EFFECTS OF VEGFR-2 SIGNALLING IN POST-NATAL HEMATOPOIESIS AND VASCULOGENESIS

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Abstract

Vascular endothelial growth factor (VEGF) and its receptors play an essential role in the formation and maintenance of the hematopoietic and vascular compartments. Activation of the kinase activity of the VEGF receptor-2 (VEGFR-2) is triggered by binding to VEGF, which affects endothelial cell proliferation, permeability, and migration. Accumulating evidence suggests that VEGFR-2 signalling may play an important role in post-natal hematopoiesis and vasculogenesis. One of the goals of this work was to study some of the biological effects triggered by VEGFR-2 in isolation, without the interference of other VEGF receptors in the contexts of post-natal hematopoiesis and vasculogenesis. By inducing expression of the full length VEGFR-2 or of a VEGFR-2 construct that can be selectively activated in fibroblasts or hematopoietic progenitors, we show that VEGFR-2 can induce activation of the Erk1/2 mitogen activated protein (MAP) kinase, p38 MAP kinase and Akt signalling pathways. Moreover, VEGFR-2 activation can elicit biological responses such as cell proliferation, migration and survival *in vitro*. Using a bone marrow transplantation model, we also show that VEGFR-2 activation promotes the expansion of myeloid cells *in vivo*, in part through the up-regulation of the hematopoietic cytokine Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF). In the second part of the thesis, we confirm the existence of early endothelial progenitors in mice. These cells originate from the bone marrow and can integrate in the vasculature of tumours, although at a low frequency. VEGF does not modulate the occurrence or mobilization of these progenitors. We also demonstrate that these cells originate from hematopoietic stem cells and that they arise by

cell differentiation, and not through cell fusion. The work presented in this thesis, by elucidating some of the effects triggered by VEGF signalling though VEGFR-2 in hematopoietic cells, could potentially lead to the development of therapies targeting the growth of malignant hematopoietic cells.

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List of Abbreviations

5-FU	5-fluorouracil
AcLDL	acetylated low density lipoprotein
AGM	aorta-gonad-mesonephros
AML	acute myeloid leukemia
BFU-E	burst forming unit erythroid
BL-CFC	blast colony forming cell
BM	bone marrow
BSA	bovine serum albumin
CD	cluster of differentiation
CFU-EC	colony forming unit endothelial cell
CFU-G	colony forming unit granulocyte
CFU-GEMM	colony forming unit granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	colony forming unit granulocyte macrophage
CFU-M	colony forming unit macrophage
CFU-S	colony forming unit spleen
CML	chronic myeloid leukemia
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
Erk	extracellular-regulated kinase
ES	embryonic stem
Ets-1	endothelial specific transcription factor
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
bFGF	basic fibroblast growth factor
FGFR	fibroblast growth factor receptor
FKBP	FK506 binding protein
GAPDH	glyceraldehydes-3-phosphate-dehydrogenase
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GM-CSFR	granulocyte-macrophage colony stimulating factor receptor
Gy	Grey
HA	hemagglutinin
HFF	human foreskin fibroblast
HIF	hypoxia inducible factor
HMEC	human microvascular endothelial cell
HSC	hematopoietic stem cell
HSP	heat shock protein
HUVEC	human umbilical vein endothelial cell
IAP	inhibitor of apoptosis
Ig	immunoglobulin

IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IRES	internal ribosomal entry site
JNK	c-Jun NH_2-terminal kinase
kDa	kiloDalton
KDR	kinase domain region
Lin^-	lineage negative
MAPK	mitogen activated protein kinase
MBP	myelin basic protein
M-CSF	macrophage colony stimulating factor
MIG	mscv ires gfp
MMP	matrix metalloproteinase
MSCV	murine stem cell virus
MTT	3-(4',5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	nitric oxide
PAS	para-aortic-splanchnopleura
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDZ	PSD- 95/Dlg/ZO-1
PI3-kinase	phosphatidylinositol-3 kinase
PK	protein kinase
PLC	phospholipase C
PlGF	placenta growth factor
RAFTK	related adhesion-focal tyrosine kinase
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SAPK	stress-activated protein kinase
SCF	stem cell factor
SCL	stem cell leukemia
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SP	side population
TGF-β	transforming growth factor β
TNF-α	tumour necrosis factor-α
Tpo	thrombopoietin
UTR	untranslated region
VE-cadherin	vascular-endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
vHL	von Hippel-Lindau
VPF	vascular permeability factor
vWF	von Willebrand factor

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Chapter 1

Introduction

1.1 Embryonic origins of hematopoiesis and vasculogenesis

1.1.1 Yolk sac hematopoiesis and vasculogenesis

The development of a functional circulatory system is an early prerequisite for the survival and growth of the mammalian embryo. The first differentiated cells to form in the mammalian embryo are those of the hematopoietic and endothelial lineages, which, along with cardiac components, are the backbone of the developing circulatory system (Ema and Rossant, 2003). Cells from the hematopoietic and endothelial lineages are mesodermal in origin and are first generated in yolk sac blood islands beginning at embryonic day 7 (E7.0) in the mouse (Haar and Ackerman, 1971) and between the second and third week of human gestation. Thereafter, hematopoiesis shifts to the fetal liver and again, around the time of birth, to the bone marrow (Moore and Metcalf, 1970). The generation of blood cells in blood islands of the yolk sac is referred as primitive hematopoiesis and results primarily in the production of large, nucleated erythroblasts (Wong et al., 1986), as well as some megakaryocytes (Xu et al., 2001) and primitive macrophages (Shepard and Zon, 2000), as opposed to definitive hematopoiesis, which can generate mature cells of all the blood lineages.

Blood islands, which consist of a central focus of hematopoietic cells surrounded by a layer of endothelial cells, arise in the mouse from proximal mesodermal cells in the visceral yolk sac between E7.0 and E7.5 (Shepard and Zon, 2000).

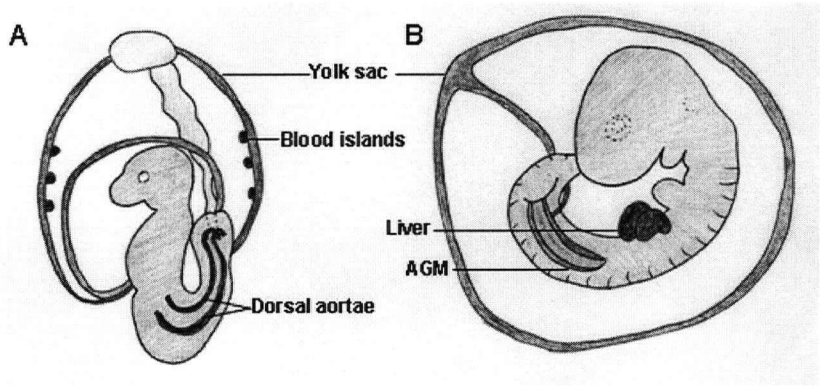

Figure 1 Hematopoietic sites in the developing mouse embryo. Schematic representation of whole mouse embryos at (a) E8.5/9 and (b) E10.5/11. Abbreviations: AGM, aorta-gonad-mesonephros. Adapted from Dzierzak et al. (Dzierzak et al., 1998).

Between E8.0 and E9.0, the cells comprising the outer layer of the blood island cell aggregates assume a spindle shape as they differentiate into endothelial cells (Shepard and Zon, 2000). The vast majority of the inner cells of the blood islands progressively lose their intercellular attachments as they differentiate into primitive erythroblasts. The simultaneous spatial and temporal appearance of hematopoietic and endothelial cells in the yolk sac blood islands has led to the concept of the hemangioblast, a common precursor for the hematopoietic and endothelial lineages (Ema and Rossant, 2003). Moreover, many known markers of endothelial cells are also expressed on hematopoietic cells (Fina et al., 1990; Kallianpur et al., 1994; Matthews et al., 1991). Recent studies with cells from chick embryos and with embryonic stem cells have led credence to this concept (Eichmann et al., 1997).

