastoma (RB) gene inhibits TGF-ß-induced but not haemin-induced erythroid differentiation of these cells (Bergh *et al.*, 1997), it is possible that FGF-2 may modulate retinoblastoma protein (pRB) or affect other components of the erythroid differentiation signalling network.

Retinoblastoma protein is involved in erythroid differentiation and RB-/- murine embryos have defective erythropoiesis and die after approximately 14 days (Hu *et al.*, 1997; Jacks *et al.*, 1992). High levels of pRB mRNA are induced during erythroid differentiation of haematopoietic progenitor cells and antisense RB oligonucleotides inhibit this process

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(Condorelli *et al.*, 1995). Treatment of murine erythroleukaemia cells with hexamethylene bisacetamide (Richon *et al.*, 1992; Zhuo *et al.*, 1995) or of K562 cells with activin A (Sehy *et al.*, 1992) or TGF-ß (Bergh *et al.*, 1997) results in induction of erythroid differentiation, a block in the cell cycle that halts the progression of G1 to S phase and an increase in the hypophosphorylated form of pRB. Furthermore, the TGF-ß-induced erythroid differentiation of K562 cells is inhibited, in a dose related manner, by the addition of RB oligonucleotides (Bergh *et al.*, 1997), indicating the relevance of pRB in this process.

Although effects of FGF-2 on pRB function in haematopoietic cells have not been described, there are reports of effects of FGF-2 on pRB in other systems: (1) FGF-2 induces an increase in the amount of the hyperphosphorylated form of pRB in human fibroblasts (Takuwa *et al.*, 1993). (2) Human aortic endothelial cells, stimulated with FGF-2, undergo hyperphosphorylation of pRB and activation of cyclin E-cdk2, resulting in reversal of G1 arrest (Nath *et al.*, 1999). (3) Addition of FGF-2 reverses the effects of induction of G1 arrest in human colon carcinoma cells and results in hyperphosphorylation of pRB, a decrease in the levels of the cyclin dependent kinase inhibitor, p27, and the suppression of apoptosis (Liu *et al.*, 2001). As TGF-ß induces an increase in the hypophosphorylated form of pRB during erythroid differentiation of K562 cells (Bergh *et al.*, 1997), it is possible that the mechanism whereby FGF-2 inhibits TGF-ß-mediated differentiation in these cells may be through the phosphorylation of pRB.

Phosphorylation of pRB is induced by cyclin dependent kinases (cdks) (Weinberg, 1995), predominantly through the activity of cyclin D/cdk4 or cdk6 complexes (Kato *et al.*, 1993; Sherr, 1995) and progression through the cell cycle is regulated by the activity of cdks and cdk inhibitors (Sherr, 1993), such as p21 and p27. TGF-ß and FGF-2 have opposing effects on cdks and on cdk inhibitors: (1)TGF-ß reduces cdk4 expression (Hu *et al.*, 2001; Kim *et al.*, 2001b), while FGF-2 promotes cdk4 expression (Kim *et al.*, 2001b). (2) Treatment with TGF-ß increases the expression of the cdk inhibitors p21 (Ducos *et al.*, 2000) and p27 (Hu *et al.*, 2001; Jonuleit *et al.*, 1933; Kim *et al.*, 2001b), while treatment with FGF-2 reduces p27 expression (Kim *et al.*, 2001a; Liu *et al.*, 2001). There

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is therefore evidence that TGF-ß and FGF-2 have opposing effects on the activity of cdks and their inhibitors as well as on the phosphorylation of pRB. These antagonistic effects of FGF-2 and TGF-ß, together with their effects on bcl-2 expression, which will be discussed later, may contribute to the opposing effects that these two growth factors have on erythroid differentiation, cell proliferation and cell survival (Table 4).

TABLE 4. OPPOSING EFFECTS OF FGF-2 AND TGF-ß ON ERYTHROID DIFFERENTIATION, CELL PROLIFERATION AND CELL SURVIVAL.

FGF-2	TGF-ß
Inhibits erythroid differentiation of K562 cells	Induces erythroid differentiation of K562 cells
Induces *hyper*phosphorylation of pRB	Induces *hypo*phosphorylation of pRB
Increases cdk expression	Decreases cdk expression
Decreases cdk inhibitor expression	Increases cdk inhibitor expression
Increases bcl-2 expression	Decreases bcl-2 expression
Decreases apoptosis	Increases apoptosis

There are other indications that FGF-2 and TGF-ß have opposing effects on haematopoietic cells. FGF-2 counteracts the suppressive effects of TGF-ß on the growth of human myeloid progenitor cells (Gabrilove *et al.*, 1993). Furthermore, TGF-ß has been shown to be a potent inhibitor of progenitor cell cycling in long term bone marrow cultures (Hayashi *et al.*, 1989) and the addition of neutralising antibodies to TGF-ß results in an increase in both the percentage of progenitor cells in S phase (Eaves *et al.*, 1991) and in progenitor cell proliferation (Waegell *et al.*, 1994). In contrast, FGFs have been noted to enhance the progenitor content of long-term bone marrow cultures (Quito *et al.*, 1996; Wilson *et al.*, 1991). This indicates that one of the functions of FGF-2 in the haematopoietic environment may be to oppose or counterbalance the effects of TGF-ß on haematopoietic cells. The ability of FGF-2 to inhibit the TGF-ß-mediated increase in glycophorin A on an approximately molar basis (Table 1, Figures 15 A,B,C) supports this concept.

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In addition to their effects on haematopoiesis, FGF-2 and TGF-ß have also been noted to have opposing effects in other systems: (1) TGF-ß prevents the growth stimulatory effects of FGF-2 on endothelial cell proliferation and invasion (Pepper *et al.*, 1990). (2) TGF-ß inhibits the FGF-2-mediated production of plasminogen activator by endothelial cells (Saksela *et al.*, 1987; Saksela and Rifkin, 1990). (3) FGF-2 negates the inhibitory effect of TGF-ß on the synthesis of DNA in endothelial cells and decreases the expression of TGF-ß receptors by these cells (Fafeur *et al.*, 1990). (4) FGF-2 inhibits the stimulatory effect of TGF-ß on the synthesis of collagen by human fibroblasts (Ichiki *et al.*, 1997). (5) FGF-2 and TGF-ß have opposing effects on the proliferation of smooth muscle cells (Berrou *et al.*, 1996) and (6) bovine retinal endothelial capillary cells (Bensaid *et al.*, 1989). (7) TGF-ß inhibits the FGF-2-induced differentiation of neural crest cells into melanocytes (Stocker *et al.*, 1991). (8) TGF-ß stimulates, whereas FGF-2 inhibits the osteochondrogenic differentiation of periosteum derived cells (Iwasaki *et al.*, 1995). (9) Coronary vascular tube formation is enhanced by FGF-2 but inhibited by TGF-ß (Tomanek *et al.*, 2001). These studies provide ample justification for assuming that the balance between levels of FGF-2 and TGF-ß may affect both differentiation and proliferation in numerous cellular systems.

The mechanism(s) by which FGF-2 antagonises TGF-ß, were not addressed experimentally in this thesis. There are, however, a number of possibilities that could account for my results. FGF-2 and TGF-ß can oppose each other by affecting receptor expression. FGF-2 has been shown to diminish the number of TGF-ß receptors expressed by endothelial cells [Fafeur, 1990 #186] and corneal myofibroblasts [Maltseva, 2001 #694]. Another possible mechanism by which TGF-ß and FGF-2 interact may be by altering the numbers of apoptotic cells. Treatment of rat neonatal pre-oligodendroglial cells with either tumour necrosis factor-alpha (TNF-α) or TGF-ß results in increased apoptosis in culture. However if the cultures are pre-treated with FGF-2, apoptosis is induced by TNF-α but not by TGF-ß, suggesting that FGF-2 interferes with TGF-ß signalling [Yu, 2000 #691]. Even when FGF-2 and TGF-ß do not oppose each other, they may act with differing kinetics or through different pathways. For example, both FGF-2

and TGF-ß induce the expression of the cdk inhibitor, p21, in mouse fibroblasts, but with very different kinetics: induction by FGF-2 is rapid, reaching maximal levels by 2 hours, while treatment with TGF-ß results in a much slower rate of p21 induction that reaches maximal levels only after 12 hours [Kivinen, 1999 #697]. Furthermore, p21 induction by TGF-ß is dependent on Ras and mitogen-activated protein kinase/extracellular signal-related kinase (MEK) pathways, whereas regulation of p21 by FGF-2 is independent of Ras and MEK [Kivinen, 1999 #697]. Differentiation, proliferation and cell survival are interrelated processes in which both FGF-2 and TGF-ß are relevant and in which these two cytokines often play opposing roles. The mechanisms involved are likely to be complex and varied.

The ability of FGF-2 alone to reduce glycophorin A and increase c-kit expression (Tables 1, 2A and 2B; Figure 8 and 12) indicates that FGF-2 inhibits erythroid differentiation and promotes a primitive phenotype in K562 cells. FGF-2 has been shown to inhibit differentiation and promote self-renewal in a number of other cell types. It inhibits the differentiation of ES cells (Amit *et al.*, 2000), as well as that of a multipotent haematopoietic cell line derived from ES cells (Anzai *et al.*, 1999) and an EB-derived primitive erythroid cell line (Yuen *et al.*, 1998). FGF-2 also inhibits the differentiation of oligodendrocyte-type-2 astrocyte progenitor cells into oligodendrocytes (Mayer *et al.*, 1993), the osteochondrogenic differentiation of periosteum-derived cells (Iwasaki *et al.*, 1995) and the terminal differentiation of skeletal muscle cells (Clegg *et al.*, 1987; Olwin and Rapraeger, 1992), as well as promoting the self-renewal of mesenchymal cells (Tsutsumi *et al.*, 2001). Inhibition of the differentiation of haematopoietic cells may result in the accumulation of immature cells, a condition that is characteristic of leukaemias. As increased production of FGF-2 is associated with CLL, HCL, CML, AML, CMML, B-CLL as well as MDS and myelofibrosis with myeloid metaplasia (Aguayo *et al.*, 2000; Gruber *et al.*, 1999; Krejci *et al.*, 2001; Le Bousse-Kerdiles *et al.*, 1996; Menzel *et al.*, 1996), it is possible that aberrant expression of FGF-2 could contribute to the aetiology of these haematological disorders by inhibiting differentiation.

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Excessive numbers of primitive cells could also result from either the increased survival or increased proliferation of progenitor cells. There is evidence that FGF-2 promotes cell survival. FGF-2 enhances the survival of endothelial (Fuks *et al.*, 1994), epithelial (Houchen *et al.*, 1999) and haematopoietic (Gallicchio *et al.*, 1991) cells exposed to radiation-induced injury and suppresses apoptosis in a variety of cells, including endothelial (Kinoshita and Shimokado, 1999) and murine fibrosarcoma cells (Gardner and Johnson, 1996). Pre-incubation of K562 cells with FGF-2 and heparin protects the cells from haemin-induced DNA fragmentation (Allouche *et al.*, 1995b). FGF-2 also reduces the toxic effects of the antiviral drug, zidovudine, on haematopoietic cells and this reduction in toxicity is abrogated by the FGF inhibitor, protamine sulphate (Gallicchio and Hughes, 1992). In addition, FGF-2 has been implicated in the protection of CLL and HCL cells from the apoptotic effects of chemotherapeutic drugs (Gruber *et al.*, 1999; Menzel *et al.*, 1996). The ability of FGF-2 to suppress apoptosis is frequently correlated with an increase in the B cell lymphoma oncogene, bcl-2 (Vaux *et al.*, 1988) and FGF-2 inhibits apoptosis and up-regulates bcl-2 expression in B cell chronic lymphocytic leukaemia cells lines (Konig *et al.*, 1997), as well as in neuronal (Liu and Zhu, 1999), rat lymphoma (Murphy *et al.*, 2001) and 3T3 cells (Wieder *et al.*, 1997). Addition of FGF-2 to cultures of serum deprived retinal neurons increases bcl-2 expression and inhibits apoptosis by binding to FGFR-1 and activation of extracellular signal-related kinase (ERK), while addition of bcl-2 antisense oligonucleotides results in a decrease in bcl-2 levels and increased apoptosis (Desire *et al.*, 2000). In contrast, TGF-ß has been shown to reduce bcl-2 levels and induce apoptosis in K562 cells (Maccarrone *et al.*, 1996) and in multipotent haematopoietic cells (Francis *et al.*, 2000). Therefore, in addition to their effects on differentiation, the opposing effects of FGF-2 and TGF-ß on bcl-2 expression may regulate cell numbers through their differing effects on apoptosis (Table 4) and the over-expression of FGF-2 may result in a growth advantage in cells that express this oncogene.

The effects of FGF-2 on the proliferation of K562 cells differed depending on whether the cells were cultured in liquid media or in semi-solid media, such as agar. Growth was slightly inhibited in liquid media (as measured by DNA synthesis and viable cell counts:

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Figures 17 and 18: and stimulated in semi-solid media (see later). It is somewhat surprising that FGF-2 does not stimulate cell proliferation of K562 cells in liquid media as members of the FGF family have been shown to stimulate the proliferation of a variety of cells (Burgess and Maciag, 1989; Johnson and Williams, 1993; Mason, 1994; Rifkin and Moscatelli, 1989; Schweigerer *et al.*, 1987) and as FGF-2 promotes the proliferation of haematopoietic cells (Gabbianelli *et al.*, 1990; Gallicchio *et al.*, 1991; Han *et al.*, 1992; Oliver *et al.*, 1990; Wilson *et al.*, 1991) as well as increasing cdks (Kim *et al.*, 2001b) and decreasing cdk inhibitors (Kim *et al.*, 2001a; Liu *et al.*, 2001). It is possible that the inhibitory effects of FGF-2 on the growth of K562 cells, that I noted in the DNA synthesis and cell proliferation experiments, may reflect a difference in the effects of FGF-2 on leukaemic as compared to normal haematopoietic cells. A study on the effects of FGF-2 and TGF-ß on the DNA synthesis of a number of leukaemic cell lines showed that the addition of FGF-2 did not stimulate growth in 14 out of 16 cell lines, nor could it abrogate the suppressive effects of TGF-ß, indicating that in many leukaemic cell lines FGF-2 does not promote cell growth (Drexler *et al.*, 1998). A possible mechanism to explain why FGF-2 does not increase the cell growth of K562 cells may be through up-regulation of bcl-2, as over-expression of bcl-2 has been shown to result in a prolongation of the G1 phase of the cell cycle (Mazel *et al.*, 1996; O' Reilly *et al.*, 1996). Experiments using transgenic mice that over-express bcl-2 have shown that bcl-2 protects haematopoietic cells from radiation-induced cell death (Domen *et al.*, 1998). Furthermore the bone marrows of bcl-2-transgenic mice contain greater numbers of haematopoietic stem cells, that have increased plating efficiency in vitro and show enhanced reconstitution of haematopoiesis in recipient animals when compared with those that received wild type haematopoietic stem cells (Domen *et al.*, 2000). K562 cells express bcl-2 (Delia *et al.*, 1992) and although there are no reports indicating that FGF-2 induces up-regulation of bcl-2 in these cells, FGF-2 has been shown to increase bcl-2 expression in B-CLL cell lines (Konig *et al.*, 1997) and in a variety of other cells (Desire *et al.*, 2000; Liu and Zhu, 1999; Murphy *et al.*, 2001; Wieder *et al.*, 1997). Over-expression of bcl-2 is often associated with CLL and increased levels of FGF-2 in serum correlate with elevated bcl-2 levels in patients with CLL (Bairey *et al.*, 2001). It is therefore likely that FGF-2 may increase bcl-2 expression in K562 cells. It is possible that FGF-2 may

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increase the expression of bcl-2 in K562 and other leukaemic cells, resulting in diminished apoptosis without affecting cell growth. The net effect of this would be an accumulation of excessive numbers of leukaemic cells.

Addition of FGF-2 to K562 cells cultured in agar or agarose resulted in an increase in colony number and size (Table 3, Figure 19). This in agreement with other studies showing that FGF-2 promotes colony formation of haematopoietic cells (Han *et al.*, 1992; Komatsu *et al.*, 1995; Ratajczak *et al.*, 1996). However, it is in contrast to the slight inhibitory effects shown by FGF-2 on DNA synthesis and cell proliferation in liquid media. A possible explanation for this discrepancy may be the inherent differences between the effects of FGF-2 in liquid versus semi-solid medium. It is possible that when cells are cultured in a matrix such as agar or agarose, growth inducing cytokines present in FCS or produced by the cells may remain associated with the cells, rather than diffusing away, resulting in the formation of a niche containing increased concentrations of cytokines that promote proliferation. Conversely, in liquid culture the concentrations of growth factors produced by the cells may be diluted due to diffusion and may be too low to promote growth or synergize with FGF-2 to stimulate proliferation. A strong synergy between FGF-2 and SCF in promoting colony formation by K562 cells has been noted (Komatsu *et al.*, 1995). The stimulatory effects of SCF on colony formation in agar by K562 cells were potentiated by an additional 3 fold by low doses of FGF-2 (≤ 1 ng/ml), compared with the effects of SCF alone (Komatsu *et al.*, 1995). A possible mechanism for this synergy may be through the ability of FGF-2 to increase the expression of c-kit (Tables 2A and 2B; Figure 12), making the cells more sensitive to its ligand, SCF. In addition to SCF, FGF-2 also synergizes with granulocyte-colony-stimulating factor (G-CSF), GM-CSF and IL-3 to promote K562 colony growth in agar (Komatsu *et al.*, 1995), suggesting that synergistic interaction between FGF-2 and these haematopoietic cytokines may promote leukaemic cell proliferation in vivo.

Normal primitive haematopoietic cells express c-kit and FGF-2 also synergizes with SCF to promote colony growth of these cells (Gabrilove *et al.*, 1994). FGF-2 acts in synergy with a number of other cytokines such as IL-3, GM-CSF, erythropoietin and

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megakaryocyte colony stimulating factor (Bruno *et al.*, 1993; Gabbianelli *et al.*, 1990; Gallicchio *et al.*, 1991) to stimulate haematopoietic colony growth, indicating that FGF-2 is able to potentiate the effects of a number of cytokines. Bone marrow stromal cells are the main source of haematopoietic cytokines, although they are also produced by haematopoietic cells themselves. FGF-2 is a mitogen for bone marrow stromal cells (Oliver *et al.*, 1990) and it also increases the secretion of macrophage colony-stimulating factor by these cells (Abboud and Pinzani, 1991). There is therefore evidence that FGF-2 is able to stimulate haematopoiesis directly (Table 3, Figure 19) (Burger *et al.*, 2002b; Komatsu *et al.*, 1995), and also indirectly by synergising with other cytokines. In addition, it may promote proliferation of progenitor cells by increasing the secretion of other stimulatory cytokines or by stimulating proliferation of bone marrow stromal cells that interact with progenitor cells to promote progenitor proliferation. Moreover, FGF-2 inhibits apoptosis and antagonises differentiation, which would also influence progenitor cell numbers. From these examples it can be seen that there are numerous ways by which FGF-2 could modulate the haematopoietic environment and affect cell differentiation and proliferation.