While histological studies indicate a restricted hematopoietic potential within the yolk sac, precursor analysis *in vitro* and transplantation studies *in vivo* have provided clear evidence that this tissue is able to generate multiple definitive lineages as well (Yoder and Hiatt, 1997). Macrophage precursors were detected in low numbers as early

as those of the primitive erythroid lineage (Takahashi and Naito, 1993). Definitive erythroid precursors were found at E8.25 and also showed a dramatic increase followed by a general decline of their numbers (Palis et al., 1999). Precursors of the mast cell lineage developed slightly later at E8.5 (Palis et al., 1999). Unlike primitive erythroblasts, definitive erythroid precursors and mast cell precursors do not mature in the yolk sac, suggesting that they are produced for export to other sites, presumably the fetal liver (Palis et al., 1999). These findings suggest a dual role for the yolk sac: the generation of a functional primitive erythroid lineage as well as the production of a cohort of definitive precursors which migrate to the fetal liver and establish the initial stage of definitive hematopoiesis in this tissue (Galloway and Zon, 2003). Yolk sac hematopoiesis is transient and shows a dramatic decline in activity between E11 and E12. This decline coincides with the onset of activity in the developing liver, which becomes the predominant hematopoietic tissue throughout the remainder of fetal life. In contrast to the restricted program observed in the yolk sac, the fetal liver is a site of multilineage definitive hematopoiesis which includes erythropoiesis, myelopoiesis and lymphopoiesis (Galloway and Zon, 2003).

1.1.2 The formation and maturation of definitive hematopoietic cells

Shortly before the onset of organogenesis the embryo starts to generate a transitory population of embryonic hematopoietic cells that serve its immediate needs. These first hematopoietic cells, consisting mainly of primitive erythroid cells, appear in the embryonic circulation in growing numbers and then colonize the initially inactive fetal liver (Medvinsky and Dzierzak, 1996). Definitive hematopoiesis, which results in the production of all hematopoietic lineages, develops slightly later and gradually forms a

massive pool in the fetal liver, which becomes the main source of hematopoietic stem cells which subsequently colonize the bone marrow (Dzierzak et al., 1998). Hematopoietic stem cells are defined within the context of stringent transplantation assays used for adult bone marrow cells and are characterized by the following properties: 1) they clonally give rise to all differentiated lineages of the blood cells; 2) they are self-renewing; 3) they possess high proliferative/expansion potential contributing to high level hematopoietic repopulation of the recipient; and 4) they are active long term/over the lifespan of the individual (Wognum et al., 2003). Definitive hematopoietic stem cells are produced both in the mature yolk sac and within the para-aortic splanchnopleura (PAS, E8.5-E9.5) and the aorta-gonad-mesonephros region (AGM, E10.5-E11.5) (Galloway and Zon, 2003). When AGM, yolk sac and fetal liver cells are directly compared throughout development for repopulation capabilities by the criteria previously mentioned, the AGM region consistently demonstrates more hematopoietic stem cells than the yolk sac or fetal liver at E10 and E11 (Muller et al., 1994). The definitive hematopoietic stem cells formed in the yolk sac and the AGM region do not mature *in situ* but instead are believed to migrate and seed the fetal liver, where they undergo terminal differentiation (Palis and Yoder, 2001). Thus, within the yolk sac, there is a temporal, if not spatial, overlap between primitive and definitive hematopoiesis.

Although it was previously believed that the definitive hematopoietic stem cells formed in the yolk sac contribute only to primitive hematopoiesis (Muller et al., 1994), it was later shown that yolk sac cells isolated around day 9 and later can engraft and repopulate recipient animals following transplantation into the livers of newborn mice (Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder et al., 1997b). Furthermore, yolk sac

hematopoietic cells have the ability to repopulate adult bone marrow following coculture on certain types of stromal cells (Matsuoka et al., 2001) or when HoxB4 is ectopically expressed (Kyba et al., 2002). Yolk sac and AGM definitive hematopoietic stem cells appear to circulate (Kumaravelu et al., 2002) and can presumably seed intraembryonic tissues such as the liver and large arteries.

1.1.3 The hemangioblast: a common progenitor of hematopoiesis and vasculogenesis

The close spatial and temporal appearance of hematopoietic and endothelial cells in the embryo has led to the hypothesis that a common precursor exists for both of these lineages. The term hemangioblast was first introduced to describe discrete cell masses that develop in chick embryo cultures and displayed both hematopoietic and endothelial potential (Haar and Ackerman, 1971). Since originally introduced, the concept of the hemangioblast has gained support from studies demonstrating that the hematopoietic and endothelial lineages share expression of a number of different genes such as MB1/QH1 in the quail (Pardanaud et al., 1987; Peault et al., 1983), CD31, CD34, SCL/Tal-1 and VEGFR-2 (Kabrun et al., 1997; Kallianpur et al., 1994; Watt et al., 1995; Young et al., 1995). Gene targeting studies in the mouse have provided further evidence for this bi-potential precursor in showing that some of these genes are essential for the development of both lineages (Robb et al., 1995; Shalaby et al., 1995; Shivdasani et al., 1995). Furthermore, overexpression of SCL/Tal-1 in zebrafish embryos results in overproduction of common hematopoietic and endothelial precursors, perturbation of vasculogenesis and concomitant loss of pronephric duct and somitic tissue (Gering et al., 1998). Analysis of knock-in embryos in which SCL/Tal-1 is expressed under the control of the VEGFR-2 locus suggests that VEGFR-2 and SCL/Tal-1 act in combination to

regulate cell fate decisions for formation of endothelial and hematopoietic cells in early development (Ema et al., 2003). Additional support for the existence of the hemangioblast comes from analysis of a specific mutation in zebrafish known as *cloche*, which affects the development of hematopoietic cells and endocardium (Stainier et al., 1995).

The most compelling and direct evidence for the presence of the hemangioblast comes from recent studies utilizing the *in vitro* ES cell system (Choi et al., 1998). Embryoid bodies differentiated for 2.5-3.5 days contain a unique cell population, the blast colony-forming cell (BL-CFC). BL-CFCs form blast colonies in the presence of vascular endothelial growth factor (VEGF) in methylcellulose cultures (Choi et al., 1998). Gene expression analysis indicates that cells within blast colonies express a number of genes common to both hematopoietic and endothelial lineages, including SCL, CD34, and the VEGF receptor-2 (VEGFR-2) (Kennedy et al., 1997a). Most importantly, BL-CFCs give rise to primitive, definitive hematopoietic and endothelial cells when replated in medium with both hematopoietic and endothelial cell growth factors (Choi et al., 1998; Kennedy et al., 1997a). Moreover, in the quail embryo, VEGFR-2^+ cells isolated from the mesoderm can give rise to cells of the hematopoietic or endothelial lineages depending on the culture conditions (Eichmann et al., 1997). Blast colonies have recently been shown to give rise to a third lineage, the smooth muscle cell (Ema et al., 2003). These characteristics of the BL-CFC suggest that it represents the *in vitro* equivalent of the hemangioblast and, as such, one of the earliest stages of hematovascular development described to date.

BL-CFCs have been detected in small numbers in dissected mouse embryos (Baron, 2003), suggesting that hemangioblasts may form during normal mouse development. The technical difficulties encountered in identifying cells with the properties of the hemangioblast in mouse embryos suggests that these cells are produced in very small numbers and for a very short time, indicating that these cells might be short-lived, differentiating soon after their formation.