Haematopoiesis is a complex process involving interactions between haematopoietic stem/progenitor cells and bone marrow stromal cells. This process is controlled by numerous cytokines that act as positive and negative regulators. Under normal conditions, there is a balance between the concentrations of cytokines that promote stem cell self-renewal and those that induce differentiation into lineage committed cells. There is evidence that specific cell lineages are localized to micro-compartments of the bone marrow (Jacobsen and Osmond, 1990; Ploemacher *et al.*, 1984; Schofield, 1983). Experiments by Lowry *et al* have indicated that SCF, produced at a high local concentration could act as an "anchor factor" in a haematopoietic niche, enabling haematopoietic stem cells to respond to very low "subliminal" cytokine concentrations (Lowry *et al.*, 1992). Gradients of cytokines produced in the marrow microenvironments (Quesenberry *et al.*, 1991) could also dictate the lineage commitment of stem cells. It is therefore possible that FGF-2 produced in niches by stromal or haematopoietic cells (Brunner *et al.*, 1993) and present in high local concentrations, might favour the

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proliferation of a "stem cell" phenotype and antagonise the influence of cytokines that promote differentiation. It is also possible that aberrant FGF-2 production might result in excessive proliferation of stem cells, resulting in leukaemias or myeloproliferative disorders.

Summary

I have shown that FGF-2 promotes a primitive phenotype in K562 cells and counteracts the inducing effects of TGF-ß on the erythroid differentiation of these cells. These are novel findings, as is my observation of the ability of FGF-2 to increase the expression of c-kit. These effects of FGF-2 on haematopoietic cells confirm the relevance of FGF-2 in haematopoiesis and may be of clinical significance. Normal haematopoiesis is a tightly regulated process under the influence of growth factors, whereby haematopoietic stem cells either undergo self-renewal, thereby remaining immature cells with multilineage repopulating potential or differentiate into more mature cells. Factors that either stimulate the growth, prevent the apoptosis, or inhibit the differentiation of haematopoietic stem cells may result in the accumulation of immature cells in the bone marrow. An imbalance in the production of opposing cytokines, such as FGF-2 and TGF-ß, may therefore interfere with normal haematopoiesis and excessive FGF-2 may result in an increase in cells with a primitive phenotype (Figure 20).

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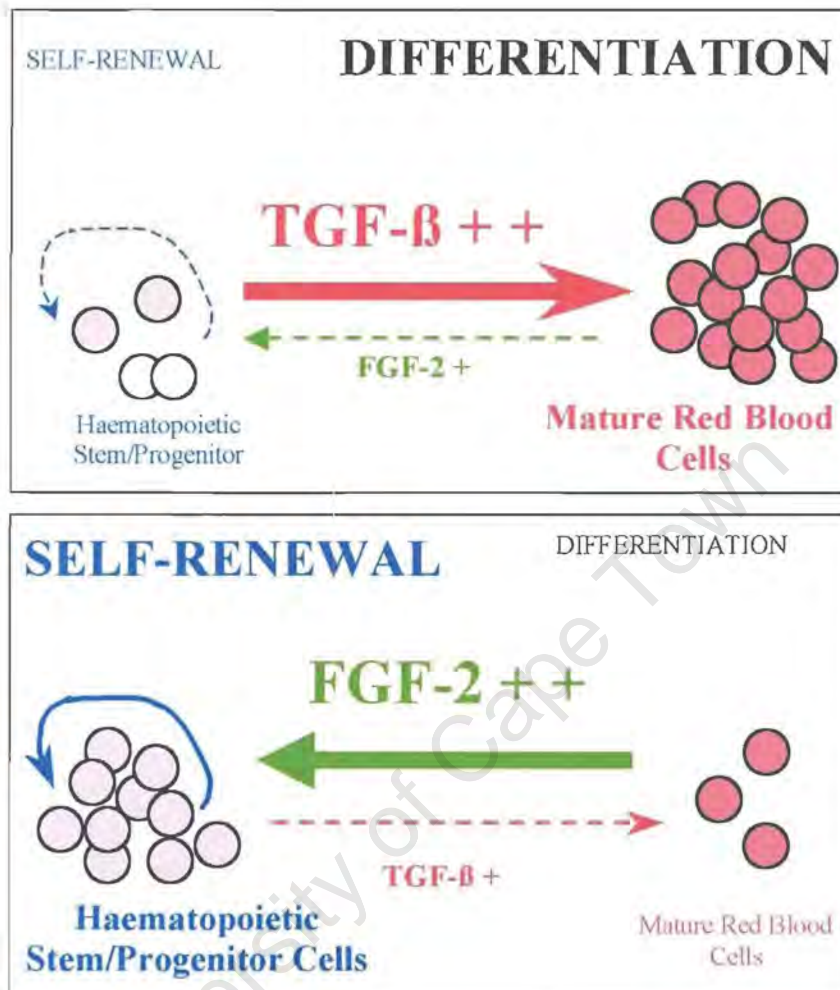

Figure 20. A hypothesis of the action of FGF-2 on haematopoietic cells. Haematopoiesis may be modulated by the activity of cytokines with opposing actions. In the case of erythropoiesis, factors such as TGF-ß that promote erythroid differentiation to mature red blood cells are opposed by cytokines such as FGF-2 that inhibit differentiation, resulting in accumulation of cells with a more primitive phenotype.

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It is possible that FGF-2 may promote the abnormal accumulation of progenitor cells that is associated with leukaemias and myeloproliferative diseases. FGF-2 is produced by K562 erythroleukaemic cells (Allouche *et al.*, 1995b), CLL (Menzel *et al.*, 1996) and HCL cells (Gruber *et al.*, 1999). Plasma levels of FGF-2 are increased in patients with B-CLL, CML, AML, CMML and myelodysplastic syndromes, compared to normal individuals (Aguayo *et al.*, 2000; Krejci *et al.*, 2001). Increased expression of both FGF-2 and the receptors, FGFR-1 and FGFR-2, is found in CD34+ cells from patients who have myelofibrosis with myeloid metaplasia as compared to normal donors (Le Bousse-Kerdiles *et al.*, 1996). Significantly elevated levels off FGF-2 were found in cells from patients with high-risk and intermediate-risk CLL compared to the levels in cells from those with low-risk disease (Menzel *et al.*, 1996). It is therefore possible that enhanced production of FGF-2 by leukaemic cells or by adjacent stromal cells could contribute to the neoplastic phenotype of leukaemic cells by opposing the effects of negative regulators or cytokines that induce differentiation.

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Suggestions for further studies

There are a number of experiments that could be performed to clarify the mechanism by which FGF-2 influences the behaviour of haematopoietic cells. As mentioned in my 'discussion' section, FGF-2 has effects on pRB in some types of cells. It would be interesting to determine the effects of FGF-2 on pRB and other proteins and transcription factors in normal haematopoietic as well as in K562 cells. It is likely that the effects of FGF-2 on haematopoietic cell growth, survival and differentiation involve the modulation of a variety of relevant processes. Elucidation of the mechanisms involved may advance our understanding of the aetiology of a number of haematological disorders.

(1) Does FGF-2 inhibit erythroid differentiation of haematopoietic cells through changes in pRB phosphorylation?

Pre-treatment of K562 cells with antisense RB oligonucleotides inhibits the ability of TGF-ß to induce the synthesis of haemoglobin (Bergh *et al.*, 1997). I have shown that FGF-2 also inhibits the TGF-ß-mediated induction of the erythroid differentiation of K562 cells. It would therefore be interesting to determine whether this inhibitory effect of FGF-2 is mediated through changes in the phosphorylation of pRB. *In vitro* studies show that differentiation of normal human haematopoietic or leukaemic cells correlates with the expression of the hypophosphorylated or dephosphorylated form of pRB (Chen *et al.*, 1989b; Furukawa *et al.*, 1990). Although effects of FGF-2 on pRB have not been described in haematopoietic cells, FGF-2 has been shown to increase the expression of the hyperphosphorylated form of pRB in a number of non-haematopoietic cells (Liu *et al.*, 2001; Nath *et al.*, 1999; Takuwa *et al.*, 1993). In order to determine the effects of FGF-2 on the phosphorylation of pRB in haematopoietic cells, K562 cells could be cultured in the presence and absence of TGF-ß and increasing concentrations of FGF-2 for various lengths of time and the expression of the hyper- and hypo-phosphorylated forms of pRB could be assessed by Western blot analysis. Briefly, cell lysates would be immunoprecipitated with a monoclonal anti-pRB antibody and the immunoprecipitates subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After electroblotting, the membrane would be treated with anti-pRB and the bands

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visualized with horseradish peroxidase-coupled goat anti-mouse antibody. Monoclonal anti-p53 antibody could be used as a control. Comparison of the expression of hyper- and hypophsporylated pRB in cells treated with FGF-2, TGF-ß, FGF-2 plus TGF-ß as well as untreated K562 cells would determine whether FGF-2 affects the phosphorylation state of pRB and whether FGF-2 and TGF-ß have opposing effects on pRB. Aliquots of the cell cultures taken before cell lysis and assayed for haemoglobin content or the expression of glycophorin A would determine whether inhibition of erythroid differentiation by FGF-2 correlates with the phosphorylation state of pRB.

(2) Does FGF-2 affect the expression of p210 bcr-abl ?

CML is associated with the presence of the hybrid gene, bcr-abl (Rowley, 1973), that encodes a chimeric protein, p210 bcr-abl, with enhanced protein tyrosine kinase activity. Cells of the leukaemic cell line K562, originally derived from a patient with CML in blast crisis, also express this oncogene (Grosveld *et al.*, 1986). Treatment of K562 cells with bcr-abl antisense oligonucleotides, indicate that bcr-abl expression has little effect on the rate of cell division but has considerable effects on the inhibition of apoptosis, possibly through bcl-2 expression (Rowley *et al.*, 1996). In addition, K562 cells can be induced to undergo erythroid differentiation by down-regulation of p210 bcr-abl expression through treatment with specific antisense oligonucleotides (Szczylik *et al.*, 1991), specific tyrosine kinase inhibitors (Anafi *et al.*, 1993) or the non-selective tryrosine kinase inhibitor, herbimycin A (Honma *et al.*, 1989). As I have shown that FGF-2 inhibits the erythroid differentiation of K562 cells, it would be interesting to determine whether treatment with FGF-2 results in an increase in p210 bcr-abl in these cells. K562 cells could be cultured in the absence and presence of increasing concentrations of FGF-2 and the expression of p210 bcr-abl determined in these cultures by immunoblotting. Briefly, cell lysates would be added to protein A-Sepharose beads coupled to anti-abl antibody 8E9 or K12. The bead-bound immune complexes would be collected by centrifugation, washed and subjected to SDS-PAGE. After transferring to a nitrocellulose membrane and incubating with anti-abl antibody, the bcr-abl bands would be visualized by enhanced chemiluminescence (LaMontagne *et al.*, 1998). In addition, as it has been suggested that the anti-apoptotic effect of bcr-abl may be mediated through up-regulation of bcl-2

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(Rowley *et al.*, 1996), the expression of bcl-2 could be determined in cell aliquots from the same cultures. Glycophorin A and bcl-2 expression could be determined simultaneously using double immunofluorescence staining and flow cytometry. The cells would first be stained with PE-conjugated monoclonal anti-glycophorin A antibody, permeablized and then incubated with a primary goat polyclonal antibody against bcl-2 followed by a FITC-conjugated mouse anti-goat secondary antibody. The results of this and the previous experiment would ascertain whether FGF-2 increases the expression of bcr-abl and bcl-2, while decreasing that of glycophorin A, indicating whether the gene products of these oncogenes are up-regulated by FGF-2 and are associated with the inhibitory effects of FGF-2 on erythroid differentiation.

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(3) Does FGF-2 increase the expression of GATA-2 in haematopoietic cells?

The transcription factor, GATA-2, is essential for the earliest stages of haematopoiesis as revealed by the death of GATA-2 knockout mice at E10-11, due to their failure to generate sufficient numbers of primitive erythroid cells (Tsai *et al.*, 1994). Over-expression of GATA-2 also has effects on primary haematopoietic stem cells and inhibits haematopoiesis (Persons *et al.*, 1999). Recently, it has been shown that over-expression of GATA-2 in K562 cells and human progenitor erythroid cells results in inhibition of erythroid differentiation as measured by benzidine staining and expression of glycophorin A (Ikonomi *et al.*, 2000). As I have demonstrated that FGF-2 inhibits erythroid differentiation of K562 cells, it would be of interest to determine whether treatment of K562 cells with FGF-2 increases GATA-2 expression. K562 cells could be cultured in the absence or presence of increasing concentrations of FGF-2 for different periods of time and the expression of GATA-2 determined by real-time quantitative RT-PCR. Briefly, polyadenylated RNA would be prepared from cell aliquots using a mRNA purification kit, after which cDNA would be synthesized using GATA-2 specific primers and SybrGreenI dye. S16 RNA would act as a control. Flow cytometric determination of the expression of glycophorin A would be performed on aliquots of the same cell cultures in order to ascertain whether there is a correlation between the effects of FGF-2 on glycophorin A and GATA-2 expression. The results would indicate whether treatment

with FGF-2 increases the level of GATA-2 while, at the same time, decreasing glycophorin A expression.

(4) Does FGF-2 affect SCL expression?

The stem cell leukaemia (SCL) gene encodes a helix-loop-helix transcription factor that is required for the development of all haematological lineages (Porcher *et al.*, 1996; Robb *et al.*, 1996; Shivdasani *et al.*, 1995). SCL-/- EBs do not express any haematopoietic specific genes, whereas expression of endothelial genes is normal, indicating that SCL is not required for endothelial differentiation of the haemangioblast but that it is an absolute requirement for haemangioblast commitment to the haematopoietic lineages (Elefanty *et al.*, 1997). SCL is expressed by multipotent haematopoietic progenitors before lineage commitment, as well as by erythroid, mast and megakaryocytic cells. Bipotential and committed erythroid precursors express high levels of SCL RNA that diminish with subsequent maturation to proerythroblasts and normoblasts (Hoang *et al.*, 1996). Experiments using the erythroleukaemic cell line, K562 show that the introduction of an antisense SCL construct into these cells resulted in spontaneous erythroid differentiation (Green *et al.*, 1991). In addition, those K562 cells that contained antisense SCL exhibited reduced clonogenicity and smaller colonies in agar than untreated cells and, on recloning, showed profound suppression of self-renewal (Green *et al.*, 1991), indicating that SCL expression is relevant for cell proliferation and self-renewal in these cells. As I have shown that treatment of K562 cells with FGF-2 promotes colony formation in soft agar and inhibits erythroid differentiation, it would be interesting to determine whether FGF-2 increases SCL expression. Furthermore, as the addition of SCF increases SCL protein levels in erythroid progenitors (Miller *et al.*, 1994) and since there is a strong synergy between SCF and FGF-2 in promoting colony formation in K562 cell cultures (Komatsu *et al.*, 1995), it would be interesting to determine whether FGF-2 acts in synergy with SCF to increase SCL levels in these cells. K562 cells could be incubated in the presence and absence of FGF-2, with and without the simultaneous addition of SCF, and the expression of SCL determined by Northern blot analysis. The results would indicate whether FGF-2 alone or in combination with SCF, modulates the expression of SCL and

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may lead to a better understanding of the mechanism whereby FGF-2 inhibits erythroid differentiation.

(5) Does FGF-2 affect integrin expression?

Adhesive interactions between the haematopoietic environment and haematopoietic stem/progenitor cells are critical for normal haematopoiesis (Simmons *et al.*, 1997). Erythroid progenitor cells express $\alpha4\beta1$ and $\alpha5\beta1$ integrins (Rosemblatt *et al.*, 1991) and treatment of normal mice with anti-$\alpha4\beta1$ antibody completely blocks erythropoiesis (Hamamura *et al.*, 1996). Fibronectin (FN) is highly expressed throughout the haematopoietic micro-environment (Yoder and Williams, 1995) and interactions between erythroid cells and FN are essential for erythropoiesis (Hamamura *et al.*, 1996). The presence of SCF increases the adherence of haematopoietic cells to FN (Kovach *et al.*, 1995; Levesque *et al.*, 1995) and erythroid cell survival, proliferation and differentiation are dependent on c-kit and integrin-mediated signalling (Broudy, 1997). As I have shown that FGF-2 inhibits erythroid differentiation and up-regulates the expression of c-kit on K562 cells, and as the integrin expression of K562 cells is modulated by erythroid differentiation (Jarvinen *et al.*, 1993), it would be interesting to determine the effects of FGF-2 on the expression of $\alpha4\beta1$ and $\alpha5\beta1$ integrins on these cells. The expression of $\alpha4\beta1$ and $\alpha5\beta1$ could be determined, in conjunction with that of glycophorin A and c-kit, on cells that had been cultured for 3 or 4 days in the presence or absence of varying concentrations of FGF-2 and/or SCF. The results would indicate whether FGF-2 modulates integrin expression as well as differentiation of K562 cells. CML progenitor cells adhere poorly to bone marrow stroma or FN and decreased $\alpha5\beta1$ integrin affinity is thought to contribute to the abnormal circulation and proliferation of malignant progenitor cells in CML (Lundell *et al.*, 1996). As the plasma levels of FGF-2 are elevated in patients with CML, compared to normal individuals (Aguayo *et al.*, 2000), it is possible that FGF-2 may contribute to the aetiology of this disease through modulation of integrin expression.

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CHAPTER 3

ISOLATION AND CHARACTERIZATION OF A POPULATION OF HAEMATOPOIETIC CELLS THAT EXPRESSES FGFR-1 AND THAT CONTAINS ENDOTHELIAL PRECURSOR CELLS

Introduction

The CD34+ population, obtained from bone marrow, cord blood and peripheral blood contains haematopoietic stem cells (Civin *et al.*, 1996; de Wynter *et al.*, 1999; Herbein *et al.*, 1994; Terstappen *et al.*, 1991). A small number of CD34+ haematopoietic progenitor cells express receptors for fibroblast growth factors (Berardi *et al.*, 1995; Burger *et al.*, 1998; Burger *et al.*, 1999; Le Bousse-Kerdiles *et al.*, 1996; Testa *et al.*, 1996) and fibroblast growth factor-2 (FGF-2) has both synergistic and direct effects on progenitor cell proliferation (Allouche, 1995a; Bruno *et al.*, 1993; Gabbianelli *et al.*, 1990; Gabrilove *et al.*, 1994; Wilson *et al.*, 1991). FGF-2 also promotes the self-renewal (Anzai *et al.*, 1999) and proliferation (Yuen *et al.*, 1998) of primitive haematopoietic cell lines, indicating a possible relevance for FGF-2 in early haematopoiesis. FGF-2 is also a potent angiogenic cytokine, stimulating endothelial proliferation (Schweigerer *et al.*, 1987) and inducing angiogenesis (Brown *et al.*, 1996; Joseph-Silverstein and Rifkin, 1987; Pepper *et al.*, 1992). FGF-2 is therefore a cytokine that effects both haematopoietic and endothelial cells.

In addition to haematopoietic stem cells, the CD34+ population contains endothelial stem/precursor cells (Asahara *et al.*, 1997; Kocher *et al.*, 2001; Nieda *et al.*, 1997; Peichev *et al.*, 2000; Schatteman *et al.*, 2000; Shi *et al.*, 1998; Takahashi *et al.*, 1999). Human CD34+ cells isolated from peripheral blood are incorporated into the endothelium of ischaemic blood vessels of recipient animals (Asahara *et al.*, 1997; Takahashi *et al.*, 1999). CD34+ cells also promote neovascularization of ischaemic myocardium, improve cardiac function (Kocher *et al.*, 2001) and accelerate the restoration of blood flow in diabetic mice undergoing neovascularization (Schatteman *et al.*, 2000). CD34+ cells purified from

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umbilical blood give rise to von Willebrand factor (vWF) expressing endothelial cells *in vitro* (Nieda *et al.*, 1997) and bone marrow derived CD34+ cells are found lining the surfaces of vascular prostheses in sex-mismatched canine grafts (Shi *et al.*, 1998). Furthermore, endothelial cells of donor origin have been cultured from the peripheral blood of human subjects who were given sex-mismatched allogeneic bone marrow transplants, thereby confirming the existence of bone marrow derived circulating progenitor endothelial cells (Ikpeazu *et al.*, 2000; Lin *et al.*, 2000). The sub-population of CD34+ cells that contains the endothelial stem cell population remains to be identified.

The observation that the CD34+ population contains both haematopoietic and endothelial stem/precursor cells is not surprising considering the close association that exists between haematopoietic and endothelial cells during ontogeny. The existence of haemangioblasts that give rise to both lineages has long been hypothesized (Murray, 1932; Sabin, 1920; Wagner, 1980) and recent research has indicated that these cells exist in the embryo. Cells capable of differentiating into both haematopoietic and endothelial cells have been identified within the Tek+ fraction of the aorta-gonad-mesonephros (AGM) region of the embryo (Hamaguchi *et al.*, 1999). To date however, cells with haemangioblast properties have not been isolated in post-natal life. Blast colony forming cells (BL-CFCs), derived from embryonic stem (ES) cells also give rise to both endothelial and haematopoietic cells (Choi *et al.*, 1998). These BL-CFCs express FGFR-1 and are stimulated to proliferate by FGF-2 (Faloon *et al.*, 2000) indicating that FGFR-1 and FGF-2 may be relevant in haemangioblast cell biology.

ES cells are undifferentiated cells that can be induced to form ectoderm, endoderm and mesoderm (Czyz and Wobus, 2001; Martin, 1981). These pluripotent cells express receptors for FGF (Schuldiner *et al.*, 2000) and vascular endothelial growth factor (VEGF) (Vittet *et al.*, 1996) (Figure 21A). FGFs are inducers of mesoderm (Amaya *et al.*, 1991; Amaya *et al.*, 1993; Slack, 1994; Slack *et al.*, 1987) and both FGF-2 (Hrabe de Angelis and Kirchner, 1993; MacNicol *et al.*, 1993) and FGFR-1 (Ciruna and Rossant, 2001; Paterno *et al.*, 2000) are involved in this process. Haemangioblasts are derived from mesoderm and although a precise phenotype has not yet been described for these cells,

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they express the following molecules (Figure 21B): (1) FGFR-1 (Faloon *et al.*, 2000), a tyrosine kinase receptor for FGFs that is also expressed by both haematopoietic (Berardi *et al.*, 1995; Bikfalvi *et al.*, 1992; Burger *et al.*, 1998; Burger *et al.*, 1999; Le Bousse-Kerdiles *et al.*, 1996; Testa *et al.*, 1996) and endothelial (Cross *et al.*, 2000; Kanda *et al.*, 1999; Tsou and Isik, 2001) cells. (2) Vascular endothelial growth factor receptor-2 (VEGFR-2/Flk-1/KDR) (Faloon *et al.*, 2000), a tyrosine kinase receptor expressed by primitive haematopoietic stem cells (Ziegler *et al.*, 1999) as well as endothelial cells (Vittet *et al.*, 1996). (3) Tunica interna endothelial kinase (Tek/Tie-2) (Hamaguchi *et al.*, 1999), a tyrosine kinase receptor that is essential for the development of haematopoiesis in the AGM region of the embryo (Takakura *et al.*, 1998) and also for angiogenesis and vascular remodeling (Dumont *et al.*, 1994; Sato *et al.*, 1995). (4) SCL (Faloon *et al.*, 2000), a transcription factor encoded by the stem cell leukaemia gene that is required for the development of all haematopoietic lineages (Porcher *et al.*, 1996; Robb *et al.*, 1996; Shivdasani *et al.*, 1995). (5) Podocalyxin-like protein1 (PCLP1) (Hara *et al.*, 1999) a highly glycosylated protein, similar to CD34, that is expressed by CD45- cells of the AGM region that are able to produce both endothelial and haematopoietic cells. Haemangioblasts have been shown to express FGFR-1 and to proliferate in the presence of FGF-2 (Faloon *et al.*, 2000) and they differentiate into both endothelial and haematopoietic progenitor cells (Choi *et al.*, 1998; Hamaguchi *et al.*, 1999) (Figure 21). FGF-2 and FGFR-1 are therefore relevant in endothelial and haematopoietic ontogeny.

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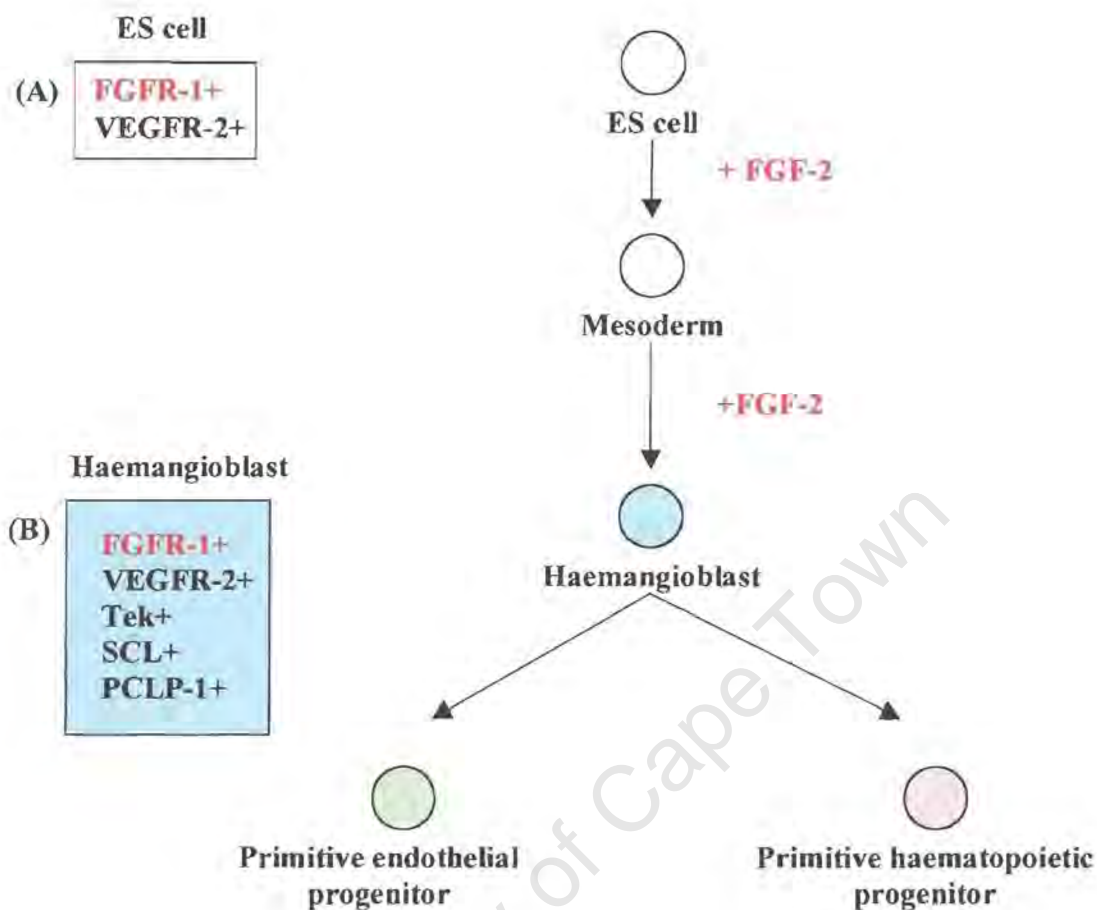

Figure 21. Antigens expressed by cells during the differentiation of ES cells into endothelial and haematopoietic progenitor cells.

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The antigens expressed by embryonic endothelial progenitor cells and mature endothelial cells are very similar: embryonic endothelial progenitor cells express CD34, VEGFR-2, platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31), Tek, vascular endothelial cadherin (VE-cadherin) and Tie 1 (Tie), (Nishikawa *et al.*, 1998b; Vittet *et al.*, 1996) (Figure 22A). In addition to CD34, VEGFR-2, CD31, Tek, VE-cadherin and Tie, mature endothelial cells also express the endothelial specific antigens, P1H12 (Lin *et al.*, 2000; Solovey *et al.*, 1997) and vWF (Zanetta *et al.*, 2000) (Figure 22B). The phenotype of post-natal circulating endothelial progenitor cells is currently a matter of intensive research. The cells obtained from human post-natal bone marrow, cord blood or peripheral blood that differentiate into endothelial cells express a number of antigens found on mature

endothelial cells i.e. CD34 (Asahara *et al.*, 1997; Boyer *et al.*, 2000; Murohara *et al.*, 2000; Nieda *et al.*, 1997; Shi *et al.*, 1998) and VEFGR-2 (Asahara *et al.*, 1997; Peichev *et al.*, 2000) (Figure 22C). However these cells also express CD133 (AC133) (Gehling *et al.*, 2000) (Figure 22C), a cell surface antigen expressed by primitive haematopoietic cells (Buhring *et al.*, 1999; de Wynter *et al.*, 1998; Majka *et al.*, 2000; Matsumoto *et al.*, 2000; Miraglia *et al.*, 1997) that is not expressed on mature endothelial cells (Peichev *et al.*, 2000; Yin *et al.*, 1997) (Figure 22B).

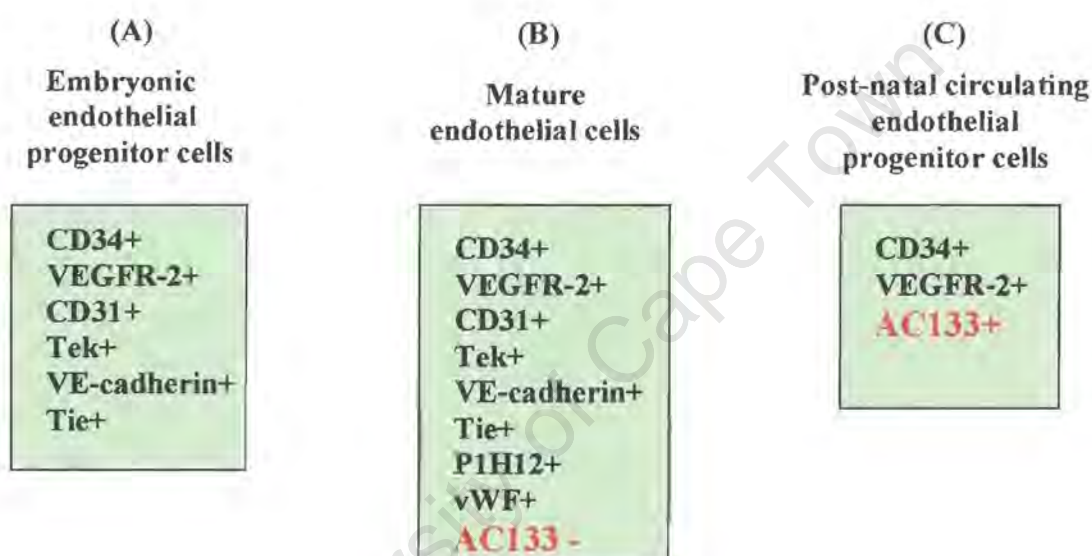

Figure 22. Antigens expressed by embryonic endothelial progenitor cells (A), mature endothelial cells (B) and circulating endothelial progenitor cells (C).

This chapter of my thesis describes the isolation of a subpopulation of CD34+ cells from bone marrow, cord blood and mobilized peripheral blood that expresses FGFR-1. FGFRs are expressed on mature endothelial cells (Cross *et al.*, 2000; Kanda *et al.*, 1999; Tsou and Isik, 2001) and on CD34+ haematopoietic progenitor cells (Berardi *et al.*, 1995; Burger *et al.*, 1998; Burger *et al.*, 1999; Le Bousse-Kerdiles *et al.*, 1996; Testa *et al.*, 1996). As the CD34+ haematopoietic population has been shown to contain endothelial precursor cells (Asahara *et al.*, 1997; Kocher *et al.*, 2001; Nieda *et al.*, 1997; Peichev *et al.*, 2000; Schatteman *et al.*, 2000; Shi *et al.*, 1998; Takahashi *et al.*, 1999), I decided to

characterize the CD34+FGFR+ population and determine whether endothelial progenitors are found among these cells. In order to determine the phenotype of the CD34+FGFR+ cells, I examined these cells for the expression of those antigens expressed by endothelial cells, as well as for cell surface antigens consistent with the phenotype of primitive haematopoietic progenitor cells (Table 5).

TABLE 5. ANTIGENS EXPRESSED BY PRIMITIVE HAEMATOPOIETIC AND/OR ENDOTHELIAL CELLS

	Antigen	Cells expressing indicated antigen
A	AC133	Primitive haematopoietic cells
	Thy-1	Primitive haematopoietic cells
	c-kit	Primitive haematopoietic cells
B	CD31	Haematopoietic and endothelial cells
	Tie	Haematopoietic and endothelial cells
	Tek	Haematopoietic and endothelial cells
	KDR	Haematopoietic and endothelial cells
C	P1H12	Endothelial cells
	VE-cadherin	Endothelial cells

I found that the CD34+FGFR+ population expresses antigens found on primitive haematopoietic cells, such as AC133 (Miraglia *et al.*, 1997), Thy-1 (Murray *et al.*, 1995) and c-kit (Katayama *et al.*, 1993) (Table 5A). The CD34+FGFR+ cells are selectively enriched for the expression of 2 antigens specific for endothelial cells, namely P1H12 (Lin *et al.*, 2000; Solovey *et al.*, 1997) and VE-cadherin (Ali *et al.*, 1997; Breier *et al.*, 1996; Gory *et al.*, 1999; Matsuyoshi *et al.*, 1997; Nishikawa *et al.*, 1998b) (Table 5C), as well as for other antigens, such as CD31, Tie, Tek and KDR that are expressed by endothelial cells (Hanahan, 1997; Sato *et al.*, 1995; Vittet *et al.*, 1996; Yong *et al.*, 1998). However as CD34, CD31, Tie, Tek and KDR are expressed by both haematopoietic and endothelial cells (Civin *et al.*, 1996; Fina *et al.*, 1990; Hanahan, 1997; Hashiyama *et al.*, 1996; Sato *et al.*, 1998; Sato *et al.*, 1995; Vittet *et al.*, 1996; Watt *et al.*, 1995; Yong *et al.*, 1998; Ziegler *et al.*, 1999) (Table 5B), it is important to establish whether circulating CD34+ cells that give rise to endothelium are endothelial precursors or merely mature endothelial cells derived from vessel walls. For this reason I examined the CD34+FGFR+ population for

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expression of AC133. AC133 is a primitive haematopoietic cell surface marker (Buhring *et al.*, 1999; de Wynter *et al.*, 1998; Majka *et al.*, 2000; Matsumoto *et al.*, 2000; Miraglia *et al.*, 1997) that is not expressed on differentiated endothelial cells (Peichev *et al.*, 2000; Yin *et al.*, 1997) and therefore co-expression of AC133 with endothelial markers can be used to distinguish endothelial precursors from mature endothelial cells. I found that the majority of CD34+FGFR+ cells expressed AC133, indicating that the CD34+FGFR+ cells are of a primitive haematopoietic or endothelial nature rather than of a mature endothelial nature. As human CD34+AC133+ cells isolated from mobilized peripheral blood differentiate into endothelial cells *in vitro* (Gehling *et al.*, 2000), I examined the ability of CD34+FGFR+ cells to give rise to endothelial cells in culture. Isolated CD34+FGFR+ cells grew slowly in culture, were stimulated by FGF-2 and vascular endothelial growth factor (VEGF) and gave rise to VE-cadherin and vWF expressing cells indicating that the CD34+FGFR+ population contains endothelial stem/progenitor cells. This chapter describes the phenotype of a subset of CD34+ cells, present in bone marrow, cord blood and peripheral blood, that expresses FGFR-1 and gives rise to endothelial cells *in vitro*. This is a novel finding that may have considerable clinical significance.

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Materials and Methods

Cell preparation

Fresh or frozen cells from bone marrow, cord blood or cytokine mobilized peripheral blood were obtained from donors after informed consent. Bone marrow or cord blood samples were diluted with 4 volumes RPMI 1640 medium containing 10% foetal calf serum (FCS). Mononuclear cells were separated on a Histopaque-1077 density gradient (Sigma Diagnostics, St. Louis, MO) and washed twice with PBS-citrate (PBS containing 13.6 mmol/L sodium citrate, 1 mmol/L adenosine and 2 mmol/L theophylline). Leukapheresis samples were not usually subjected to Histopaque-1077 density gradient centrifugation and were washed twice with PBS-citrate. PBS-citrate washed cells (from all sources) were filtered through 40 μm nylon cell strainers (Becton-Dickinson, New Jersey), resuspended in 2 ml PBS-citrate and overlaid on a 3 ml PBS-citrate/10% bovine serum albumin (BSA) cushion and subsequently centrifuged for 10 minutes at 200 g at room temperature, to remove platelets (Thoma *et al.*, 1994). If necessary, deoxyribonuclease 1 (DNAse) (Sigma Chemicals, St. Louis, MO) was added to increase recovery of viable cells from frozen samples.

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Two methods were used to purify primitive cells:

(1) Samples were enriched for CD34+ cells by a positive selection technique using magnetic beads coated with an antibody to CD34. For this immunomagnetic separation I routinely used CD34 MACS microbeads, magnetic columns and the MiniMACS system (Miltenyi Biotec, Auburn, CA) in preference to the Dynal CD34 Progenitor Cell selection system (Dynal A.S., Norway). The reason for this is that the CD34+ cells selected using MACS beads can be assayed by FACS without having to remove the beads as the beads are very small (50 nm in diameter) and do not affect the light scatter of the cells or interfere with performance of the flow cytometer, whereas Dynal beads which are much larger (4.5μm in diameter) need to be detached from the selected cells before FACS analysis.

(2) Samples were enriched for CD34+ cells by lineage depletion. Lineage depletion is a negative selection technique in which mature lineage positive cells are coated with

antibodies and magnetically removed, resulting in the isolation of a primitive population of cells enriched for CD34+ progenitor cells. This method has some advantages over positive selection methods, as the isolated primitive population is not coated with antibodies, allowing subsequent immunomagnetic selection and characterization of other surface antigens using monoclonal antibodies. In addition, dead cells, being sticky are removed non-specifically by the immunomagnetic beads during the lineage depletion process. Therefore in some experiments, I depleted the sample of mature cells expressing antigens found on T cells, B cells, monocytes, macrophages, megakaryocytes, platelets, natural killer cells, granulocytes and erythrocytes. This was done using a cocktail of lineage specific antibodies (CD2, CD3, CD14, CD16, CD19, CD24, CD41, CD56, CD66b and glycophorin A) (Bhatia *et al.*, 1998) obtained from StemCell (StemCell Technologies Inc., Vancouver, Canada), goat anti-mouse magnetic beads (Miltenyi) or sheep anti-mouse magnetic beads (Dynal) and immunomagnetic separation.