1.2 Hematopoiesis and Vasculogenesis in the adult

1.2.1. The hematopoietic stem cell

The scale of the hematopoietic system is quite remarkable, considering that daily some 2 x 10^{11} erythrocytes and 5 x 10^{10} granulocytes, in addition to platelets, lymphocytes, and monocytes enter the circulation (Finch et al., 1977). Despite the magnitude of this production system, dysregulation is uncommon and external influences can rapidly induce changes in the blood cell count of a specific lineage. For example, hypoxia can induce an increase in erythrocyte production, but does not affect the neutrophil count, whereas the opposite is true in an acute bacterial infection. The regulation of hematopoiesis must be exquisitely fine to be able to maintain blood cells counts within a relatively narrow range. This prodigious output of mature cells is ultimately dependent on the precise regulation of primitive hematopoietic stem cells, which, in turn, give rise to an ordered series of transit populations of progenitor and precursor cells of progressively more restricted proliferative and differentiative potentiality. By definition, a hematopoietic stem cell is a cell isolated from the blood or bone marrow that can renew itself, can differentiate to a variety of specialized cells, can

mobilize out of the bone marrow into circulating blood and can undergo apoptosis (Krause, 2002). Definitive proof of the existence of a hematopoietic stem cell requires the demonstration of its ability to produce a long-lasting multilineage clone in vivo. Identifying and characterizing the properties of hematopoietic stem cells has been a formidable task, since it is estimated that about 1 in every 10,000 to 15,000 bone marrow cells is thought to be a stem cell (Coulombel, 2004). In the bloodstream, the proportion falls to 1 in 100,000 blood cells (Coulombel, 2004).

Despite the immense burden of producing over 10^{11} cells per day in the human adult, the great majority of stem cells are not dividing at any one time (Lajtha et al., 1971). The current picture of the stem cell pool is that of a small, but potent group of cells, able to maintain tremendous hematopoietic cell supplies through the division of a small fraction of its members, keeping the remainder of the stem cells in reserve. There appear to be two kinds of hematopoietic stem cells. If bone marrow cells from a transplanted mouse can, in turn, be transplanted to another lethally irradiated mouse and restore its hematopoietic system over some months, they are considered to be *long-term stem cells* that are capable of self-renewal (Coulombel, 2004). Other cells from bone marrow can immediately regenerate all the different types of blood cells, but under normal circumstances cannot renew themselves over the long term, and these are referred to as short-term progenitor or precursor cells (Coulombel, 2004). Progenitor or precursor cells are relatively immature cells that are precursors to a fully differentiated cell of the same tissue type. They are capable of proliferating, but they have a limited capacity to differentiate into more than one cell type, as hematopoietic stem cells do (Messner, 1998).

The primitive hematopoietic stem cells and their immediate progeny are morphologically indistinguishable (Messner, 1998). Despite continued refinement of multiparameter cell separation strategies, unique phenotypic markers that are able to unmistakably characterize, resolve, and purify hematopoietic stem cells have not been identified. However, several makers have been used in combination in order to separate hematopoietic stem cells. Some of the markers most frequently used to separate human and murine hematopoietic stem cells are listed in Table 1 (Wognum et al., 2003).

Table I Commonly used markers for the purification of hematopoietic stem cells in human and mouse. Lin: Lineage markers (CD11b, B220, Gr-1, TER119)

Mouse	**Human**
CD34 $^{low/-}$	CD34 $^{+}$
Sca-1 $^{+}$	CD59 $^{+}$
Thy1 $^{+/low}$	Thy1 $^{+}$
CD38 $^{+}$	CD38 $^{low/-}$
c-kit $^{+}$	c-kit $^{-/low}$
Lin $^{-}$	Lin $^{-}$
FGFR $^{+}$	CD133
CD201	VEGFR-1
VEGFR-1	VEGFR-2

Fluorescent dye staining approaches have also been used to define hematopoietic stem cells based on efflux capacity and staining profile. The combination of both Rhodamine 123 and Hoechst 33342 staining has demonstrated that Hoechst^{low} –

Rhodamine^{low} cells are highly enriched for hematopoietic stem cell activity (Goodell et al., 1996). A dual-wavelength flow cytometric analysis of bone marrow based on differential Hoechst 33342 staining that identifies a "side population" (SP) which gives rise to all mature blood lineages in transplanted mice has attracted much interest in the field of hematopoietic stem cells (Goodell et al., 1996).

To detect hematopoietic stem cell functional activity, *in vivo* transplantation is generally considered to be the most appropriate assay because the ability of cells to reconstitute blood cell production in a myeloablated recipient measures the capacity of stem cells for both extensive proliferation and multilineage differentiation over extended time periods (Coulombel, 2004).

1.2.2 Hematopoietic hierarchy

The bone marrow contains a bewildering array of dividing and intermediate or immature cell types. These immature types can be recognized by morphology, antibodies to specific surface markers or stains. In this way, it is possible to arrange the immature cells into lineages and construct a path by which an immature cell becomes fully mature.

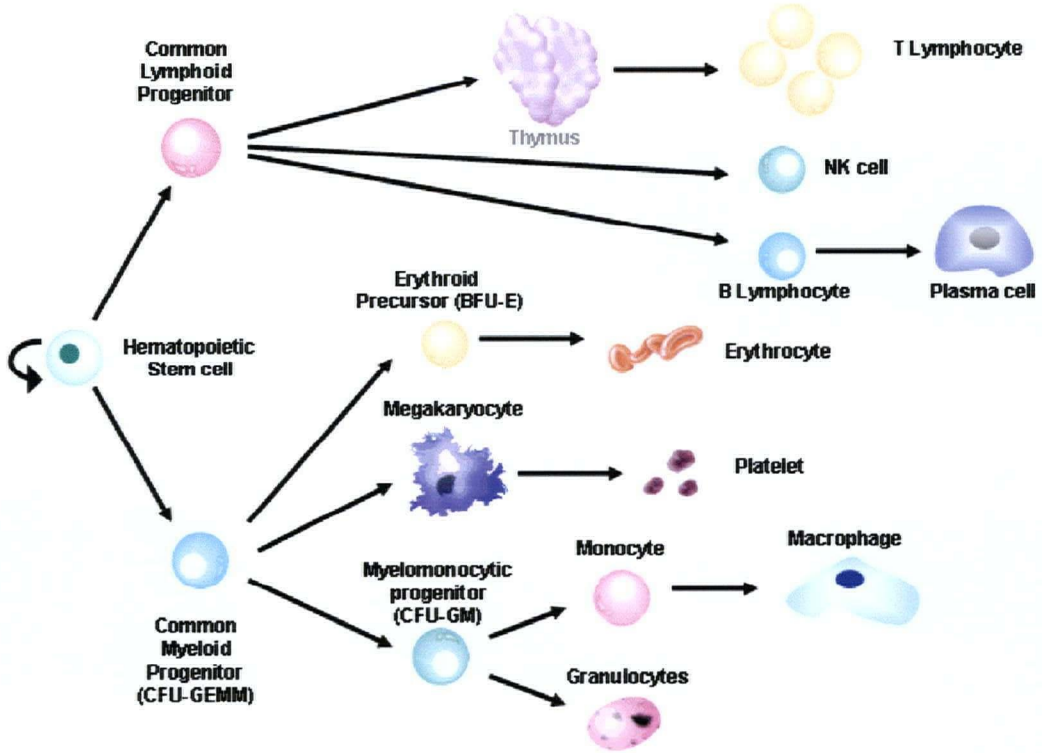

Figure 2 Schematic representation of the hematopoietic hierarchy: all the final mature blood cells (extreme right) are derived from a multipotential hematopoietic stem cell (extreme left). Self-renewal potential decreases from left to right co-ordinately with increasing differentiation; therefore, cells on the left are more primitive than those on the right.