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Antibodies and Reagents

The following antibodies (all antibodies were mouse anti-human monoclonal antibodies unless otherwise stated) and reagents were used for immunofluorescent staining: Thy-1-FITC and Thy-1-PE (Immunotech, France); goat anti-mouse-FITC, goat-anti-rabbit-FITC, mouse IgG, goat IgG, rabbit IgG and deoxyribonuclease 1 (DNAse) (Sigma Chemicals, St. Louis, MO); CD31-FITC (Caltag Laboratories, Burlingame, CA); CD31-PE (Becton-Dickinson, San Jose, CA); biotinylated goat-anti-human KDR (R&D Systems Inc., Minneapolis, MN); goat anti-mouse IgG2a-PE (Southern Biotechnology Associates, Inc., Birmingham, Alabama); human IgG (Massachusetts Public Health Biologic Laboratories, Boston, MA); AC133-PE (Miltenyi Biotec, Auburn, CA). CD34-APC, mouse IgG-APC and mouse IgG Biotin were obtained either from Caltag or Becton-Dickinson. The following antibodies were purchased from Dako (Dako A/S, Glostrup, Denmark): CD34-FITC, CD34-PE, CD34-PE-CY5, c-kit-PE, CD38-FITC, mouse IgG-FITC, mouse IgG-PE, mouse IgG-PE-Cy5, mouse IgG2a, rabbit anti-human von Willebrand factor, streptavidin-PE and goat anti-mouse-PE. Additional antibodies used were: unconjugated VE-cadherin (Hec 1.2) and VE-cadherin-FITC (Dr. W. A. Muller, Cornell University, New York, NY); Tie-FITC and biotinylated Tek (Dr. T. Suda, Kumamoto University, Kumamoto, Japan);

P1H12-FITC and biotinylated P1H12 (Dr. R. P. Hebbel, University of Minnesota, Minneapolis, MN); FGFR-1 antibody (Dr. W. L. McKeehan, Texas A and M University, Houston, TX) and QED Bioscience Inc. (San Diego, CA). Anti-FGFR-1-FITC was either purchased from QED or prepared in our laboratory. Anti-FGFR-1 was conjugated to allophycocyanin (APC) in our laboratory. Conjugation of the antibody to APC was performed using a Phycolink conjugation kit purchased from Prozyme (San Leandro, CA). Recombinant human FGF-2 was obtained from Scios Nova (Mountain View, CA), VEGF and stem cell factor (SCF) from PeproTech Inc (Rocky Hill, NJ).

Purification of FGFR-1 antibody

2F12 hybridoma cells, producing a monoclonal antibody against FGFR-1, were a generous gift from Dr W.L. McKeehan (Texas A & M University, Houston, TX, USA). The cells were cultured and expanded in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FCS in tissue culture plastic dishes at 37°C with 5% CO_2, in a humidified atmosphere. Once sufficient numbers of cells had been generated, the cells were pooled and washed three times in PBS in order to remove medium and serum. Balb/c mice that had been primed with pristane (0.5 ml/mouse) 14 days previously, were injected intraperitoneally with 5 x 10^6 viable 2F12 cells/mouse. After 7-9 days, ascitic fluid was collected from the mice into tubes containing the protease inhibitor, aprotinin (20 µg/ml), in order to prevent degradation of the antibody by proteases present in the ascitic fluid. Ascites was centrifuged at ± 700g for 10 minutes, the supernatant was collected, re-centrifuged and filter sterilized through a syringe tip Dynagard filter (Microgon Inc., Laguna Hills, CA, USA) and either stored frozen at -80°C or immediately added to a Protein G Sepharose column for isolation of antibody to FGFR-1. Immunoglobulins were isolated from ascitic fluid by column chromatography, using a Protein G Sepharose 4 Fast Flow column (Pharmacia LKB Biotechnology, Uppsala, Sweden). The purified FGFR-1 antibody was eluted from the column with 0.1M glycine pH 2.7 and concentrated on an Amicon B15 Macrosolute concentrator (Amicon Inc., MA, USA).

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Fluorescent labelling of FGFR-1 antibody

I prepared two fluorochrome conjugates of the FGFR-1 antibody in order to allow greater flexibility in the choice of antigens that could be examined in conjunction with FGFR-1 and CD34. Antibody to CD34 was commercially available as fluorescein isothiocyanate (FITC), phycoerythrin (PE), cyanine Cy5 dye coupled to PE (PE-CY5) and allophycocyanine (APC) conjugates, with emission spectra in FACS channels FL 1, FL 2, FL 3 and FL 4 respectively. I could therefore determine CD34 expression in combination with antibodies conjugated to fluorochromes with different emission spectra. I had access to antibodies against VE-cadherin, P1H12 and Tie as FITC conjugates only and to the antibody to AC133 only as a PE conjugate. It was therefore helpful to have the antibodies to FGFR-1 conjugated to FITC and to APC, in order to determine the combinations of antigens present on CD34+FGFR+ cells.

FITC conjugation of the FGFR-1 antibody was performed as described on the web site http://www.drmr.com/abcon/FITC1.html. Briefly, purified antibody to FGFR-1 was dialysed against 500mM sodium carbonate buffer pH 9.5 and the protein concentration, measured on a spectrophotometer at 280nm, was adjusted to 2 mg/ml A solution of FITC isomer I (Sigma Chemicals, St. Louis, MO) (10 mg/ml) was prepared in anhydrous dimethylsulfoxide (DMSO) and this was added to the antibody solution such that the final concentration of FITC was 80 µg/mg antibody. The reagents were mixed immediately in a small tube, wrapped in foil and incubated with rotation for 1 hour at room temperature. The FITC conjugated FGFR-1 antibody was dialysed against 10mM Tris buffer pH8.2, containing 150mM NaCl as well as the preservative pentachlorophenol (Sigma Chemicals, St. Louis, MO) (5 mg/ml).

A Phycolink APC conjugation kit (Prozyme, San Leandro, CA, USA) was used for the conjugation of APC to the FGFR-1 antibody. The details of the method are available from the website http://www.prozyme.com/phycolink/pj25k.html. Briefly, purified FGFR-1 immunoglobulin (IgG) was dialysed against 50mM sodium phosphate buffer pH 7.0, containing 1mM EDTA, and adjusted to 4 mg/ml protein. FGFR-1 IgG was treated with dithiothreitol (DTT) (20 µM/ml IgG solution) for 30 minutes at room temperature in order

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to expose free sulphydryl groups, after which excess DTT was removed by gel filtration. The kit provided sufficient succinimidyl 4-{N-maleimidomethyl}-cyclohexane-1-carboxylate-activated cross-linked allophycocyanin (SMCC-xlAPC) for conjugation of 1 mg of IgG. The cross-linked APC was covalently coupled to the FGFR-1 antibody by reaction of the maleimide groups with the free sulfhydryl groups on the IgG. This reaction was allowed to continue for 60 minutes at room temperature at which time N-ethylmaleimide (NEM) (34 µg/mg IgG) was added for 20 minutes at room temperature in order to block remaining free sulfhydryl groups. The APC conjugated FGFR-1 antibody was then dialysed against 10mM Tris buffer pH8.2, containing 150mM NaCl and the preservative pentachlorophenol (Sigma Chemicals, St. Louis, MO) (5 mg/ml) in order to remove free APC.

As free fluorochrome was still present after dialysis, the anti-FGFR-1 FITC and anti-FGFR-1 APC conjugates were further purified on Sephadex G200 (Pharmacia LKB Biotechnology, Uppsala, Sweden). After column chromatography to remove unreacted fluorochromes, the fluorescent conjugated FGFR-1 antibodies were concentrated on an Amicon B15 Macrosolute concentrator (Amicon Inc., MA, USA) to a concentration of approximately 1 mg/ml, dialysed against storage buffer (10mMTris, 150mM EDTA, 5 µg/ml pentachlorophenol pH 8.2) and stored in the dark at 4°C. The specificity and activity of the unconjugated, FITC- and APC- conjugated antibodies were determined using 3T3 cells transfected with FGFR-1 (3T3 *Flg* 26 cells) (Dionne *et al.*, 1990). Cells transfected with a neomycin-resistant gene (3T3 Neo cells) were used as a control.

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Cell Staining and Flow Cytometry

MACS-selected or Dynal-selected CD34+ cells or Lin- cells were resuspended in fluorescence-activated cell sorting (FACS) buffer which comprised PBS supplemented with bovine serum albumin (0.1%), sodium azide (0.01%) and aprotinin (20 µg/ml). Fc receptors and non-specific binding of immunoglobulins to cell surfaces were blocked with human IgG. The cells were also blocked with mouse IgG when monoclonal antibodies were being used or with goat IgG in the case of goat polyclonal antibodies. In the majority of experiments, staining of FGFR+ cells was performed using directly labelled FGFR-1-

APC, although FGFR-1-FITC was used in some cases. In a few experiments unconjugated FGFR-1 was used together with either goat anti-mouse-PE or goat anti-mouse IgG2a-PE as secondary antibodies.

As the Class II CD34 antibody, QBEND10, was used for the CD34 selection procedure, a fluorochrome-conjugated antibody to a different CD34 epitope was required for staining and FACS analysis of the cells. The Class III CD34 antibody, HPCA-2, directly conjugated to either FITC, PE, PE-CY5 or APC was therefore employed for cell staining; the choice of fluorochrome being dependent on the combination of FITC-labelled or APC-labelled FGFR-1 antibody with the other fluorescent antibodies being used. I had access to the following conjugated antibodies: antibodies to CD38, Tie, VE-cadherin and P1H12 were FITC conjugated, whereas anti-AC133 and anti-c-kit were PE conjugated. Antibodies to Thy-1 and CD31 were available as both FITC and PE conjugates. Antibodies to KDR and to Tek were only available as biotinylated conjugates and staining with these antibodies required an additional staining step, using streptavidin-PE. Fluorochrome conjugated isotype matched antibodies were used as control antibodies in every instance for the detection of non-specific binding. Cells were incubated with appropriate antibodies for 30 minutes on ice, using FACS buffer to wash the cells between staining steps. Cell aliquots were also prepared with combinations of fluorochrome labelled antibodies and isotype matched control antibodies in order to set the electronic compensation of the flow cytometer i.e., to compensate for the breakthrough of fluorescence from one fluorochrome into the analysis window of another, as described at the following web site: http://www.drmr.com/compensation. Briefly, for four colour FACS analysis, four tubes containing cell aliquots were prepared, each with a single fluorescent antibody expected to be expressed on the cells, such as CD34, as well as the appropriate isotype matched control antibodies (Table 6). Compensation was set by aligning the median fluorescences of the positive and control cell populations for each fluorochrome. Depending on the number of cells available, an optional fifth tube, containing cells stained with all four fluorochromes was prepared and used after performing single colour compensation, to confirm that the settings were optimal.

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TABLE 6. CELL STAINING SCHEME FOR SETTING THE COMPENSATION FOR FOUR COLOUR FACS ANALYSIS.

Tube	FITC	PE	PE-Cy5	APC
1	**VE-cadherin FITC**	Mouse IgG PE	Mouse IgG PE-Cy5	Mouse IgG APC
2	Mouse IgG FITC	**AC133 PE**	Mouse IgG PE-Cy5	Mouse IgG APC
3	Mouse IgG FITC	Mouse IgG PE	**CD34 PE-Cy5**	Mouse IgG APC
4	Mouse IgG FITC	Mouse IgG PE	Mouse IgG PE-Cy5	**FGFR-1 APC**

Cells were analysed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA), equipped with an argon laser to excite FITC, PE and RPE-CY5 fluorochromes and a helium-neon diode, with time delay adjusted according to manufacturer's recommendations, for excitation of APC. 30,000 to 150,000 CD34+ or Lin- selected cells were analysed using CellQuest software (Becton-Dickinson, San Jose, CA). The dye 7-aminoactinomycin D (7-AAD), at a final concentration of 1 µg/ml, was added 5 minutes before flow cytometry in order to identify dead cells. This was done to exclude analysis of non-viable 7AAD-containing cells (Philpott *et al.*, 1996) and ensure the viability of all CD34+FGFR+ cells analysed.

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Culture of CD34+FGFR+ cells

(a) Growth of haematopoietic colonies in methylcellulose

Low density mononuclear cells, enriched for CD34+ cells by immunomagnetic selection, were incubated with antibodies to CD34 and FGFR-1 and sorted on an Epics Elite Cell Sorter (Beckman Coulter Inc., Fullerton, CA) into CD34+FGFR+, CD34+ FGFR- and unfractionated CD34+ populations*, using antibodies to different epitopes of the CD34 molecule for selection and staining i.e., the CD34 antibody QBEND/10 was used for the selection process, while the CD34 antibody HPCA-2 conjugated to a fluorochrome was used for fluorescent labelling of the cells and subsequent FACS sorting. The unfractionated CD34+ population was passed through the sorter in order to have control cells for these experiments that had been subjected to the same stresses as the sorted FGFR+ and FGFR- populations. The three different populations were seeded into MethoCult GF+H4435

medium (StemCell Technologies Inc., Vancouver, BC, Canada), containing methylcellulose (0.9%), FCS (30%), BSA (1%), 2-mercaptoethanol (10^{-4} M), L-glutamine (2mM), recombinant human (rh) SCF (50 ng/ml), rh erythropoietin (3 unit/ml), rh GM-CSF (20 ng/ml), rh IL-3 (20 ng/ml), rh IL-6 (20 ng/ml) and rh G-CSF (20 ng/ml) in Iscove's modified Dulbecco's medium (IMDM). As cell viability is adversely affected by the sorting procedure, the viability of the cells was determined by trypan blue exclusion so that equal numbers of viable cells were seeded for each cell population. Viable cells from the different populations were seeded (300-1000 cells/well) into 12 or 24 well tissue culture plates, in duplicate or triplicate, in the absence or presence of FGF-2 (100 ng/ml) and incubated at 37°C with 5% CO_2 in a humidified atmosphere. After 2 weeks, colonies were scored according to their morphology and expression of haemoglobin and divided into burst forming unit-erythroid (BFU-E), colony forming unit-erythroid (CFU-E), colony forming unit-granulocyte erythrocyte macrophage megakaryocyte (CFU-GEMM), colony forming unit-macrophage (CFU-M) and colony forming unit-granulocyte macrophage (CFU-GM) colonies.

(b) Cell growth in the presence of endothelial growth factors

CD34+ enriched cells were selected by MACS selection and sorted into CD34+FGFR+, CD34+ FGFR- and unfractionated CD34+ populations* and seeded into fibronectin-coated (50μg/ml) wells of 96 well plates at 1000 cells/well in long term culture medium (Myelocult H5100 from StemCell Technologies Inc., Vancouver, Canada) containing 12.5% horse serum and 12.5% FCS, in the presence or absence of FGF-2 (10 ng/ml) or FGF-2 and VEGF (10 ng/ml). Cells were fed twice weekly by replacing half the medium with fresh growth factor-containing medium. Growth was assessed by determining the number of viable cells/well present after various times in culture.

(c) Growth on OP9 feeder layers and determination of VE-cadherin expression

Sorted CD34+FGFR+ and CD34+FGFR- cells* were also seeded on OP9 feeder layers (1000 viable cells/well in a 96 well tissue culture plate) in long term culture medium containing FGF-2 (10 ng/ml) and VEGF (10 ng/ml). The cells were cultured on OP9 feeder layers as OP9 cells support the growth of endothelial progenitors in the presence of factors

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known to promote endothelial proliferation (Hirashima *et al.*, 1999). Cells were fed twice weekly by replacing half the medium with fresh growth factor-containing medium. After 12 weeks, cells were fixed with 1% paraformaldehyde in PBS and examined for expression of VE-cadherin using antibodies to VE-cadherin (40 μg/ml) and secondary goat anti-mouse FITC conjugated antibodies.

* At the time that this work was being undertaken, I did not have access to a FACS machine with cell sorting capabilities and therefore the experiments involving the use of FACS sorted cells were performed in New York in conjunction with Dr E L Wilson and Ms S Coetzee.

(d) Growth on fibronectin/gelatin slides and determination of vWF expression

Purified Lin-CD34+FGFR+ cells were examined for evidence of endothelial maturation after culture in endothelial growth medium (EGM-2 medium from Clonetics Inc., San Diego, CA) supplemented with SCF (50 ng/ml). The cells were cultured in fibronectin-coated (50 μg/ml in 0.1% gelatin) wells (Lab-Tek chamber slides from Miles Scientific, Naperville, IL) and incubated in a humidified incubator in the presence of 5% CO_2. Half the medium was removed and replaced with fresh medium once weekly. After 8 weeks, the cells were fixed with 1% paraformaldehyde in PBS, permeabilized with ice-cold acetone/methanol (50/50) and examined for expression of vWF, using rabbit anti-human vWF antibodies (28 μg/ml) and goat anti-rabbit-FITC antibodies (11 μg/ml). Control wells were incubated with goat anti-rabbit-FITC antibodies without previous treatment with rabbit anti-human vWF antibody. Human umbilical vein endothelial cells (HUVECs) were used as a positive control for vWF expression. In another experiment, Lin-CD34+FGFR+ cells were cultured in fibronectin-coated plates, as described above, for 5 weeks and the ability of the cultured cells to incorporate acetylated low density lipoprotein (ac-LDL) was determined. This was done to further confirm the endothelial nature of the cells. As vWF is expressed by endothelial cells and megakaryocytes only (Zanetta *et al.*, 2000) and as ac-LDL is only taken up by endothelial cells and macrophages (Voyta *et al.*, 1984), double positive cells would be endothelial in nature. Incorporation of ac-LDL was determined by incubating the cells with ac-LDL labelled with the fluorescent probe 1, 1'- dioctadecyl-3, 3,

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3', 3'-tetramethyl-indocarbocyanine perchlorate (DiI) (Molecular Probes Inc., Eugene, OR, USA). Cultured cells were incubated with DiI-ac-LDL (10 µg/ml) for 4 hours at 37° C (Voyta *et al.*, 1984). The cells were washed twice with PBS to remove free DiI-ac-LDL, fixed with 3% paraformaldehyde in PBS and subsequently permeabilized with ice-cold acetone/methanol (50/50) and treated with vWF antibodies as described above. The secondary antibody used in this instance was Alexa Fluor labelled goat anti-rabbit IgG (Molecular Probes Inc., Eugene, OR, USA) (20 µg/ml). Dual fluorescence (Alexa Fluor 488 = green fluoresence, DiI = red fluorescence) indicating simultaneous vWF expression and ac-LDL incorporation, would confirm the endothelial nature of the cells.

Statistical analysis

Data are expressed as the mean ± SD (standard deviation). When comparing the data from two populations, paired, two-tailed Student *t* tests were used to determine levels of significance. A p value of < 0.05 was considered statistically significant.

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Results

The phenotype of the CD34+FGFR+ population

In order to characterize the CD34+FGFR+ population, purified CD34+ cells (Figure 23A) were examined for evidence of FGFR-1 expression. CD34+ cells were incubated with 7AAD to ensure that only viable cells that excluded 7AAD (R2, Figure 23B) were analysed for expression of FGFR-1 and other antigens. CD34+ cells from bone marrow, umbilical cord blood and mobilized peripheral blood contained a distinct population of FGFR-1 expressing cells (R3, Figure 23C). These cells comprised 4.5 ± 2.1% of the total CD34+ population (Table 7). The CD34+FGFR+ cells have low forward (FSC) and side scatter (SSC) properties (Figure 23D), indicating that they are small cells with little granularity. As significant numbers of CD34+FGFR+ cells are found in a region of the FSC/SSC dot plot that is often excluded from FACS analysis (as this region contains dead cells), the use of a dye such as 7AAD or propidium iodide is important to ensure the analysis of viable cells.