Successive divisions of the hematopoietic stem cell generate a series of lineage-limited divisions, and differentiation generates the end cells. The mature bone marrow cells within each cell lineage are believed to derive from a small pool of undifferentiated cells (progenitors), whose destiny is to divide and differentiate along a single hematopoietic pathway, resulting in the unique production of mature, differentiated cells (Messner, 1998). When hematopoietic progenitors are placed into *in vitro* cultures in methylcellulose in the presence of optimal combinations of hematopoietic growth factors, each progenitor cell repeatedly divides and differentiates, giving rise to colonies of one or

more hematopoietic lineages. Progenitor cells constitute approximatively 0.1% of total bone marrow mononuclear cells (Messner, 1998). Progenitors for B and T cells appear to diverge early and follow separate modes and sites of differentiation: progeny of B-committed progenitors differentiate partly in the bone marrow, then migrate to the lymph nodes. T cell progenitors migrate to the thymus where independent proliferation, differentiation, and selection for immune function take place (Petrie, 2003). The common myeloid progenitors (CFU-GEMM) are unipotential progenitors committed to granulocytopoiesis, erythropoiesis and megakaryocytopoiesis. The granulocytic colonies which emerge *in vitro* are usually made up of both neutrophils and monocytes and originate from a single bipotential cell, named CFU-GM (Barreda et al., 2004). Progenitor cells committed to the erythroid cell line will also grow *in vitro* but will differentiate to haemoglobin-containing erythroblasts only if erythropoietin is present (Koury et al., 2002).

1.2.3 Regulation of hematopoiesis

The regulation of lineage commitment and expansion is clearly physiologically critical, since this determines what sorts of blood cells are finally produced by the bone marrow. Hematopoietic stem cells either self-renew, thereby maintaining stem cell properties, or alternatively give rise to cells increasingly committed to differentiation into various hematopoietic lineages. It is unclear if this cell fate decision is controlled by a purely stochastic mechanism or is the result of environmental cues mediated at least in part through specific receptor-ligand interaction. It is likely that cell fate is influenced both by the stochastic nature of gene expression and by soluble factors and cell-cell interactions.

The processes of self-renewal and differentiation occur *in vivo* within bone marrow microenvironments. These niches are populated by densely packed differentiating hematopoietic cells and by stromal endothelial cells, fibroblasts and adipocytes, as well as macrophages and lymphocytes (Sensebe et al., 1997). These stromal cells not only provide physical support and points of adhesion, but actually direct the processes of hematopoietic differentiation, by secreting positive hematopoietic growth factors such as colony stimulating factors, tyrosine kinase receptor ligands, or negative regulators such as transforming growth factor β (TGF-β) (Ogawa, 1993; Sensebe et al., 1997). These cytokines can act independently or in combination to maintain or stimulate hematopoietic stem cell lineage commitment by binding to cell surface receptors. Some cytokines are known to have various biological functions on different types of progenitors, but how they elicit these functions and what signal transduction pathways they activate remains unclear.

In addition to cytokines, lineage commitment and maturation of hematopoietic cells is driven by the action of a few lineage-specific transcription factors (Friedman, 2002). As cells differentiate, there is an orchestrated silencing of some genes and activation of others. Experimental data suggest that pluripotent stem/progenitor cells are primed to differentiate down several different lineages by low level transcription of many genes that are characteristic of multiple independent discrete lineages. Over the past years, gene targeting experiments have demonstrated the essential functions of individual transcription factors in specifying commitment to the mature blood lineages. Transcription factors such as SCL/Tal-1, LMO2 and GATA-2 are required for the formation and maintenance of stem cells (Robb et al., 1996; Tsai et al., 1994; Yamada et

al., 1998). GATA-1 and PU.1 serve as the dominant factors for erythroid/megakaryocytic and myeloid development, respectively (Nerlov and Graf, 1998; Rekhtman et al., 1999). Other transcription factors such as GATA-3, Ikaros (T-lymphoid cells) and Pax-5 (B-lymphoid cells) are required for normal lymphopoiesis (Busslinger, 2004; Pai et al., 2003; Smith and Sigvardsson, 2004).

Other proteins, such as the Notch receptors, play a central role in the fate decisions of multipotent precursor cells. The Notch pathway is an evolutionarily conserved mechanism that plays a fundamental role in regulating cell-fate decisions of various types of progenitors in both invertebrates and vertebrates (Harper et al., 2003). Notch signalling is involved in multiple developmental processes, including neurogenesis, myogenesis, eye development, and oogenesis (Artavanis-Tsakonas et al., 1999). Hematopoietic cells and bone marrow stromal cells have been shown to express Notch receptors and their ligands, and Notch signalling affects the survival, proliferation and fate choices of progenitors at various stages of hematopoietic development (Ohishi et al., 2003), including whether hematopoietic stem cells self-renew or differentiate (Varnum-Finney et al., 2000), whether common lymphoid precursors undergo T or B cell differentiation (Izon et al., 2002), and whether monocytes differentiate into macrophages or dendritic cells (Ohishi et al., 2001). These observations suggest that Notch signalling can play a fundamental role in regulating hematopoietic development.

1.2.4 The angioblast and evidence of vasculogenesis in the adult

Until recently, neovascularization in the adult was thought to occur by angiogenesis only, which represents the sprouting of new blood vessels from pre-existing blood vessels (Tonini et al., 2003). The other means of neovascularization,

vasculogenesis, which refers to the *de novo* formation of blood vessels from endothelial progenitors (angioblasts), was thought to occur only in the embryo (Figure-3).

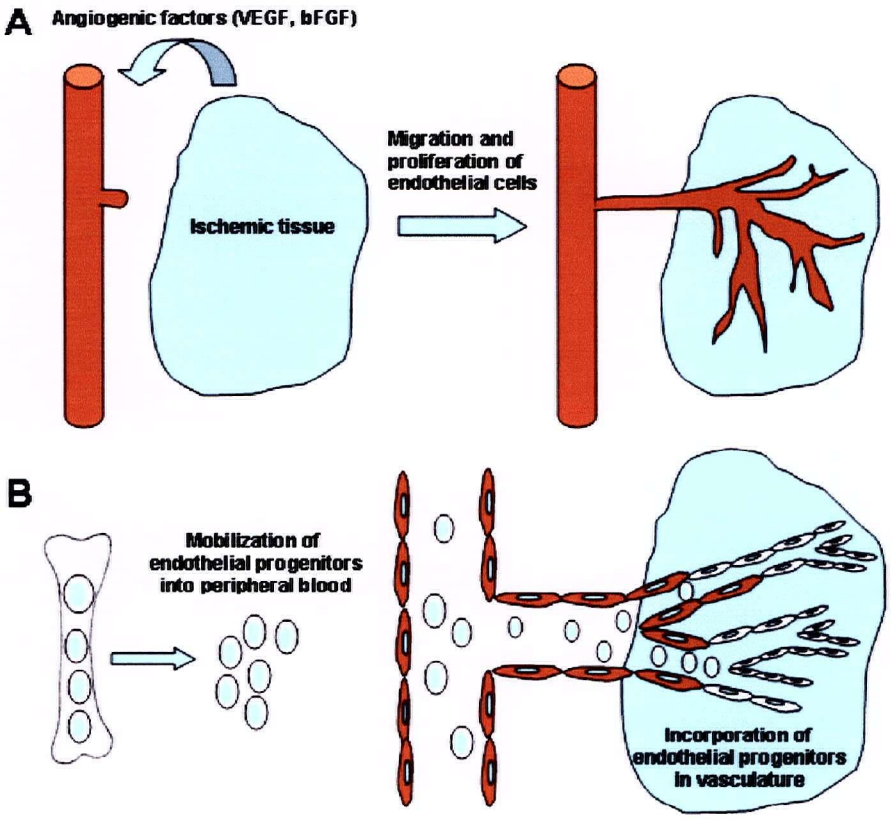

Figure 3 Current concepts for neovascularization in adult ischemic tissues. Angiogenesis (A) and vasculogenesis (B).

In recent years, several groups have shown that such endothelial progenitors can be isolated from adult sources such as peripheral blood, bone marrow and umbilical cord blood mononuclear cells (Asahara et al., 1997; Nieda et al., 1997; Peichev et al., 2000; Shi et al., 1998). Likewise hematopoietic cells positive for the hematopoietic stem cell marker CD133 (previously named AC133) are capable of differentiating into endothelial cells *in vitro* (Gehling et al., 2000).

Under the current status, it is impossible to differentiate endothelial progenitors from hematopoietic cells or endothelial cells, since the markers used to isolate endothelial progenitors are also expressed on subsets of hematopoietic cells (CD133, CD34) and endothelial cells (VEGFR-2, VE-cadherin) (Bhatia, 2001). In circulation, the endothelial progenitors are considered to be included in the cell population expressing CD133 and VEGFR-2 markers within the subset of $CD34^+$ cells (Gill et al., 2001). When isolated and cultured *in vitro*, $CD133^+VEGFR\text{-}2^+$ endothelial progenitors loose expression of CD133, but acquire features of mature endothelial cells, such as cobblestone morphology, uptake of acetylated low density lipoproteins (AcLDL) and expression of von Willebrand factor (Peichev et al., 2000). Mature endothelial cells derived *in vitro* from endothelial progenitors also display higher proliferative potential than endothelial cells such as HUVECs (human umbilical vein endothelial cells) (Quirici et al., 2001). It has also been reported that VEGFR-3 and CD133 identify a population of lymphatic endothelial progenitors in fetal liver, bone marrow and peripheral blood (Salven et al., 2003). Table II displays some of the markers found to be expressed on endothelial progenitors, and how these markers overlap with markers found on endothelial cells and subsets of hematopoietic cells.

Table II Common markers expressed on human endothelial progenitors, mature endothelial cells and subsets of hematopoietic cells.

Surface antigen	Hematopoietic cells	Endothelial progenitors	Mature endothelial cells
CD133	Subset (primitive hematopoietic stem cells)	+	-
CD117 (cKit)	Subset (Hematopoietic stem/progenitor cells)	+	+
CD34	Subset (Hematopoietic stem/progenitor cells)	+	+
VEGFR-1	Subset	?	+
VEGFR-2	Subset	+	+
VEGFR-3	?	Lymphatic endothelial progenitors	+ (lymphatic endothelial cells)
Tie-1	?	?	+
Tie-2	Subset	-	+
VE-cadherin	-	+	+
CD31 (PECAM)	+	+	+
AcLDL uptake	Subset (mainly monocytes/macrophages)	+	+
E-selectin	-	-	+ (activated endothelium)

Some groups have demonstrated in a mouse model that the recruitment of bone marrow-derived endothelial precursors plays an essential role in tumour vascularization and tumour growth (Lyden et al., 2001). These bone marrow-derived endothelial cells are thought to have the capacity to form new vessels in cancer and ischemic tissues. The relative contribution of bone marrow-derived endothelial cells to tumour vasculature remains unclear however. Studies report a relative contribution of bone marrow-derived endothelial progenitors to neovessel formation ranging from 5 to 25% in response to granulation tissue formation (Crosby et al., 2000) or growth factor-induced neovascularization (Murayama et al., 2002). In tumour neovascularization, the reported ranges are from 35-65% higher than the former events (Hilbe et al., 2004; Lyden et al.,

2001; Rafii et al., 2002). This issue remains controversial however, as recent studies report no significant contribution of bone marrow-derived cells to tumour vascularization (Gothert et al., 2004a; Machein et al., 2003; Rajantie et al., 2004).

1.3 Vascular Endothelial Growth factor (VEGF)

The formation and maintenance of the vascular system is a fundamental requirement for organ development and differentiation during embryonic life and for processes such as wound healing and reproduction in the adult. Many types of cancer cells secrete angiogenic factors that attract endothelial cells to neovascularize solid tumours (Dvorak, 2002; Folkman, 1995). Angiogenesis is the formation of a new vascular network from preexisting vessels. Other than cancer, abnormal angiogenesis is implicated in diseases such as rheumatoid arthritis, diabetic retinopathy and psoriasis (Folkman, 1995). In contrast, in ischemic diseases such as angina and peripheral vascular disease, the relative lack of neovasculature may worsen symptoms. Inhibition or promotion of angiogenesis may therefore offer a new approach for the treatment of many diseases.

Many secreted molecules have been shown to promote angiogenesis, including transforming growth factors (TGF) α and β, tumour necrosis factor α (TNF-α), angiogenin, interleukin 8 (Folkman and Shing, 1992; Risau, 1997), angiopoietins (Maisonpierre et al., 1997; Suri et al., 1996), members of the fibroblast growth factor family (FGF) (Mergia et al., 1989) and vascular endothelial growth factor (VEGF) (Neufeld et al., 1999). These factors can induce a diverse array of signals and initiate downstream intracellular pathways that affect the survival, proliferation, transcription,

adherence, permeability and migration of endothelial cells. Some of these signals can be elicited through transmembrane tyrosine kinase receptors that are expressed at high levels in endothelial cells, including VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1), VEGFR-3 (flt-4), Tie-1 and Tie-2/Tek (Mustonen and Alitalo, 1995; Terman et al., 1992).

VEGF was first purified in 1989 by Ferrara and Henzel (Ferrara and Henzel, 1989). Cloning and expression of VEGF revealed that it was the same molecule as vascular permeability factor (VPF) (Keck et al., 1989), a protein first described in 1983 (Senger et al., 1983) which was able to promote extravasation of proteins from tumour-associated blood vessels. It was later reported that the VEGF gene could produce several isoforms by alternative splicing to form active disulfide-linked homodimers (Poltorak et al., 1997; Robinson and Stringer, 2001; Tischer et al., 1989; Tischer et al., 1991). Studies have shown that the loss of a single VEGF allele results in embryonic lethality, pointing to the indispensable role of this protein in development and maintenance of the whole organism (Carmeliet and Collen, 1999; Carmeliet et al., 1996; Ferrara et al., 1996; Patterson et al., 1996).

Over the past few years, several members of the VEGF gene family have been identified, including placenta growth factor (PlGF) (Maglione et al., 1991), VEGF-B (Olofsson et al., 1996a; Olofsson et al., 1996b), VEGF-C (Joukov et al., 1996), VEGF-D (Achen et al., 1998) and VEGF-E, a viral protein that binds specifically to VEGFR-2 (Meyer et al., 1999; Ogawa et al., 1998; Wise et al., 1999).