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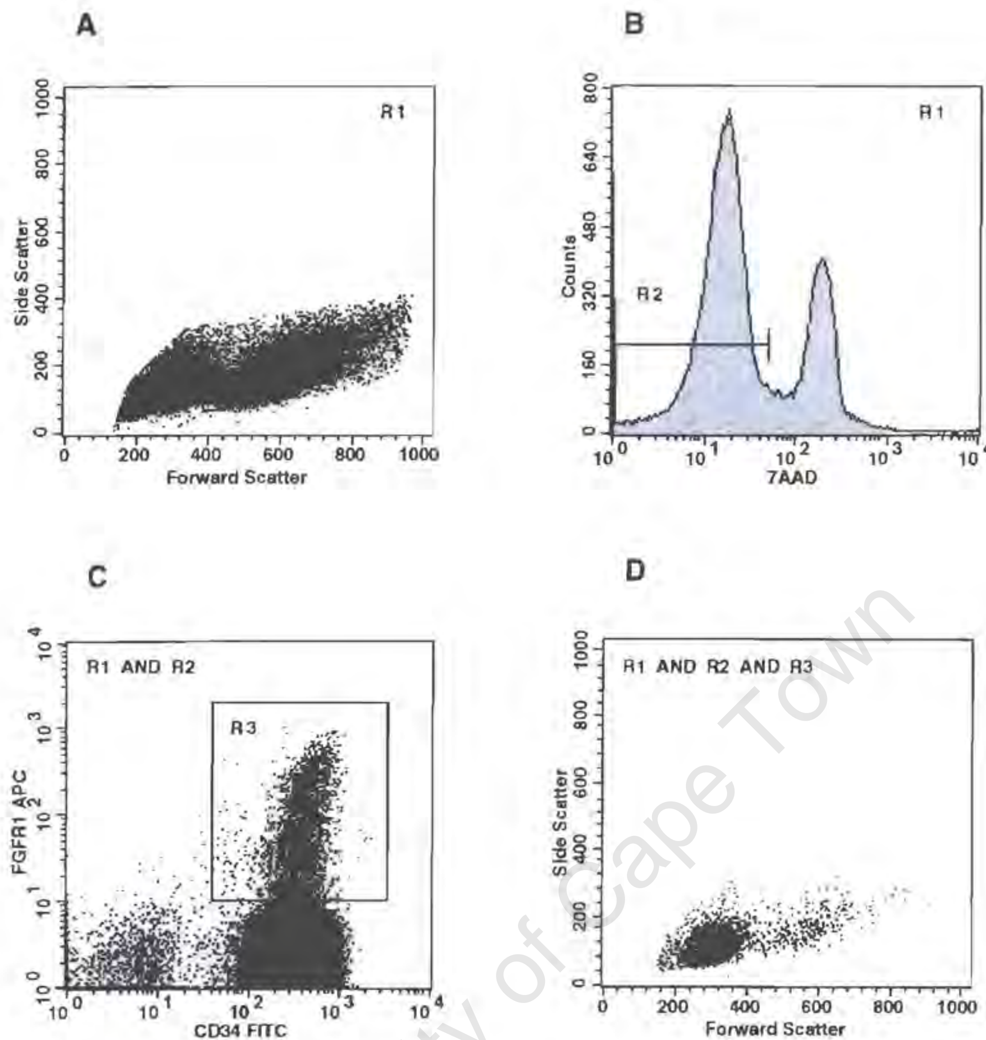

Figure 23. Flow cytometry of the CD34+FGFR+ population. (A) Forward and side scatter analysis of CD34+ enriched cells isolated from mobilized peripheral blood. R1 defines a region that excludes debris with low FSC and cells with high SSC. (B) R2 defines viable (7AAD-) cells. (C) R3 defines viable CD34+FGFR+ cells. (D) Boolean gating is used to illustrate the FSC/SSC characteristics of viable CD34+FGFR+ cells and shows that the majority of these cells are small (low FSC) and display little granularity (low SSC).

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The incidence of FGFR-1 expression on CD34+ cells was similar for bone marrow, cord blood and mobilized peripheral blood samples, being 3.5 ± 2.0% (n=2), 3.7 ± 2.9% (n=4) and 4.8 ± 1.9% (n=13) respectively and 4.5 ± 2.1% (n=19) overall (Table 7).

TABLE 7. % CD34+ CELLS THAT EXPRESS FGFR-1

Experiment #	Source	Selection Method	%CD34+FGFR+ cells
1	BM	DYNAL	4.9
2	BM	MACS	2.1
3	CB	DYNAL	5.8
4	CB	MACS	1.3
5	CB	MACS	6.5
6	CB	MACS	1.1
7	mPB	DYNAL	3.2
8	mPB	MACS	7.9
9	mPB	MACS	3.6
10	mPB	MACS	1.8
11	mPB	MACS	6.5
12	mPB	MACS	3.3
13	mPB	MACS	8.2
14	mPB	MACS	3.6
15	mPB	DYNAL	4.7
16	mPB	DYNAL	5.3
17	mPB	MACS	3.5
18	mPB	DYNAL	5.6
19	mPB	DYNAL	5.3
		Mean	**4.5**
		SD	± 2.1
		n	19

mPB = mobilized peripheral blood
BM = bone marrow
CB = cord blood

SD = standard deviation
n = number of samples

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During the course of the study, I decided to concentrate on mobilized peripheral blood as a sample source rather than bone marrow or cord blood. The reasons for this choice were (a) to reduce the risk of sample contamination with mature endothelial cells from vessel walls that may accompany harvesting from bone marrow and (b) the difficulty I

experienced in obtaining sufficient cord blood to isolate enough CD34+ cells for the determination of FGFR-1 expression in conjunction with multiple other antigens.

In order to determine the phenotypic nature of the CD34+FGFR+ population, I examined viable CD34+FGFR+ cells isolated from mobilized peripheral blood for the expression of antigens expressed by primitive haematopoietic and endothelial cells. The expression of CD38, AC133, Thy-1, c-kit, CD31, Tie, Tek, VEGFR-2/KDR, P1H12 and VE-cadherin by CD34+FGFR+ cells was therefore determined (Table 8, Figure 24 and 25). This was done using four color flow cytometry: three different fluorochromes were used for the staining of CD34, FGFR-1 and one of each the 10 antigens, while the dye, 7AAD (for viability) comprised the fourth color. The number of CD34+FGFR+ cells obtained in each experiment limited the number of antigens that could be examined per sample.

TABLE 8. COMPARISON OF THE EXPRESSION OF THE INDICATED ANTIGENS BY CD34+ FGFR+ AND CD34+ CELLS

Antigen	%CD34+FGFR+ cells expressing antigen (mean±SD)	%CD34+ cells expressing antigen (mean±SD)	Number of samples (n)	Enrichment (fold)	*p* Value
CD38	94.9 ± 5.7	95.6 ± 2.9	4	0	-
AC133	75.3 ± 6.4	79.7 ± 6.4	6	0	-
c-kit	84.5 ± 11.3	77.4 ± 18.3	6	0	-
CD31	84.0 ± 22.6	84.5 ± 18.3	6	0	-
Thy-1	63.9 ± 10.9	12.0 ± 8.9	8	5.3	< 0.00001
Tie	55.8 ± 15.1	4.4 ± 2.4	7	12.6	< 0.0002
Tek	60.0 ± 18.5	3.6 ± 2.6	6	16.6	< 0.0004
P1H12	58.0 ± 17.1	4.2 ± 2.0	7	13.8	< 0.0003
VE-cadherin	87.8 ± 10.3	8.4 ± 4.0	4	10.4	< 0.0003
KDR	75.5 ± 16.0	5.2 ± 1.9	3	14.5	< 0.014

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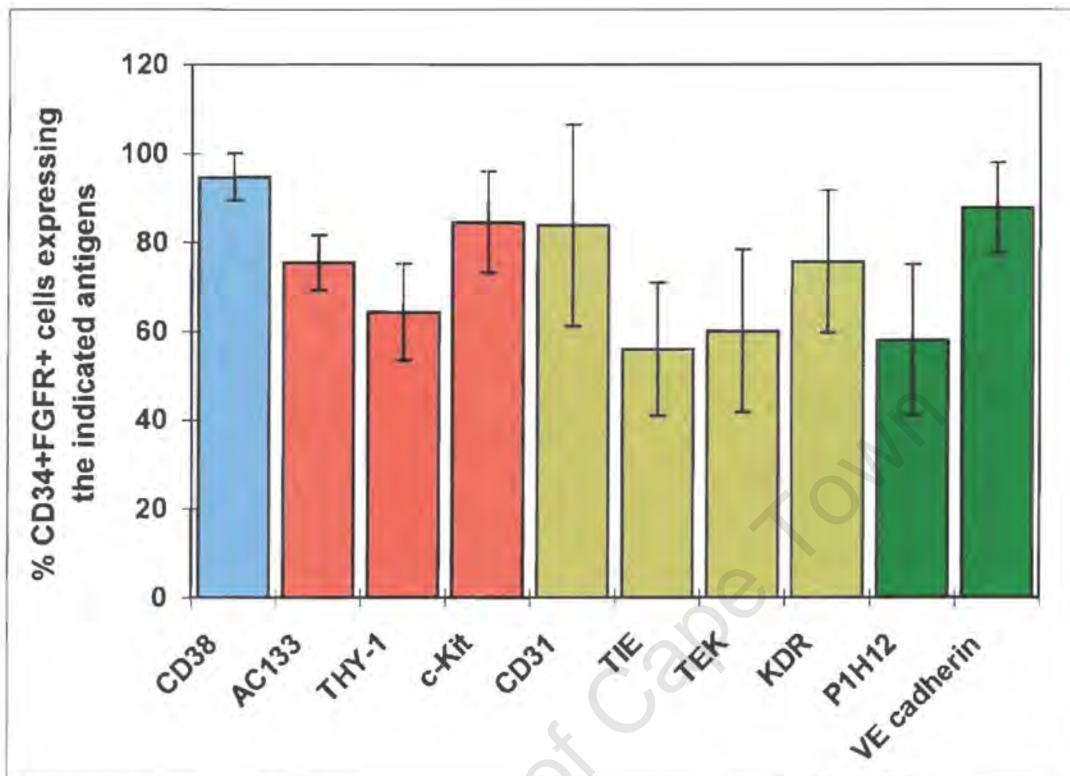

Figure 24. The expression of antigens by CD34+FGFR+ cells. Isolated CD34+ cells were examined for the presence of FGFR-1 together with the expression of one of the following antigens: CD38 (■), AC133, Thy-1, c-kit (primitive haematopoietic cell surface antigens■), CD31, Tie, Tek, KDR (antigens expressed by both endothelial and haematopoietic cells■), P1H12 or VE-cadherin (endothelial specific antigens ■).

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The majority of CD34+FGFR+ cells expressed cell surface antigens that are present on primitive haematopoietic cells, such as AC133 (Buhring *et al.*, 1999; de Wynter *et al.*, 1998; Majka *et al.*, 2000; Matsumoto *et al.*, 2000; Miraglia *et al.*, 1997) (75%, Table 8, Figure 24, Figure 25B), c-kit (Katayama *et al.*, 1993; Simmons *et al.*, 1994) (85%, Table 8, Figure 24, Figure 25C) and Thy-1 (Murray *et al.*, 1995) (64%, Table 8, Figure 24, Figure 25D). In addition, significant numbers of CD34+FGFR+ cells expressed the endothelial specific markers VE-cadherin (Ali *et al.*, 1997; Breier *et al.*, 1996; Gory *et al.*, 1999; Matsuyoshi *et al.*, 1997; Nishikawa *et al.*, 1998b) (88%, Table 8, Figure 24, Figure 25I) and P1H12 (Lin *et al.*, 2000; Solovey *et al.*, 1997) (58%, Table 8, Figure 24, Figure 25J). A number of antigens that are expressed by both haematopoietic and endothelial cells, were also present on the CD34+FGFR+ population: CD31 (Watt *et al.*, 1995; Yong *et al.*, 1998) (84%, Table 8, Figure 24, Figure 25E), Tie (Hanahan, 1997; Hashiyama *et al.*, 1996) (56%, Table 8, Figure 24, Figure 25H), Tek (Sato *et al.*, 1998; Sato *et al.*, 1995) (60%, Table 8, Figure 24, Figure 25L) and KDR (Vittet *et al.*, 1996; Ziegler *et al.*, 1999) (76%, Table 8, Figure 24, Figure 25M). CD38 was found on 95% of CD34+FGFR+ cells (Table 8, Figure 24, Figure 25G), indicating that 5% of CD34+FGFR+ cells do not express CD38. As CD34+CD38- cells represent very primitive haematopoietic cells capable of multilineage re-population in immunodeficient mice (Bhatia *et al.*, 1997), the absence of CD38 on a subset of CD34+FGFR+ cells indicates that these cells may be precursor cells that differentiate into CD34+FGFR+CD38+ cells in an analogous manner to that observed for the differentiation of CD34+CD38- cells into CD34+CD38+ haematopoietic cells (Civin *et al.*, 1996; Tajima *et al.*, 2001). Isotypic control antibodies were used to verify the specificity of the staining for each antigen (Figure 25A, F, K). CD34+FGFR+ cells isolated from bone marrow and cord blood samples showed a similar pattern of expression of haematopoietic and endothelial markers to that found on cells isolated from mobilized peripheral blood (data not shown). These results demonstrate that significant numbers of viable CD34+FGFR+ cells express antigens that are found on both early haematopoietic and endothelial cells.

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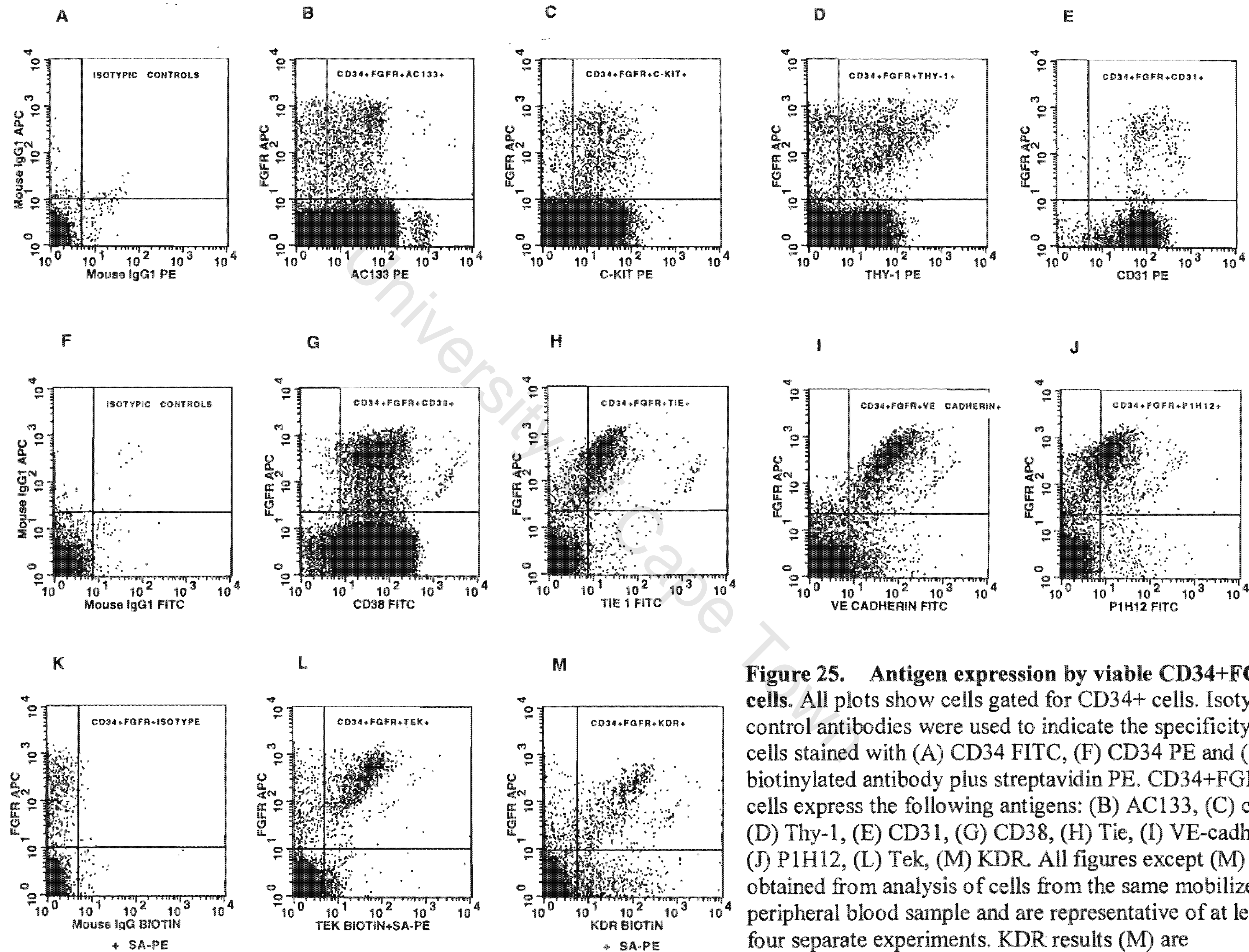

Figure 25. Antigen expression by viable CD34+FGFR+ cells. All plots show cells gated for CD34+ cells. Isotype control antibodies were used to indicate the specificity of cells stained with (A) CD34 FITC, (F) CD34 PE and (K) biotinylated antibody plus streptavidin PE. CD34+FGFR+ cells express the following antigens: (B) AC133, (C) c-kit, (D) Thy-1, (E) CD31, (G) CD38, (H) Tie, (I) VE-cadherin, (J) P1H12, (L) Tek, (M) KDR. All figures except (M) were obtained from analysis of cells from the same mobilized peripheral blood sample and are representative of at least four separate experiments. KDR results (M) are representative of three experiments

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The incidence of expression of endothelial and haematopoietic antigens on CD34+FGFR+ cells was compared with that of the CD34+ population to determine if any of these antigens were preferentially expressed on CD34+FGFR+ cells. I found that CD38, AC133, c-kit and CD31 were expressed to similar extents by CD34+ and CD34+FGFR+ cells (Table 8). In contrast, significantly increased numbers of CD34+FGFR+ cells expressed Thy-1, Tie, Tek, P1H12, VE-cadherin and KDR compared with the CD34+ population (Table 8), showing that the CD34+FGFR+ population is enriched for antigens expressed on primitive haematopoietic (Thy-1), and endothelial (Tie, Tek, P1H12, VE-cadherin, KDR) cells. Thy-1 is expressed by 64% of CD34+FGFR+ cells compared with 12% of the CD34+ cells (Table 8). This indicates that there is a 5.3 fold enrichment of Thy-1+ cells in the CD34+FGFR+ subset as compared to the whole CD34+ population ($p < 0.00001$, Table 8). The differences in expression of the endothelial specific markers, VE-cadherin and P1H12 between these two populations are even more striking, with a 10-14 fold increase in expression by the CD34+FGFR+ population compared with the CD34+ population ($p < 0.0003$, Table 8). Similar significant increases in expression (12-16 fold) of Tie ($p < 0.0002$), Tek ($p < 0.0004$) and KDR ($p < 0.014$) were noted by the CD34+FGFR+ cells compared with the CD34+ cells (Table 8). The increased numbers of cells expressing the endothelial specific markers, P1H12 and VE-cadherin, as well as the antigens Tie, Tek, KDR and Thy-1, found within the CD34+FGFR+ population compared with that of the CD34+ population, indicate that the CD34+FGFR+ subset is enriched for cells that express endothelial antigens and the primitive haematopoietic antigen Thy-1.

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In order to determine whether CD34+FGFR+ cells co-expressed both primitive and endothelial antigens on the same cells, the dye 7AAD was excluded from analysis so that simultaneous examination of the cells for the expression of AC133 and VE-cadherin could be performed. This was necessary as I was limited to four colour FACS analysis and wished to determine the expression of CD34, FGFR-1, AC133 and VE-cadherin simultaneously on the same cells. I found that 76% of CD34+FGFR+ cells co-expressed AC133 and that 74% co-expressed both AC133 and VE-cadherin (Table 9, Figure 26), indicating that almost all of the CD34+FGFR+AC133+ population expressed the endothelial specific marker VE-cadherin. To determine whether other primitive

haematopoietic and endothelial antigens were co-expressed by CD34+FGFR+ cells, the cells were also examined for concomitant expression of Thy-1 with either Tek or VE-cadherin. I found that 65% of CD34+FGFR+ cells expressed both Thy-1 and VE-cadherin while 57% expressed both Thy-1 and Tek (Table 9), confirming co-expression of primitive haematopoietic (Thy-1) and endothelial specific (VE-cadherin) antigens by CD34+FGFR+ cells. As the majority (68%) of the cells that expressed AC133 also expressed Thy-1 (Table 9), this indicates that significant numbers of CD34+FGFR+ cells co-express AC133, Tek, VE-cadherin and Thy-1, thereby having the phenotype CD34+FGFR+AC133+Thy-1+Tek+VE-cadherin+.

TABLE 9. CO-EXPRESSION OF PRIMITIVE HAEMATOPOIETIC AND ENDOTHELIAL ANTIGENS BY CD34+FGFR+ CELLS

Phenotype	% CD34+FGFR+ cells expressing the phenotype
CD34+FGFR+AC133+	76
CD34+FGFR+AC133+VE-cadherin+	74
CD34+FGFR+Thy-1+VE-cadherin+	65
CD34+FGFR+Thy-1+Tek+	57
CD34+FGFR+AC133+Thy-1+	68

These results show that CD34+FGFR+ cells co-express antigens found on primitive haematopoietic cells (AC133, Thy-1) as well as those specific for endothelial cells (VE-cadherin). Furthermore, as AC133 is not expressed by mature endothelial cells, the CD34+FGFR+ population that co-expresses AC133 and VE-cadherin may represent an endothelial progenitor cell population. To ensure that meaningful numbers of quadruple-stained cells were assayed, sufficient numbers of CD34+ cells were analysed to obtain at least 1000 labelled CD34+FGFR+AC133+VE-cadherin+ cells.

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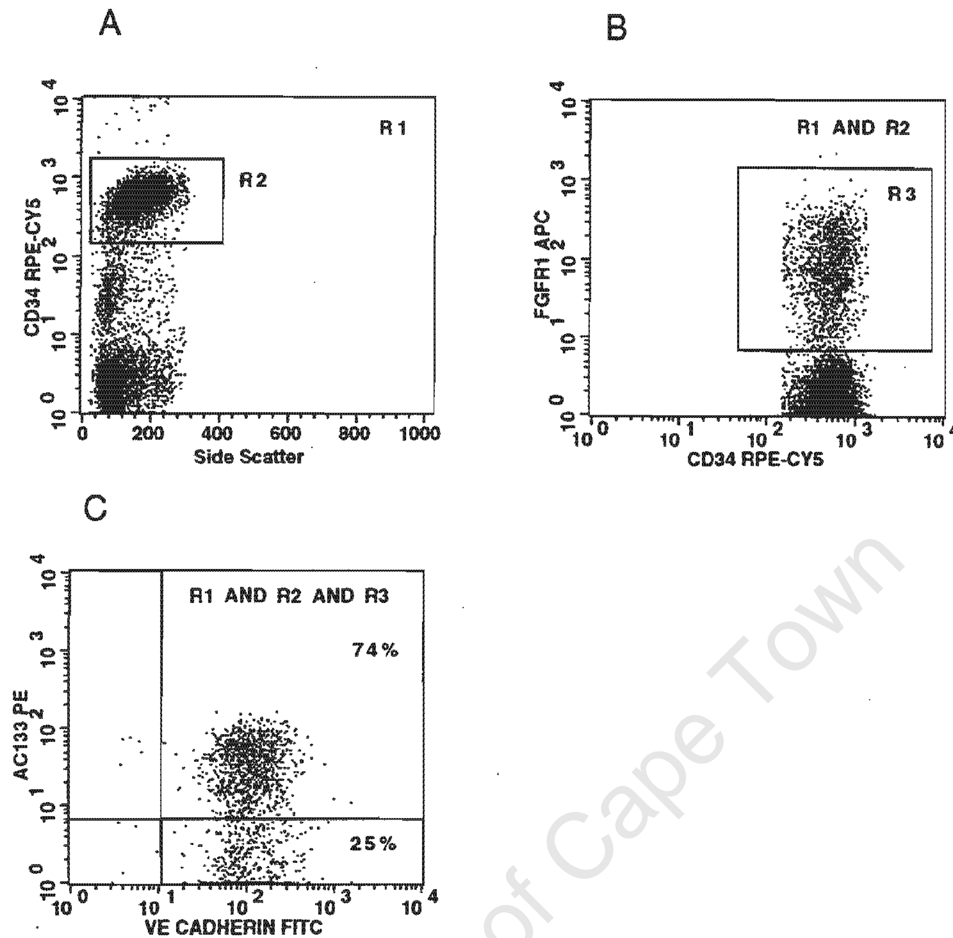

Figure 26. CD34+FGFR+ cells co-express AC133 and VE-cadherin. CD34+ enriched cells were gated to exclude debris with low FSC and cells with high SSC (R1). (A) CD34+ cells with the highest intensity of CD34 expression were gated (R2) and (B) were analysed for the expression of FGFR-1. (C) CD34+FGFR+ cells were analysed for the simultaneous expression of AC133 and VE-cadherin. These plots are representative of 3 separate experiments.

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To confirm the co-expression of haematopoietic and endothelial markers on **viable** cells, I examined Lin- cells for the simultaneous co-expression of FGFR-1, the primitive haematopoietic marker, AC133 and the endothelial specific marker, VE-cadherin. As I was limited to four color flow cytometry, the use of Lin- cells enabled me to examine a progenitor population for the co-expression of 2 surface antigens on viable (7AAD-) FGFR-1+ cells. This is in contrast to experiments described earlier, where CD34+FGFR+

cells were analysed for the co-expression of either one surface antigen on viable cells (Table 8; Figures 24 and 25) or two surface antigens on cells where viability staining was omitted (Table 9; Figure 26). Viable Lin-FGFR+ cells have a low SSC (Figure 27C) similar to that of viable CD34+FGFR+ cells (Figure 23D) and 68% of Lin- FGFR+ cells co-express both AC133 and VE-cadherin (Figure 27D) similar to the incidence (74%) of co-expression of AC133 and VE-cadherin on CD34+FGFR+ cells (Table 9; Figure 26C), suggesting that the Lin-FGFR+AC133+VE-cadherin+ and CD34+FGFR+AC133+VE-cadherin+ populations are comparable. As the majority (>99%) of AC133+ cells express CD34 (Buhring *et al.*, 1999; Matsumoto *et al.*, 2000), the viable Lin-FGFR+AC133+VE-cadherin+ population (upper right quadrant in Figure 27D) must also be CD34+. These data confirm that **viable** CD34+FGFR+ cells simultaneously express both early haematopoietic and endothelial antigens on their surface. As AC133 is not found on mature endothelial cells, including circulating endothelial cells that have been shed from vessel walls (Peichev *et al.*, 2000; Yin *et al.*, 1997), the expression of AC133 with VE-cadherin on CD34+FGFR+ cells indicates that these cells may be endothelial stem/precursor cells.

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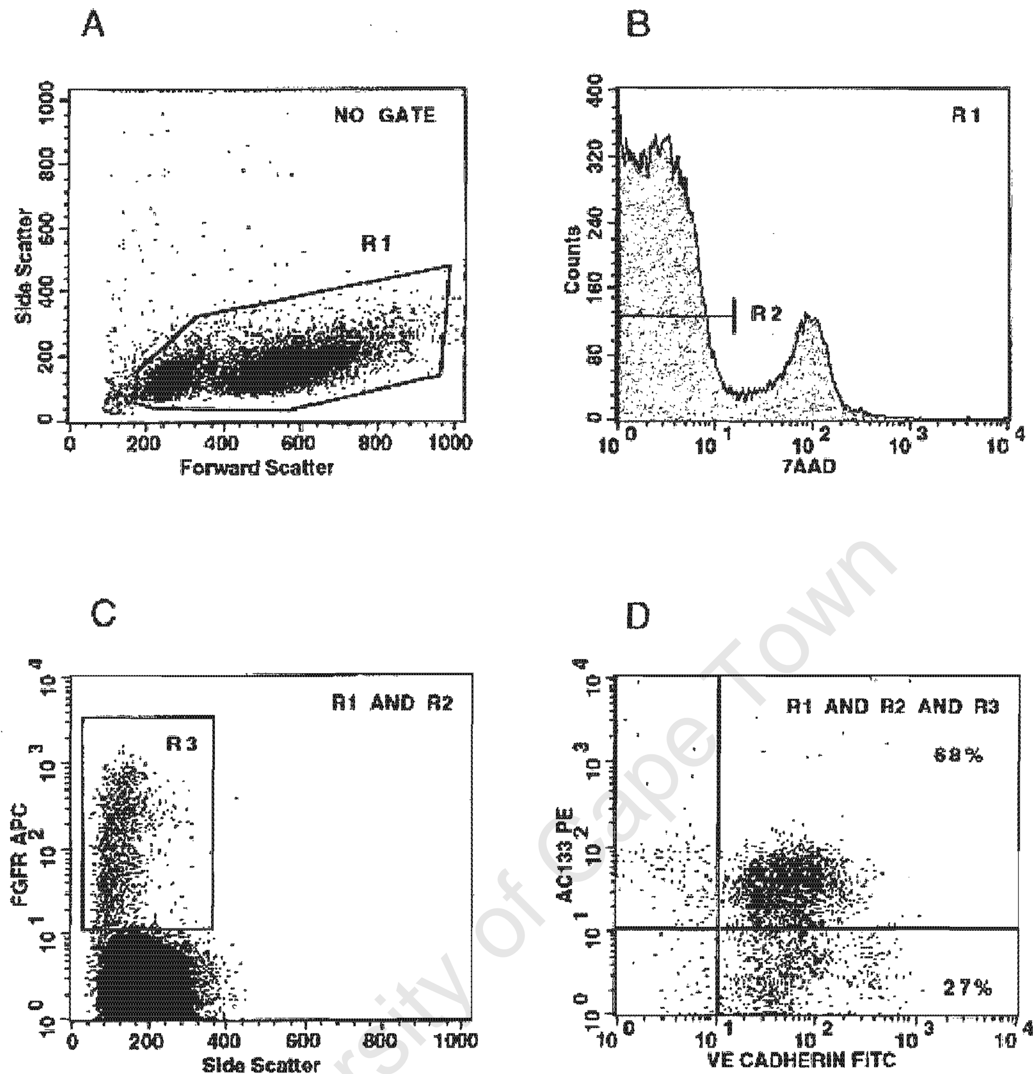

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Figure 27. Viable (7AAD-) Lin-FGFR+ cells co-express both primitive haematopoietic (AC133) and endothelial-specific (VE-cadherin) cell surface markers. CD34+ enriched cells were isolated from lineage depleted mobilized peripheral blood and gated (A) to exclude debris with low FSC and cells with high SSC (R1) and (B) to include only viable (7AAD-) cells (R2). (C) R3 defines viable Lin-FGFR+ cells and shows that the majority of viable Lin-FGFR+ cells have low SSC. (D) Boolean gating is used to show co-expression of both AC133 and VE-cadherin by viable Lin-FGFR+ cells. These plots are representative of 2 separate experiments.

These results show that approximately 4.5% of CD34+ cells from bone marrow, cord blood and mobilized peripheral blood express FGFR-1 and that they simultaneously express antigens found on both primitive haematopoietic and endothelial cells. The expression of Thy-1, Tie, Tek, KDR, P1H12 and VE-cadherin is enriched in the CD34+FGFR+ population as compared to the CD34+ population. As two of these antigens (VE-cadherin, P1H12) are endothelial cell specific, the FGFR-1+ cells may represent an endothelial stem/progenitor cell population or a population of cells containing precursors with a combined haematopoietic/endothelial nature that have at least two tissue potentialities. These observations are of significance as they describe, for the first time, the existence of a subset of CD34+ cells that co-expresses FGFR-1, primitive haematopoietic cell surface antigens and endothelial specific antigens and identify a possible phenotype for circulating endothelial precursor cells.

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Growth characteristics of CD34+FGFR+ cells

In order to determine the nature of the population that I had characterized, I studied the growth properties of the cells in two ways. First, I used the well described methylcellulose assay for determining whether the numbers and types of haematopoietic progenitors differed between CD34+FGFR+ and CD34+FGFR- populations. Second, I determined whether the isolated populations could differentiate into endothelial cells *in vitro*.

(a) Growth of haematopoietic colonies in methylcellulose

Cells obtained from mobilized peripheral blood samples were enriched for CD34+ cells by immunomagnetic selection and sorted into CD34+FGFR+, CD34+FGFR- and unfractionated CD34+ populations. Cells (450 cells/well) were seeded in MethoCult GF+H4435 (IMDM containing FCS and cytokines as described in Materials and Methods) in 12 well tissue culture plates and the numbers and types of colonies formed by the different populations was determined in the absence and presence of FGF-2 (100 ng/ml). After 14 days, BFU-E, CFU-E, CFU-GEMM, CFU-M and CFU-GM colonies were enumerated according to their morphology. There was no significant difference between the numbers of BFU-E, CFU-E, CFU-GEMM, CFU-M or CFU-GM colonies formed in the presence or absence of FGF-2, either within each cell population or between the various populations seeded (Figure 28). Similar results were found in 10 separate experiments, indicating that the CD34+FGFR+ population is capable of forming multiple lineages and that it does not differ in this respect from the CD34+FGFR- population or the unfractionated CD34+ population.

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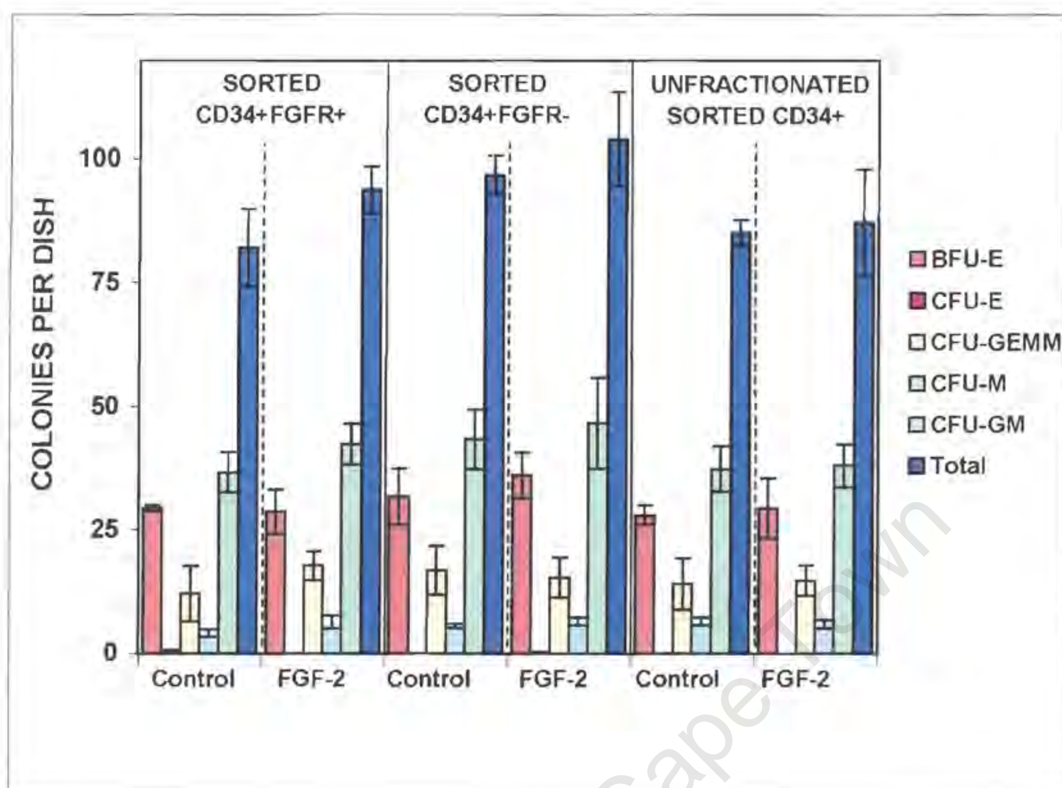

Figure 28. Growth of haematopoietic colonies. FACS sorted CD34+FGFR+, CD34+FGFR- and unfractionated CD34+ populations, isolated from mobilized peripheral blood, were cultured in methylcellulose in the presence and absence of FGF-2 (100 ng/ml). The numbers of haematopoietic colonies of various lineages were counted. Each bar represents the mean result from triplicate wells.

(b) Cell growth in the presence of endothelial growth factors

In order to determine whether CD34+FGFR+ cells respond to endothelial growth factors and give rise to endothelial cells, cells were isolated and cultured under conditions that promote endothelial cell growth. CD34+FGFR+, CD34+FGFR- and unfractionated CD34+ populations, isolated on the FACS, were cultured in the absence and presence of FGF-2 alone or FGF-2 and VEGF together. As very few viable CD34+FGFR+ cells were obtained after FACS sorting, we were limited by the numbers of viable cells recovered after sorting and therefore were not able to determine the effect of VEGF treatment alone. As VEGF alone is a poor mitogen for endothelial cells, we considered that omitting VEGF was a reasonable option. In the absence of growth factors, the CD34+FGFR+ and CD34+FGFR- populations increased by 1.7 fold and 1.2 fold respectively, after 19 weeks in culture

(Table 10, Experiment 1). However, in the presence of FGF-2 (10 ng/ml) the CD34+FGFR+ population increased by 11.9 fold whereas the CD34+FGFR- population increased by only 2.4 fold (Table 10, Experiment 1). As the sorted CD34+FGFR+ cells show a 5 fold increase in growth compared to the CD34+FGFR- population in the presence of FGF-2, the CD34+FGFR+ cells must express functional receptors for FGF-2. In a second experiment, CD34+FGFR+ cells showed an increase in growth of 3.2 fold in the absence of growth factors, 8 fold in the presence of FGF-2 (10 ng/ml) and 17 fold in the presence of both FGF-2 (10 ng/ml) and VEGF (10 ng/ml) after 5 weeks in culture, while the corresponding CD34+FGFR- increased only by approximately 1.5, 2.1 and 3.1 fold respectively (Table 10, Experiment 2). These results demonstrate that CD34+FGFR+ cell growth is stimulated by factors, such as FGF-2 and VEGF, known to promote endothelial cell proliferation.

TABLE 10. FGF-2 AND VEGF PROMOTE GROWTH OF CD34+FGFR+ CELLS

Experiment #	Incubation time (weeks)	Population	* Control	* FGF-2 (10ng/ml)	* FGF-2 + VEGF (both at 10ng/ml)
1•	19	CD34+FGFR+	1734	11978	ND
		CD34+FGFR-	1244	2500	ND
2•	5	CD34+	1617	1457	2311
		CD34+FGFR+	3227	8057	17140
		CD34+FGFR-	1493	2098	3093

• Wells were seeded with 1000 cells
* = cells/well
ND = Not determined

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In addition to demonstrating the effects of FGF-2 and VEGF on the growth of the FGFR positive and negative subsets of the CD34+ population, these data show that the sorted populations grow slowly in culture (Table 10). There may be a number of reasons for this: (1) the culture conditions may not be optimal for the promotion of cell growth i.e., the medium or the added factors used may not be optimal for growth or the starting cell density may be too low, especially if factors produced by the cells themselves are required to condition their medium, (2) since primitive cells are known to be dormant (Ogawa, 1993), the slow expansion of CD34+FGFR+ cells may be due to the fact that they are slow-growing primitive cells, and (3) the sorting procedure may damage the cells, thereby affecting their ability to grow.