1.3.1 Biological activities of VEGF

VEGF is a secreted mitogen whose target cell specificity is mainly restricted to endothelial cells (Partanen et al., 1999). VEGF was first purified from conditioned

medium of several cell lines as a glycosylated homodimer of 46 to 48 kDa (Ferrara and Henzel, 1989; Leung et al., 1997). Differentially spliced mRNA transcripts encode five VEGF transcripts coding for polypeptides of 121, 145, 165, 189 and 206 amino acids (Houck et al., 1991; Poltorak et al., 1997; Tischer et al., 1989). $VEGF_{165}$ is the predominant VEGF isoform produced by a variety of normal and transformed cells, whereas $VEGF_{121}$, $VEGF_{145}$, $VEGF_{189}$ and $VEGF_{206}$ are rarer forms encountered in some of the tissues expressing the VEGF gene (Houck et al., 1991; Poltorak et al., 1997). $VEGF_{165}$ is a basic, homodimeric glycoprotein of 45 kDa which has affinity for heparan sulfates, and is partially sequestered in the pericellular matrix. In contrast, $VEGF_{121}$ is a weakly acidic polypeptide which lacks the heparin binding domain, and is secreted as a freely diffusible protein (Houck et al., 1992). $VEGF_{189}$ and $VEGF_{206}$ are more basic, and bind heparan sulfates with greater affinity than $VEGF_{165}$ (Houck et al., 1992). These two molecules are almost completely sequestered in the extracellular matrix, but can be released as soluble forms by heparinase or by plasmin, which generate a bioactive proteolytic fragment (Park et al., 1993). This suggests that VEGF may become available to endothelial cells in two ways: as freely diffusible proteins ($VEGF_{121}$ and $VEGF_{165}$) or following protease activation and cleavage of the two longer isoforms ($VEGF_{189}$ and $VEGF_{206}$). Thus, plasminogen activation and generation of plasmin could play an important role in angiogenesis.

Although VEGF is a mitogen for vascular endothelial cells, it lacks significant mitogenic activity for most other cell types, because the expression of VEGF receptors is mainly restricted to endothelial cells (Barleon et al., 1994; Plouet and Bayard, 1994). Moreover, it can act as a survival factor for endothelial cells cultured in serum-deprived

conditions (Karsan et al., 1997). The mechanisms of the VEGF cytoprotective effect have not been well-studied but may involve the PI3-kinase pathway and upregulation of members of the Bcl-2 and IAP (Inhibitor of Apoptosis) family of anti-apoptotic proteins (Tran et al., 1999). VEGF also promotes expression of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 in endothelial cells, which can result in the adhesion of activated leukocytes to endothelial cells (Melder et al., 1996a; Melder et al., 1996b). In hematopoiesis, VEGF can inhibit the maturation of antigen-presenting cells, such as dendritic cells, thereby inhibiting the ability of the immune system to recognize and target tumour cells (Gabrilovich et al., 1998; Kusmartsev and Gabrilovich, 2002; Takahashi et al., 2004).

In vivo, VEGF is known for its ability to induce vascular leakage, thus causing the exudation of plasma proteins (Bates et al., 1999). This leakage results in the laying down of a provisional matrix of fibrin and fibronectin allowing endothelial migration (Dvorak et al., 1995). VEGF possesses a potent ability to increase microvessel permeability to a 50,000-fold higher level than histamine (Dvorak et al., 1995).

1.3.2 Regulation of VEGF expression

VEGF is a key regulator of angiogenesis, and its expression is regulated by many external factors. Many cytokines, which do not promote angiogenesis, can modulate angiogenesis indirectly by regulating VEGF expression in specific cell types. Molecules that can increase VEGF expression include epidermal growth factor (EGF), insulin-like growth factor 1, fibroblast factor 4 (FGF-4) (Deroanne et al., 1997), TGFβ (Pertovaara et al., 1994), TNFα (Ryuto et al., 1996), PDGF (Finkenzeller et al., 1997) and IL-6 (Cohen et al., 1996). On the other hand, cytokines such as IL-10 and IL-13 can inhibit VEGF

expression (Matsumoto et al., 1997). In addition, hydrogen peroxide, which is produced by neutrophils that invade a wound in the healing process, or UV-B radiation also potentiate VEGF production by keratinocytes (Bielenberg et al., 1998; Brauchle et al., 1996; Mildner et al., 1999). Nitric oxide can also increase VEGF production, which, in turn upregulates the production of nitric oxide in a positive feedback loop (Frank et al., 1999; Kroll and Waltenberger, 1999; Shen et al., 1999).

Hypoxia is also a major stimulator of VEGF expression. Since cells in a solid tumour are often hypoxic, this could account, at least in part, for the increased VEGF expression in many types of cancer cells (Richard et al., 1999). The transcription of the VEGF gene, under hypoxic conditions is mediated by the binding of hypoxia-inducible factor 1 (HIF-1) to a hypoxic responsive element localized in the VEGF promoter (Levy et al., 1995; Liu et al., 1995; Semenza et al., 1999). Hypoxia also stabilizes the VEGF transcript by inducing proteins that bind and stabilize the 3' untranslated region (UTR) of VEGF mRNA (Stein et al., 1998; Stein et al., 1995).

Specific transforming events can also result in induction of VEGF gene expression. For example, oncogenic mutations or amplification of ras lead to VEGF mRNA stabilization (Okada et al., 1998). The von Hippel-Lindau (vHL) tumour suppressor gene has also been implicated in up-regulation of VEGF (Brieger et al., 1999; Siemeister et al., 1996). Wild type vHL inhibits the production of several hypoxia-regulated proteins such as VEGF at the post-transcriptional level. vHL is known to inhibit the activity of protein kinase C zeta and delta (Pal et al., 1997). In the absence of wild-type vHL, these kinases remain active and VEGF mRNA is stabilized as a result of

constititutive interactions of various proteins that are normally induced by hypoxia with the 3'UTR (Pal et al., 1997).

Finally, loss of the wild-type tumour suppressor p53 also correlates with up-regulation of VEGF expression, and with increased angiogenesis in developing tumours (Kieser et al., 1994; Saito et al., 1999).

1.3.3 VEGF receptors

The VEGF family members are ligands for a set of mammalian tyrosine kinase receptors (VEGF receptors, VEGFRs) (Figure 4). Three VEGF tyrosine kinase receptors have been identified and cloned: VEGFR-1 also known as flt-1 (fms-like tyrosine kinase) (de Vries et al., 1992), VEGFR-2 (KDR/flk-1) (Terman et al., 1992) and VEGFR-3 (flt-4) (Neufeld et al., 1994).