(c) Growth on OP9 feeder layers and determination of VE-cadherin expression

Next I determined whether CD34+FGFR+ cells could differentiate into adherent endothelial cells in culture, as evidenced by the expression of the endothelial antigens, VE-cadherin and vWF. VE-cadherin is expressed only by endothelial cells (Ali *et al.*, 1997; Breier *et al.*, 1996; Gory *et al.*, 1999; Matsuyoshi *et al.*, 1997; Nishikawa *et al.*, 1998b), whereas vWF is expressed by endothelial cells and megakaryocytes (Zanetta *et al.*, 2000). As OP9 cells have been shown to promote the differentiation of progenitor cells to mature endothelial cells (Hirashima *et al.*, 1999), CD34+FGFR+ cells were cultured on OP9 feeder layers. Phase contrast microscopy shows that sorted CD34+FGFR+ cells adhered to the OP9 feeder layer in the presence of FGF-2 and VEGF (Figure 29A). The expression of VE-cadherin in wells seeded with CD34+FGFR+ and CD34+FGFR- cells was examined using a mouse monoclonal antibody to VE-cadherin and a goat anti-mouse FITC conjugated antibody. Wells seeded with CD34+FGFR+ cells contained significant numbers of VE-cadherin expressing cells (Figure 29B) whereas cells in wells containing CD34+FGFR- cells lacked expression of VE-cadherin (Figure 29D).

(d) Growth on fibronectin/gelatin slides and determination of vWF expression

The fact that sorted CD34+FGFR- cells did not give rise to any adherent VE-cadherin cells, indicates that the CD34+FGFR- population is unlikely to contain endothelial progenitor cells. However, since the majority of CD34+FGFR+ cells express VE-cadherin

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(Table 8; Figure 24 and 25I), the observation that the cultured sorted CD34+FGFR+ population expressed VE-cadherin is not proof that the CD34+FGFR+ population differentiates into endothelial cells, as it could also indicate that cells maintaining a primitive phenotype continue to express VE-cadherin in culture. I therefore determined whether cultured CD34+FGFR+ cells express vWF, a cell surface antigen found only on endothelial and megakaryocytic cells (Zanetta *et al.*, 2000). OP9 feeder layers could not be used for these experiments as it was found that the OP9 cells were induced by VEGF to express vWF and therefore one could not readily distinguish between vWF-expressing OP9 and endothelial cells. CD34+FGFR+ cells were therefore cultured in fibronectin-coated (50μg/ml in 0.1% gelatin) wells in EGM-2, a special endothelial growth medium (Clonetics Inc., San Diego, CA) and examined for evidence of vWF expression. When incubated with rabbit anti-human vWF antibodies and goat anti-rabbit FITC conjugated antibodies, many of these cells displayed vWF (Figure 29F), whereas no staining was found in wells that received goat anti-rabbit FITC conjugated antibodies alone (Figure 29H). The cultured CD34+FGFR+ cells grew in small groups, clusters or "bead-like" strings of round cells, approximately 6 μm in diameter (Figure 30A, B, C). As none of the cells had the morphology of megakaryocytes, positive vWF staining identified the cells as endothelial cells (Zanetta *et al.*, 2000) (Figure 30D, E, F). In addition, the expression of vWF by cultured cells that also incorporated DiI-ac-LDL (Figure 30F, G) confirmed that these cells are endothelial in nature as vWF is expressed by endothelial and megakaryocytic cells only (Zanetta *et al.*, 2000), while ac-LDL is only taken up by endothelial cells and macrophages (Voyta *et al.*, 1984). As freshly isolated CD34+FGFR+ cells were not found to express vWF or to incorporate ac-LDL (results not shown), these data indicate that CD34+FGFR+ cells give rise to endothelial cells.

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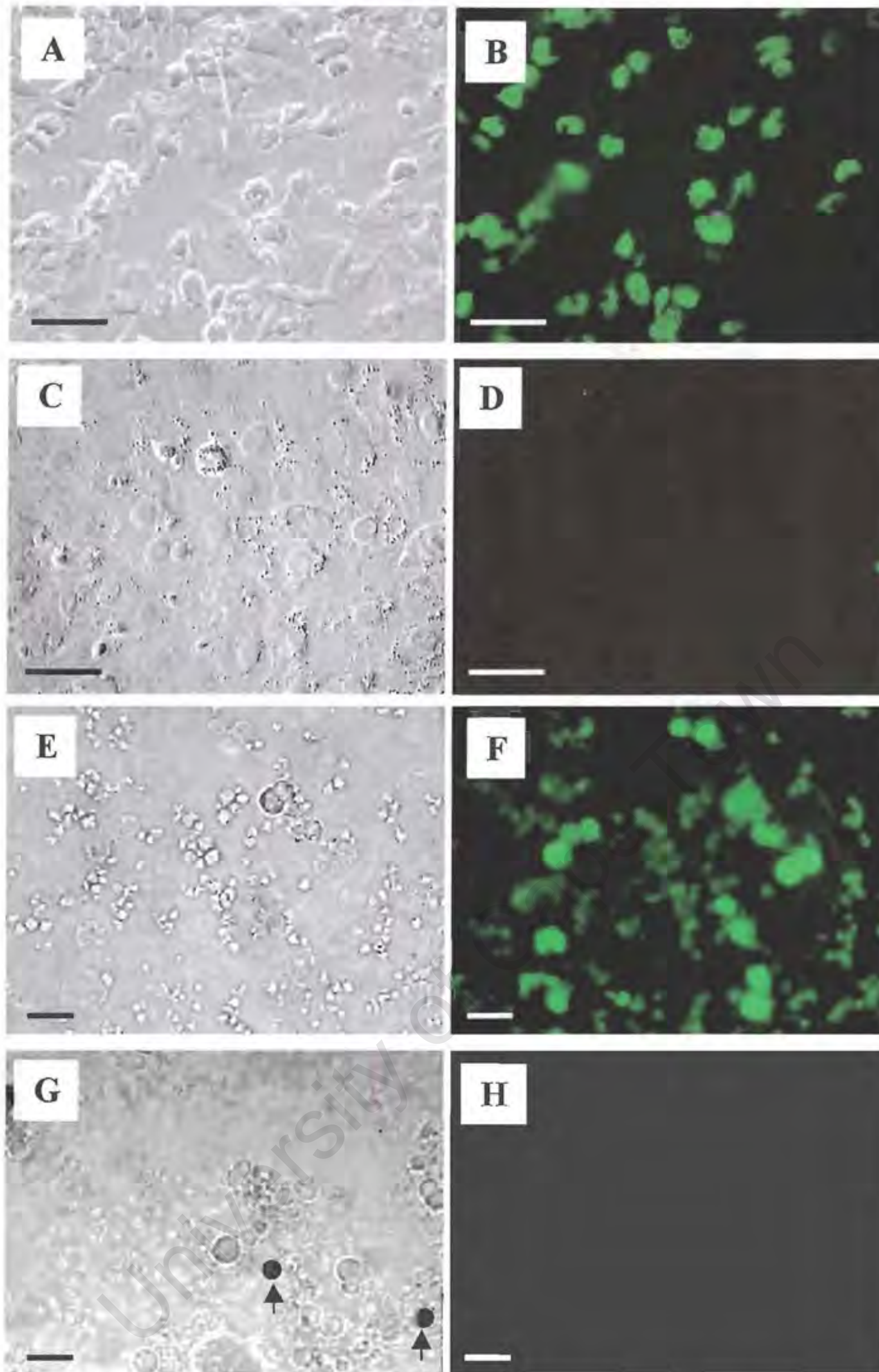

Figure 29. CD34+FGFR+ cells give rise to VE-cadherin and vWF expressing cells in culture. Sorted CD34+FGFR+ (A, B) and CD34+FGFR- (C, D) cells were cultured on OP9 feeder layers in the presence of FGF-2 and VEGF. Wells seeded with CD34+FGFR+ cells contained significant numbers of adherent VE-cadherin expressing cells (B) whereas cells in wells containing CD34+FGFR- cells lacked expression of VE-cadherin (D). Phase contrast microscopy of the same fields is shown in (A) and (C) respectively. CD34+FGFR+ cells, cultured in fibronectin-coated wells in the presence of FGF-2 and VEGF, gave rise to cells that express vWF (F). Negative control i.e. secondary FITC conjugated antibody but no primary vWF antibody (H). Arrows (G) indicate Dynal beads with a diameter of 4.5 μm. Phase contrast photographs (E, G) show the same fields as the immunofluorescence photographs (F, H). Scale bars = 10 μm.

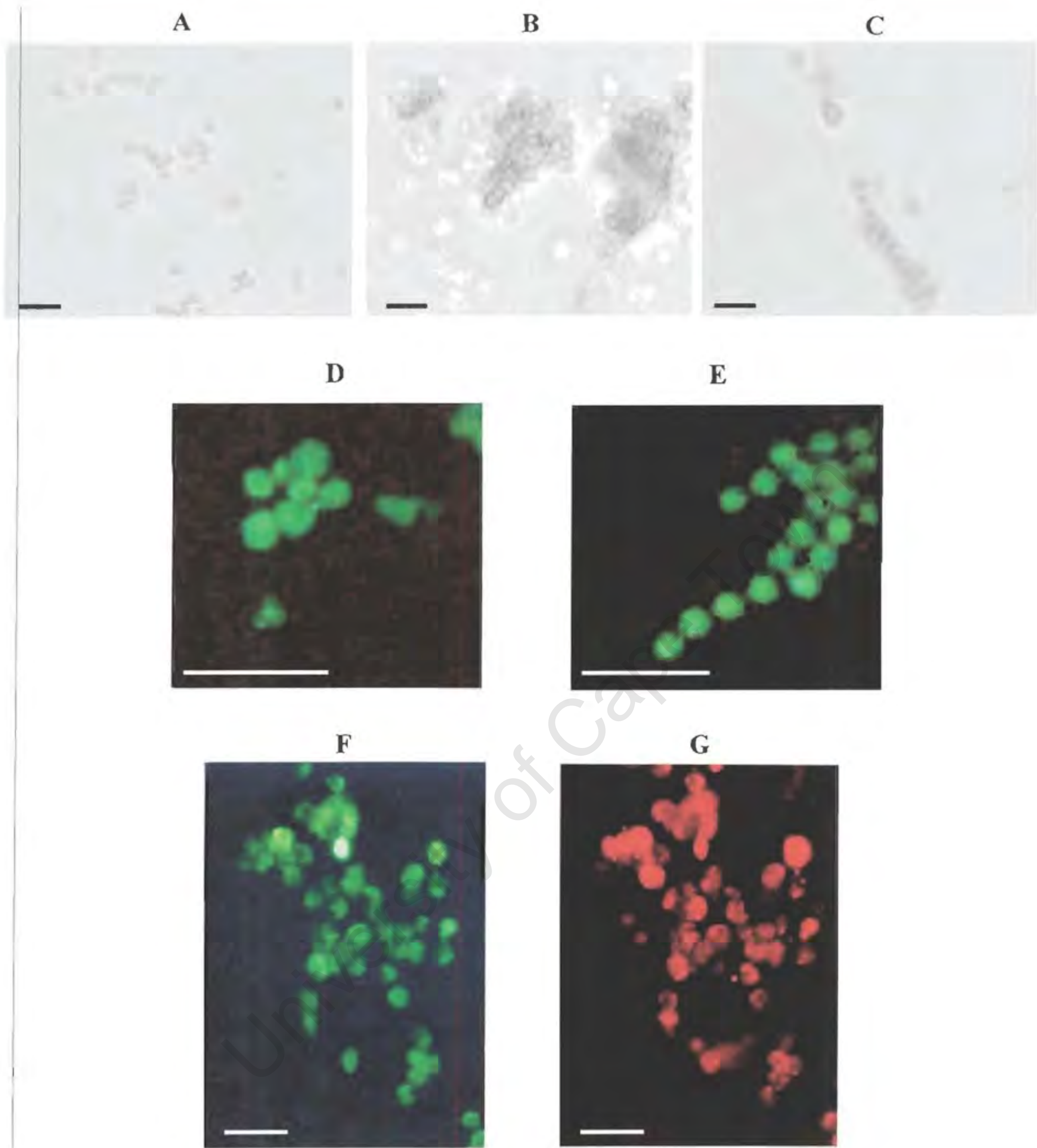

Figure 30. Cultured CD34+FGFR+ cells display various morphologies, express vWF and incorporate DiI-ac-LDL. CD34+FGFR+ cells, cultured in fibronectin-coated wells, grew as (A) single cells, (B) "piled up colonies"(C) or "bead-like strings" of cells and expressed vWF (D, E, F). Cells that expressed vWF (F) also incorporated DiI-ac-LDL (G). Scale bars = 20µm.

These results show that the CD34+FGFR+, CD34+FGFR- and unfractionated CD34+ populations form similar numbers of primitive and other types of haematopoietic colonies when cultured under conditions that promote haematopoietic colony growth. The presence of FGF-2 does not affect the numbers or types of haematopoietic colonies formed in methylcellulose by any of the populations. Using culture conditions that promote endothelial growth, the CD34+FGFR+ population grows slowly in an FGF-2 and VEGF dependent manner, indicating that these cells are stimulated by angiogenic factors. In addition, the cultured CD34+FGFR+ cells give rise to adherent cells that incorporate DiI-ac-LDL and that express vWF and VE-cadherin, antigens expressed by endothelial cells, indicating definitively that the CD34+FGFR+ population contains cells that differentiate into endothelial cells.

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Discussion

These results indicate that endothelial precursor cells reside within a population of FGFR+ progenitor cells. FGFR-1 is expressed on 4.5 ± 2.1% of CD34+ cells (Table 7, Figure 23) that co-express primitive haematopoietic and endothelial cell surface antigens (Tables 8 and 9; Figures 24, 25, 26 and 27), form haematopoietic colonies in methylcellulose (Figure 28) and give rise to endothelial cells in culture (Figures 29 and 30). The CD34+FGFR+ population is considerably enriched (10-16 fold) with respect to the expression of many antigens that are expressed by endothelial cells (VE-cadherin, P1H12, Tie, Tek, KDR) (Table 8) compared with the CD34+ population. The CD34+FGFR+ population also contains 5 fold more cells expressing Thy-1, a primitive haematopoietic marker (Murray *et al.*, 1995), than the CD34+ population, indicating that Thy-1 is also expressed on a cell with both haematopietic and endothelial potential or on a majority of endothelial progenitor cells. As 75% of CD34+FGFR+ cells express AC133, a primitive haematopoietic antigen (Miraglia *et al.*, 1997) that is not expressed on mature endothelial cells (Peichev *et al.*, 2000; Yin *et al.*, 1997) and as FACS sorted CD34+FGFR+ cells give rise to haematopoietic colonies in methlycellulose culture (Figure 28), these cells cannot be circulating endothelial cells that have been shed from the surfaces of blood vessels. Since VE-cadherin (Ali *et al.*, 1997; Breier *et al.*, 1996; Gory *et al.*, 1999; Matsuyoshi *et al.*, 1997; Nishikawa *et al.*, 1998b) and P1H12 (Lin *et al.*, 2000; Solovey *et al.*, 1997) are expressed exclusively on endothelial cells and as Thy-1 (Murray *et al.*, 1995) and AC133 (Miraglia *et al.*, 1997) are expressed on early haematopoietic cells, the CD34+FGFR+ population that co-expresses these markers represents an endothelial stem/progenitor cell population.

I have combined the data from four colour fluorescence staining (Table 9; Figures 26C and 27D) with that of the incidence of expression of antigens on CD34+FGFR+ cells (Table 8), to propose the following phenotype for endothelial progenitor cells: CD34+FGFR+CD38+VE-cadherin+c-kit+CD31+KDR+AC133+ (Figure 31). As fewer cells express Thy-1, Tie, Tek and P1H12 it is possible that these antigens may be expressed on endothelial progenitor cells of different stages of maturity in a manner

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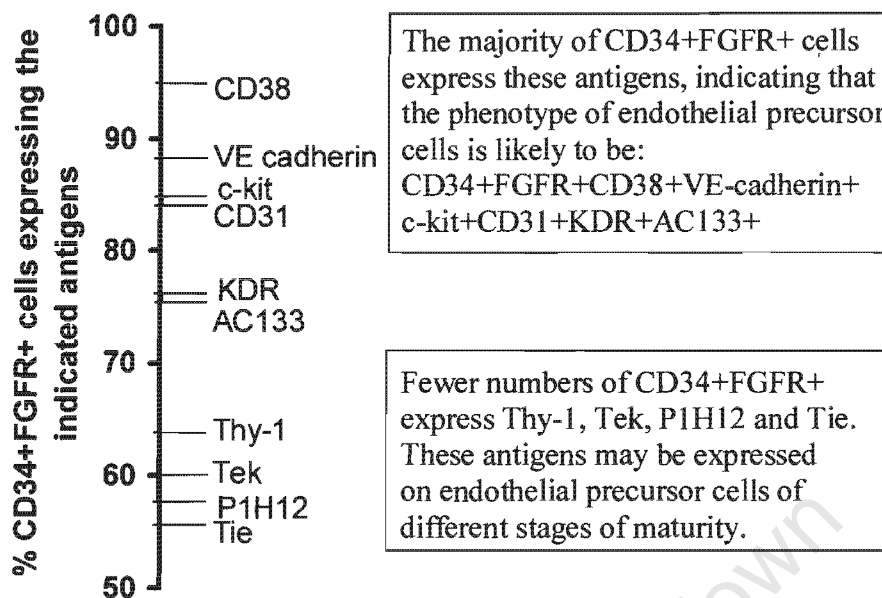

Figure 31. Phenotype of endothelial stem/precursor cells. The incidence of cell surface antigen expression was used to construct a probable phenotype of endothelial stem/precursor cells.

analogous to the expression of various lineage specific markers on haematopoietic cells as they mature along a particular lineage. It is possible that as Thy-1 is expressed by primitive haematopoietic cells, this antigen may be found on immature endothelial progenitors, whereas Tie, Tek and P1H12 may be expressed by endothelial progenitor cells of a more mature nature. During endothelial differentiation of ES cells various antigens are expressed in a sequential fashion e.g., VE-cadherin is expressed earlier than Tie or Tek (Vittet *et al.*, 1996) indicating that Tie and Tek may be expressed by more mature cells. Since P1H12 is expressed by mature endothelial cells as well as by circulating endothelial cells (Solovey *et al.*, 1997), the presence of P1H12 may also be associated with endothelial precursor cells of a more mature nature.

The growth of haematopoietic colonies in methylcellulose medium containing 30% FCS and recombinant human cytokines, indicates that the sorted CD34+FGFR+, CD34+FGFR- and unfractionated CD34+ populations form colonies of various lineages in methylcellulose cultures: erythroid (mainly BFU-E colonies), monocytic (CFU-M),

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mixed granulocytic-monocytic (CFU-GM) and multi-lineage (CFU-GEMM) colonies. The results also show that the CD34+FGFR+, CD34+FGFR- and unfractionated CD34+ populations contain cells with an equivalent repertoire of progenitors (Figure 28). However, it is possible that the CD34+FGFR+ population may contain dormant multipotent progenitor cells that would not form colonies under the assay conditions employed. In order to examine this possibility, the CD34+FGFR+ and CD34+FGFR- populations would need to be cultured under conditions that promote the growth of dormant (stem) cells, such as those used for the blast cell colony assay (Leary and Ogawa, 1987). In a blast cell colony assay, cells are first seeded in primary culture, in methylcellulose medium containing a low concentration of FCS (2%) and growth factors, such as IL-3, are only added after 2 weeks of culture (Leary and Ogawa, 1987). Culturing the cells for the initial 2 weeks in the absence of growth factor results in the death of the progenitor cells, whereas the dormant primitive stem cells survive these harsh culture conditions. After a further 3-4 weeks in culture, cells obtained from blast cell colonies are replated in methylcellulose medium containing 30% FCS and cytokines, similar to the medium used in the experiments described previously, and cultured for 2 weeks before determining the numbers of colonies representing the different haematopoietic lineages. Culture of CD34+FGFR+ and CD34+FGFR- populations in a blast colony assay would determine if CD34+FGFR+ cells contain increased numbers of dormant haematopoietic progenitor cells.