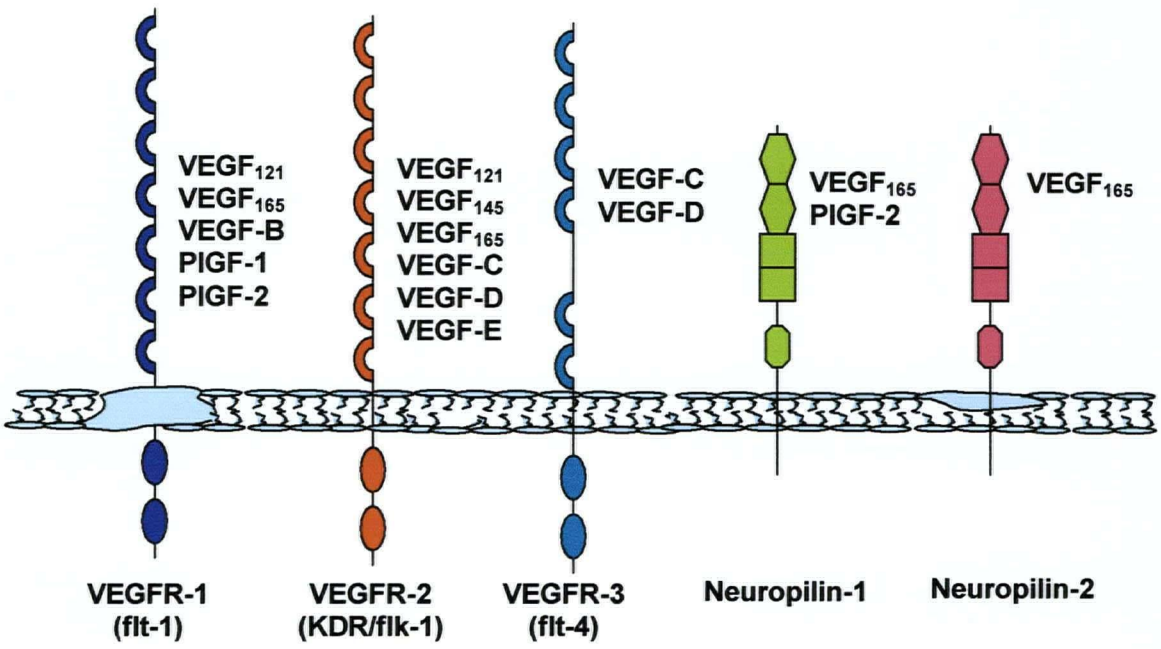

Figure 4 Receptors of the VEGF family. These receptors include three tyrosine kinase receptors: VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1) and VEGFR-3 (flt-4). Other receptors for VEGF also include neuropilin-1, which binds $VEGF_{165}$ and PlGF-2, and neuropilin-2, which binds $VEGF_{165}$. Ligand binding causes dimerization and transphosphorylation of VEGF receptors.

These receptors form a subfamily distinguished by the presence of immunoglobulin-like loops in their extracellular portion, a single transmembrane region and a split tyrosine-kinase domain in the intracellular region. The presence of the split kinase domain places these receptors in the subfamily of class III receptor tyrosine kinases, which includes c-Fms, c-Kit and the α and β chains of the PDGF receptor (Shibuya et al., 1999). VEGF binds and activates both VEGFR-1 (de Vries et al., 1992) and VEGFR-2 (Kendall et al., 1999), whereas PlGF (Athanassiades and Lala, 1998; Shore et al., 1997) and VEGF-B (Olofsson et al., 1998) bind only VEGFR-1. VEGF-C (Joukov et al., 1996; Kukk et al., 1996) and VEGF-D (Achen et al., 1998) bind VEGFR-2 in addition to VEGFR-3 (Figure 4). VEGFR-1 has a higher binding affinity for VEGF (Kd of 10-20 pM) than VEGFR-2 (Kd of 75-125 pM) (de Vries et al., 1992; Terman et al., 1992). VEGFR-3 binds VEGF-C and VEGF-D (Achen et al., 1998; Ristimaki et al., 1998).

VEGFR-1 and VEGFR-2 are expressed mainly on endothelial cells, although other cell types have also been shown to express these receptors. VEGFR-1 has been shown to be expressed in monocytes (Barleon et al., 1996), megakaryocytes (Casella et al., 2003) and hematopoietic stem cells (Hattori et al., 2002) whereas VEGFR-2 is also expressed in hematopoietic stem cells (Ziegler et al., 1999) and megakaryocytes (Katoh et al., 1995). In addition, some tumour cells, such as melanoma cells, leukemic blasts, lymphoma cells and multiple myeloma cells, express VEGFR-1 or VEGFR-2 (Bellamy et al., 1999; Gitay-Goren et al., 1993; Graeven et al., 1999; Herold-Mende et al., 1999; Padro et al., 2002; Ria et al., 2003; Zhang et al., 2004). VEGFR-3, which is expressed mainly in lymphatic endothelium, has been shown to be involved in the regulation of lymphatic angiogenesis (Jussila et al., 1998). However, recent evidence indicates that

VEGFR-3 is required for vasculogenesis in mouse embryos, thus indicating that this receptor plays an essential role in the development of the cardiovascular system (Dumont et al., 1998).

To provide evidence of the proposed role of VEGFR-1 and VEGFR-2 in vascular development, the genes encoding VEGFR-1 and VEGFR-2 have been disrupted in mice. Because VEGFR-2 is expressed in the primitive blood islands, it has been proposed that this receptor may play an essential role during the initial stages of vasculogenesis, when both endothelial and blood progenitor cells are presumed to differentiate from a common precursor, the hemangioblast (Hatva et al., 1996). Embryos lacking wild-type VEGFR-2 died *in utero* by day 9. Morphological analysis revealed an absence of blood islands and hemopoietic progenitor cells. These embryos were also characterized by the absence of mature endothelial cells (Shalaby et al., 1997; Shalaby et al., 1995). Hence, VEGFR-2 is required for the formation, migration and/or proliferation of endothelial and hemopoietic cells during vasculogenesis. In contrast to the critical role of VEGFR-2 in vasculogenesis, endothelial and hemopoietic cell differentiation was found to occur in embryos lacking the VEGFR-1 gene. However, the organization of vascular structures was grossly abnormal, and the mutant embryos also died *in utero* by day 9. Instead of fusing into typical blood vessels, the endothelia in VEGFR-1 mutants formed abnormal vascular channels, characterized by large lumens containing trapped endothelial cells mixed with blood cells. Moreover, an excessive number of endothelial cells accumulated in the VEGFR-1 knockout embryos (Fong et al., 1996; Fong et al., 1995; Fong et al., 1999). These studies suggest that VEGFR-1 is necessary to repress excessive endothelial cell proliferation.

As with VEGF, the expression of VEGFR-1 and VEGFR-2 is reported to be affected by hypoxia, although to a lesser extent than VEGF (Gerber et al., 1997; Tuder et al., 1995). VEGFR-1 transcription is upregulated by hypoxia (Suzuki et al., 1999) whereas VEGFR-2 production is also up-regulated by hypoxia, but at the post-transcriptional level (Waltenberger et al., 1996). This hypoxia-induced upregulation of VEGFR-1 and VEGFR-2 may be indirectly triggered, since VEGF is known to potentiate the expression of these two receptors (Kremer et al., 1997; Shen et al., 1998; Tsopanoglou and Maragoudakis, 1999).

Of the seven IgG-like domains of the extracellular portion of VEGFR-1 and VEGFR-2, only domains 2 and 3 are required for the tight binding of VEGF to these two receptors (Fuh et al., 1998). Several studies have mapped the binding site for VEGF to the second Ig-like domain of the receptors.

VEGF also interacts with the neuronal cell-guidance receptors, neuropilin-1 and neuropilin-2 (Miao et al., 1999; Soker et al., 1998). These receptors were initially described to bind several types of semaphorins, which are factors that act as axonal repellents (Chedotal et al., 1998; Chen et al., 1998; He and Tessier-Lavigne, 1997). The neuropilins, which are known to be expressed in neurons, some cancer cell lines and endothelial cells, bind to $VEGF_{165}$, but not $VEGF_{121}$ (Neufeld et al., 1999; Soker et al., 1998). In addition to $VEGF_{165}$, neuropilin-1 has also been found to bind the heparin binding form of PlGF, PlGF-2 (Migdal et al., 1998). The neuropilins have only a short intracellular domain. It has recently been shown that the cytoplasmic domain of neuropilin-1 contains a central PSD- 95/Dlg/ZO-1 (PDZ) domain, which may act as a protein interaction module (Jelen et al., 2003), and a C-terminal acyl carrier protein