The culture of CD34+FGFR+ and CD34+FGFR- populations under conditions that promote endothelial growth shows a definite difference between these two populations (Table 10; Figure 29). The isolated CD34+FGFR+ cells respond to the angiogenic cytokines, FGF-2 and VEGF (Table 10), and give rise to adherent cells that incorporate DiI-ac-LDL (Figure 30G) and express vWF and VE-cadherin in culture (Figures 29 and 30D, E, F), confirming that this population contains endothelial stem/progenitor cells.

Cells that have the capacity to develop into endothelial cells have recently been isolated from the circulation (Asahara *et al.*, 1997; Fernandez Pujol *et al.*, 2000; Gehling *et al.*, 2000; Ikpeazu *et al.*, 2000; Lin *et al.*, 2000; Murohara *et al.*, 2000; Nieda *et al.*, 1997;

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Peichev *et al.*, 2000; Shi *et al.*, 1998). Previous studies indicating the presence of endothelial precursor cells in cord blood or peripheral blood have used either unfractionated mononuclear cells (Ikpeazu *et al.*, 2000) or samples enriched for CD34+ (Asahara *et al.*, 1997; Boyer *et al.*, 2000; Murohara *et al.*, 2000; Nieda *et al.*, 1997; Peichev *et al.*, 2000), AC133+ (Gehling *et al.*, 2000), P1H12+ (Lin *et al.*, 2000) or CD14+ (Fernandez Pujol *et al.*, 2000) cells. Currently, the phenotype for endothelial progenitor cells is poorly described, as cell surface expression has been determined by either single or two colour flow cytometry. Gehling et al (Gehling *et al.*, 2000) reported that AC133+ cells isolated from leukapheresis samples differentiated into endothelial cells *in vitro*. In this study, AC133+ cells were isolated using an AC133 cell isolation kit (Mitenyi Biotec, Bergisch-Gladbach, Germany) and analysed for the expression of CD34 by two colour FACS, while the presence of additional antigens (KDR, vWF, VE-cadherin, P1H12) on these cells was determined by indirect staining. Indirect staining for the expression of KDR, vWF, VE-cadherin and P1H12 was carried out by the addition of the appropriate primary monoclonal antibodies followed by a biotinylated rabbit anti-mouse secondary antibody and visualized with a streptavidin-alkaline phosphatase conjugate and fast blue stain (Gehling *et al.*, 2000). However, as the magnetic beads used in the AC133 isolation kit are coated with a monoclonal antibody to AC133 and as these beads would still be attached to freshly isolated cells, the results obtained by the indirect staining of the cells reported in this paper would be incorrect, as the secondary (rabbit anti-mouse) antibody (used to detect the various antigens listed above) would also bind to the AC133 monoclonal antibody already present on the beads.

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The results presented in this chapter, using four colour flow cytometry, provide the first detailed description of the phenotype of circulating endothelial precursor cells and indicate for the first time that these primitive cells express FGFR-1. The identification of progenitor cells that express FGFR-1 as well as primitive haematopoietic and endothelial cell antigens and that give rise to endothelial cells *in vitro* is not too surprising as haematopoietic and endothelial cells have a close association during ontogeny. A common embryonic precursor, the haemangioblast, that differentiates into both haematopoietic and endothelial cells has been identified (Hamaguchi *et al.*, 1999) and

experiments using murine embryonic stem cell lines carrying mutations for FGFR-1 show that FGF-2-mediated signalling is essential for haemangioblast proliferation (Faloon *et al.*, 2000) (Figure 21). FGFRs are expressed on pluripotent human embryonic stem cells and embryoid bodies (Schuldiner *et al.*, 2000) as well as on CD34+ haematopoietic cells (Berardi *et al.*, 1995; Burger *et al.*, 1998; Burger *et al.*, 1999; Le Bousse-Kerdiles *et al.*, 1996; Testa *et al.*, 1996) and on leukaemic cell lines, such as K562, U937, Hel, Daudi, MO7E, HL60, Jurket, Molt 3 and TF-1 (Allouche *et al.*, 1995b; Liuzzo and Moscatelli, 1996; Ratajczak *et al.*, 1996). FGF-2 has been shown to promote the self-renewal of a multipotent haematopoietic cell line (Anzai *et al.*, 1999) and the growth of a primitive erythroid cell line (Yuen *et al.*, 1998). FGF-2 also stimulates the growth of early haematopoietic progenitors in synergy with other cytokines (Bruno *et al.*, 1993; Gabbianelli *et al.*, 1990; Gabrilove *et al.*, 1994; Wilson *et al.*, 1991). In addition to its effects on embryonic and haematopoietic cells, FGF-2 is also a potent angiogenic cytokine. It stimulates endothelial cell proliferation (Schweigerer *et al.*, 1987) and is a potent inducer of angiogenesis (Brown *et al.*, 1996; Joseph-Silverstein and Rifkin, 1987; Pepper *et al.*, 1992). FGF-2 is able to induce uncommitted mesoderm to differentiate into endothelial precursors (angioblasts) in the quail embryo (Cox and Poole, 2000; Poole *et al.*, 2001) and is required for the organization of vascular endothelial cells into functional networks in the murine embryo (Leconte *et al.*, 1998). FGF-2 has also been shown to induce the development of blood islands and endothelial cells from dissociated quail epiblasts (Flamme and Risau, 1992) and it stimulates the growth of both endothelial and haematopoietic cells from embryonic avian mesoderm (Pardanaud and Dieterlen-Lievre, 1999). Furthermore, experiments using three-dimensional spheroid models of endothelial cell differentiation have shown that FGF-2 is a survival factor for immature endothelial cells, inhibiting the apoptosis of these cells (Korff and Augustin, 1998). There is therefore considerable evidence that indicates that FGF-2 is a relevant cytokine that affects endothelial cells as well as haemangioblasts and early haematopoietic cells.

Although regarded as an endothelial-specific marker of mature endothelial cells, VE-cadherin is also expressed by embryonic cells that have haemangioblast potential (Nishikawa *et al.*, 1998a; Ogawa *et al.*, 1999) and has been shown to be expressed by day

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5 during endothelial differentiation of ES cells (Vittet *et al.*, 1996). As FGFR-1 and FGF-2 are required for haemangioblast proliferation (Faloon *et al.*, 2000), it is possible that the CD34+FGFR+ cells that express VE-cadherin may be primitive cells that have haemangioblast potential. The simultaneous expression of AC133, a primitive haematopoietic marker (Buhring *et al.*, 1999; de Wynter *et al.*, 1998; Majka *et al.*, 2000; Matsumoto *et al.*, 2000; Miraglia *et al.*, 1997) that is not expressed on differentiated endothelial cells (Peichev *et al.*, 2000; Yin *et al.*, 1997) by CD34+FGFR+VE-cadherin+ cells (Table 9; Figure 26) indicates the primitive nature of these cells. However it is currently unknown whether haemangioblasts exist in post-natal life. In order to determine whether CD34+FGFR+AC133+VE-cadherin+ cells have haemangioblast potential, FACS isolated single cells would have to be examined after culture for evidence of cells of both haematopoietic and endothelial origin.

Summary

I have isolated and characterized a subset of CD34+ cells that expresses FGFR-1, as well as primitive haematopoietic and endothelial cell surface antigens. The CD34+FGFR+ population is present in bone marrow, cord blood and mobilized peripheral blood where it comprises 4.5% of the CD34+ population. As CD34+FGFR+ cells are able to differentiate into endothelial cells under culture conditions that promote endothelial growth, the CD34+FGFR+ population must contain endothelial stem/progenitor cells. Since endothelial stem/progenitor cells have been used to repair sites of vascular injury (Kalka *et al.*, 2000), improve cardiac function (Kocher *et al.*, 2001), and enhance blood flow in diabetic mice after hindlimb ischaemia (Schatteman *et al.*, 2000), CD34+FGFR+ cells may be of clinical benefit in treating cardiovascular disease and diabetes. Recent literature shows that the inoculation of cultured and factor VIII transfected human endothelial progenitor cells into NOD/SCID mice results in the successful engraftment of these cells into the bone marrow and spleen of the recipient animals (Lin *et al.*, 2002). These results suggest that autologous endothelial cells, engineered to produce factor VIII, may be used for treating patients with haemophilia A. The use of engineered endothelial progenitor cells may also be an effective strategy for treating patients with cancer, using gene therapy. CD34+FGFR+ cells may home to the expanding vascular bed of tumours and the isolation

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of these cells from syngeneic individuals may enable the delivery of autologous cells, engineered with angiostatic or anti-tumour agents, to the tumour vasculature. It is therefore possible that this newly described population of cells may be of considerable benefit for the treatment of cancer as well as other diseases in which the vasculature has been damaged, such as diabetes and cardiac disease.

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Suggestions for further studies

The isolation and characterization of a subpopulation of human CD34+ cells that expresses FGFR-1 and that is capable of differentiating into endothelial cells *in vitro*, is a novel finding that can be further explored using a number of approaches.

(1) Can the culture conditions be optimized?

Although CD34+FGFR+ cells grow in an FGF-2- and VEGF-dependent manner, the cells grow very slowly in culture and the culture conditions need to be optimized. In order to do this CD34+FGFR+ cells could be cultured at different cell densities in 96 well plates coated with fibronectin, collagen and /or gelatin to promote endothelial cell adherence. Cells could be cultured in the presence of combinations of varying concentrations of cytokines known to promote endothelial cell proliferation, such as FGF-2, VEGF, SCF, insulin growth factor-1 (IGF-1) and stem cell growth factor (SCGF). Cells could also be seeded on irradiated or mitomycin C treated feeder layers of bone marrow or OP9 cells. The ability of the CD34+FGFR+ population cultured alone or on stromal layers to form endothelial sprouts/tubes in collagen gels, Matrigel and methylcellulose, in the presence and absence of various cytokines would be assessed. The ability of the cells to take up DiI-ac-LDL, an endothelial and monocyte specific process (Voyta *et al.*, 1984) and to express vWF, VE-cadherin, P1H12, CD31, Tie and Tek could be used to determine optimal culture conditions necessary to promote endothelial differentiation. CD34+FGFR- and CD34+ cells would be used as control cell populations.

As well as optimizing the conditions necessary for endothelial differentiation, optimal conditions for the promotion of immature cell proliferation need to be determined. The *in vitro* expansion of CD34+FGFR+ cells would permit the growth of increased numbers of syngeneic endothelial progenitor cells that could be used to coat valves or other surgically implanted devices, repair sites of damaged endothelium or serve as vectors for genetic engineering. CD34+FGFR+ cells could be engineered so as to be able to (1) deliver angiostatic or anti-tumour agents to the rapidly expanding vascular bed of tumours without affecting the endothelium of existing blood vessels, (2) secrete plasminogen activator to

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help prevent re-stenosis after balloon angioplasty, (3) secrete products such as adenosine deaminase or clotting factors, in disorders where there is a deficiency of these factors. The development of methodology for the expansion of syngeneic endothelial progenitor cells may therefore be of benefit to patients with a variety of disorders and may be useful for the therapy of cardiovascular disease, diabetes and cancer.

(2) Can CD34+FGFR+ cells home to damaged endothelium?

It would be interesting to determine whether CD34+FGFR+ cells are able to "home" to damaged endothelium *in vivo*. Human CD34+ cells are incorporated into sites of active angiogenesis in immunodeficient animals (Asahara *et al.*, 1997). In order to ascertain whether CD34+FGFR+ cells can similarly incorporate into damaged endothelium, human CD34+FGFR+ cells, sorted by FACS or immunomagnetically enriched, could be injected into immunodeficient animals in which the femoral artery of one hind limb had previously been ligated to create a unilateral hindlimb ischaemic model. Animals inoculated with CD34+FGFR- or unfractionated CD34+ cells could be used as controls. Experiments showing that CD34+ or FLK-1+ cells contribute to neoangiogenesis used 500,000 or 10,000 cells respectively (Asahara *et al.*, 1997) and therefore the feasibility of performing similar experiments using CD34+FGFR+ cells may be limited by the ability to obtain sufficient viable sorted CD34+FGFR+ cells. As 4.5% of the CD34+ population is CD34+FGFR+ (Table 7) it would be expected that 10^6 CD34+ cells would yield 45,000 CD34+FGFR+ cells. However, as significant numbers of cells are lost and damaged during the sorting procedure, the number of viable sorted CD34+FGFR+ cells obtained from each isolation is only approximately 2,000-10,000/10^6 CD34+ cells. Assuming that the number of CD34+FGFR+ cells available was sufficient for this experiment, dual immunofluorescence could be used to identify the presence of HLA-ABC+ cells of human origin (Berardi *et al.*, 1997) that express endothelial markers such as CD31, Tie, Tek, KDR, VE-cadherin and P1H12, in sections of ischaemic tissue. Other tissues could also be examined to determine whether CD34+FGFR+ cells could function as "plastic" stem cells in which case they may be incorporated into a variety of tissues, as has been noted for other stem cell populations (Gussoni *et al.*, 1999; Jackson *et al.*, 2001; Krause *et al.*, 2001; Lagasse *et al.*, 2000; Theise *et al.*, 2000a). In addition, CD34+FGFR+ cells could be

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labelled before inoculation with the fluorescent probe DiI, which could be used to identify tissue areas to which the CD34+FGFR+ cells home. These experiments would indicate whether CD34+FGFR+ cells could incorporate into areas of damaged endothelium *in vivo* and whether they were "plastic" in the sense that they were able to incorporate into a variety of tissue types, other than the endothelium.

(3) Can CD34+FGFR+ cells be incorporated into sites of tumour angiogenesis?

It has been shown, by the co-inoculation of AC133+ cells and human tumour cells in NOD/SCID mice, that AC133+ progenitor cells give rise to human endothelial cells in the vasculature of murine tumours (Gehling *et al.*, 2000). Similar *in vivo* studies could be performed to show whether CD34+FGFR+ cells can incorporate into sites of tumour angiogenesis and form new tumour blood vessels. Human CD34+FGFR+ cells could be inoculated subcutaneously together with human tumour cells into NOD/SCID mice. After 8 weeks, two colour immunofluorescence could be used to identify human cells expressing endothelial markers. These experiments would determine whether blood vessels in the resulting tumours are of human or murine origin. If CD34+FGFR+ cells are incorporated into tumour vasculature, a future use for these cells may be as genetically engineered vectors for delivering anti-tumour agents to the vascular bed of tumours.

(4) Do CD34+FGFR+ cells contain stromal progenitors?

Based on the expression of STRO-1, an antibody that identifies bone marrow stromal progenitor cells, approximately 5% of bone marrow CD34+ cells are stromal progenitor cells, (Simmons and Torok-Storb, 1991). Human bone marrow derived STRO-1+ cells are multipotential (Dennis *et al.*, 2002; Pittenger *et al.*, 1999) and are able to differentiate into adipocytes, osteoblasts and chondrocytes. It is possible that the CD34+FGFR+ population may contain stromal and/or haematopoietic stem/progenitor cells, in addition to endothelial progenitor cells. To test this hypothesis CD34+FGFR+ cells could be inoculated into sublethally irradiated immunodeficient NOD/SCID mice and thereafter the mouse tissue would be examined for evidence of endothelial, fibroblast and haematopoietic cells of human origin. As sublethally irradiated NOD/SCID mice have been haematopoietically reconstituted with human haematopoietic cells after transplantation of as few as 180-1000

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stem cells (Bertolini *et al*., 1997; Bhatia *et al*., 1997; Larochelle *et al*., 1996), availability of sufficient numbers of viable CD34+FGFR+ cells to perform these experiments should not be problematic. However, as the incidence of stromal progenitors within the CD34+FGFR+ population is currently unknown, sufficient stromal progenitors for reconstitution may not be present in this population.

(5) Are CD34+FGFR+ cell haemangioblasts?

As FGFR-1 is expressed by haemangioblasts (Faloon *et al*., 2000) and as the CD34+FGFR+ population expresses antigens associated with primitive haematopoietic and endothelial cells, it would be interesting to determine whether these cells express other haemangioblast-related cell surface antigens or transcription factors. The expression of PCLP1, a marker for haemangioblasts (Hara *et al*., 1999) or CD166 (HCA/ALCAM), an adhesion molecule that is involved in capillary tube formation and haemangioblast differentiation (Cortes *et al*., 1999; Ohneda *et al*., 2001; Uchida *et al*., 1997) by CD34+FGFR+ cells could be determined. In addition the expression of transcription factors associated with ES cells and early haematopoietic cells, such as SCL/Tal 1, LMO 2, Gata-1, Gata-2 and AML1 (Kaufman *et al*., 2001; Robertson *et al*., 2000) by CD34+FGFR+ could be studied. In order to ascertain whether CD34+FGFR+ cells are, in fact, haemangioblasts one could seed sorted CD34+FGFR+ cells, by single cell deposition, into microtitre wells with medium containing factors promoting haematopoietic and endothelial growth, such as IL-3 and angiopoietin-1 (Hamaguchi *et al*., 1999), as well as FGF-2 and VEGF. Wells would be examined for the presence of haematopoietic colonies and the expression of surface antigens of the granulocytic, monocytic and erythroid lineages would be determined. In addition evidence of endothelial differentiation i.e., expression of vWF and/or uptake of DiI-ac-LDL (Voyta *et al*., 1984) would be assessed. The ability of single CD34+FGFR+ cells to give rise to both haematopoietic and endothelial cells would indicate that these cells are haemangioblasts and would be a novel finding, as there is no current evidence indicating that haemangioblasts exist in post-natal life.

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(6) Are CD34+FGFR+ cells primitive "plastic" stem cells that can home to a variety of organs and give rise to differentiated progeny?

Recent reports show that haematopoietic stem cells possess amazing plasticity and are able to give rise to liver, skeletal muscle, cardiac muscle, vascular endothelium and epithelial cells of lung, gastrointestinal tract, and skin (Gussoni *et al.*, 1999; Jackson *et al.*, 2001; Krause *et al.*, 2001; Lagasse *et al.*, 2000; Theise *et al.*, 2000a). In order to determine whether the CD34+FGFR+ population may be similarly pluripotent, the potential plasticity of these cells could be assessed. FACS sorted human CD34+FGFR+ cells could be injected into NOD/SCID mice and evidence of cells of human (HLA-ABC+) origin determined in histological sections of mouse tissue, at various times after transplantation. This would determine whether CD34+FGFR+ cells home to a variety of organs and differentiate into a number of tissues. In addition, CD34+FGFR+ cells, isolated from the bone marrow of adult green fluorescent protein (GFP) (Hadjantonakis *et al.*, 1998)-expressing or ß-galactosidase (Soriano *et al.*, 1986)-expressing mice could be transplanted into blastocysts flushed from the uteri of newly pregnant mice. The extent to which the transplanted cells contribute to the development of various organs of the developing progeny could be ascertained by examing host cells for evidence of GFP or ß-galactosidase.

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(7) Are CD34+FGFR+ cells primitive "ES-like" cells?

In order to determine whether CD34+FGFR+ cells are as primitive as ES cells, human or murine CD34+FGFR+ cells could be cultured under conditions used to grow ES cells, on embryonic fibroblast feeder layers in the presence of leukaemia inhibitory factor (LIF) and FGF-2 (Chen, 1992; Keller, 1995). The putative "ES cells" could be cultured without LIF to determine whether these cells are capable of forming "embryoid bodies" ("EBs"). If obtained, the "EBs" could be dissociated with collagenase after 4-10 days of culture and grown as adherent cells in the presence of various growth factors that promote differentiation into cells that express ectodermal (skin, brain), endothelial (liver, pancreas, gut) or mesodermal (endothelial, fibroblastic, myoblastic or haematopoietic) properties.

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