domain (Cai and Reed, 1999), thus indicating that neuropilin-1 might be able to transduce a signal. However, no responses to $VEGF_{165}$ were observed when cells expressing neuropilin-1 but no other VEGF receptors were stimulated with $VEGF_{165}$ (Soker et al., 1998). It is not known whether VEGF binding is sufficient for signal transduction via a neuropilin-1-associated hypothetical signal-transducing polypeptide chain although the enhanced mitogenic signalling of VEGFR-2 in neuropilin-1-overexpressing cells suggests such a possibility. Mouse embryos lacking a functional neuropilin-1 die *in utero* because their cardiovascular system fails to develop properly, indicating that this receptor is probably an important regulator of blood vessel development (Kawasaki et al., 1999; Kitsukawa et al., 1997). Furthermore, overexpression of neuropilin-1 under the β-actin promoter is lethal because of severe anomalies of the nervous and cardiovascular systems (Kitsukawa et al., 1995). The cardiovascular defects may result from modulation of VEGF bioactivity and VEGF-induced angiogenesis by abnormal neuropilin-1 levels. Therefore, it has been proposed that neuropilin-1 acts as a $VEGF_{165}$ co-receptor. This postulate is supported by studies showing that VEGFR-2 binds $VEGF_{165}$ more efficiently in cells expressing neuropilin-1, resulting in a better migratory response to $VEGF_{165}$ (Soker et al., 1998). However it does not seem that neuropilin-1 acts as a VEGFR-1 co-receptor, since PlGF-1 and PlGF-2, which both bind VEGFR-1, promote migration of endothelial cells equally well (Clauss et al., 1996). Neuropilin-2 also binds $VEGF_{165}$, but its expression pattern is different from that of neuropilin-1 or VEGFR-1, in that it is absent from endothelial cells of capillaries (Chen et al., 1997; Giger et al., 1998).

1.3.4 VEGF signal transduction

Studies of receptor tyrosine kinases in a number of different systems have clearly shown that the pathways activated by these molecules can lead to drastic changes in cells, including their fate, survival, proliferation, migration and adhesion (van der Geer et al., 1994). Although VEGFR-1 and VEGFR-2 are both activated by the same ligand, their downstream signalling pathways cause different cellular responses.

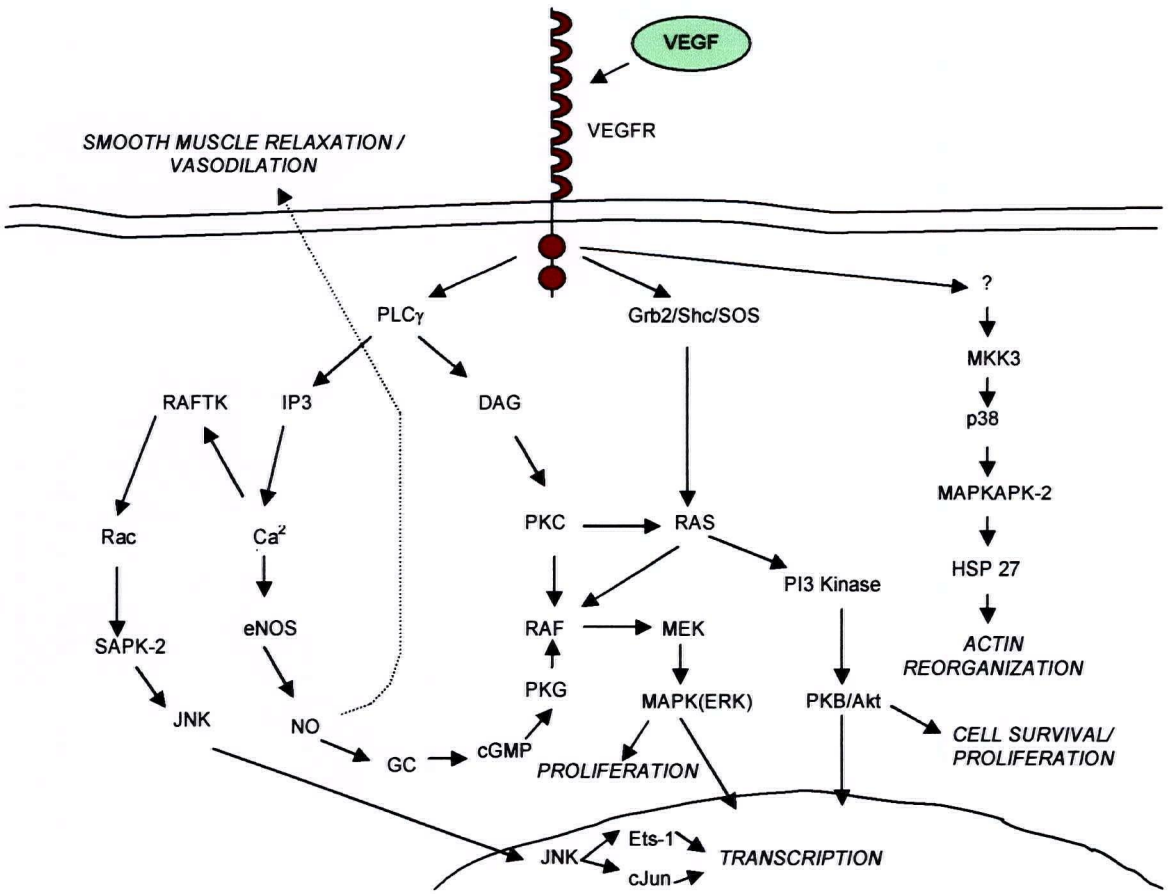

Figure 5 Signalling pathways induced by VEGF activation of tyrosine kinase receptors in endothelial cells. See text for abbreviations. VEGF binding causes dimerization and phosphorylation of tyrosine kinase receptors and further activation of intracellular mediators.

VEGFR-2 activation is required for endothelial and hemopoietic cell fate determination and proliferation (Shalaby et al., 1997; Shalaby et al., 1995), whereas VEGFR-1 activation is more important for proper regulation of endothelial cell migration and adhesion and blood vessel organization (Fong et al., 1996; Fong et al., 1995; Fong et al., 1999). However, the information regarding the signalling cascades induced by VEGFR-1 and VEGFR-2 is still limited. The situation is further complicated by the ability of certain members of the VEGF family to form heterodimers: PlGF-VEGF (DiSalvo et al., 1995) and VEGF-VEGF-B (Olofsson et al., 1996b) for example. VEGFR-1 and VEGFR-2 may also form heterodimers (Bates et al., 1999), thus adding another level of complexity.

VEGF stimulation of endothelial cells in culture results in the activation of VEGF receptors 1 and 2 and phosphorylation of a number of downstream proteins, including phospholipase Cγ (PLCγ), PI3-kinase, the adaptor protein Nck, and the Ras GAP complex (Guo et al., 1995; Seetharam et al., 1995) (Figure 5). VEGF-receptor binding triggers a signalling cascade that results in tyrosine phosphorylation and the formation of protein-protein complexes through SH2 domains (Guo et al., 1995). Activation of PLCγ is accompanied by increases in inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) production and increased PI3-kinase activity (Xia et al., 1996). However, other investigators do not report increased PI3-kinase activity following VEGF stimulation of endothelial cells (Abedi and Zachary, 1997). PI3-kinase is a heterodimer of an 85 kDa adaptor subunit (p85) and a 110 kDa catalytic subunit (p110). It is activated by most growth factors and has been implicated as a critical factor in the control of cell proliferation and cell survival (Stephens et al., 1993). PI3-kinase phosphorylates the D-3 position of the inositol ring of phosphoinositides, which in turn act as second messengers.

The p85 subunit contains two SH2 domains, which bind to tyrosine-phosphorylated receptors after stimulation of cells with growth factors and in this manner recruits the p110 catalytic subunit into the complex at the cell membrane (Stephens et al., 1993).

Production of IP3 by PLCγ stimulates the release of calcium from intracellular stores (Berridge, 1993). This is consistent with studies showing that VEGF can increase cytosolic calcium (Bates and Curry, 1997; Brock et al., 1991). On the other hand, production of DAG is well known to activate protein kinase C (PKC). It has been postulated that activation of PKC is an important intracellular signalling pathway for cellular growth. Several reports have shown that PKC plays an important role in the VEGF-initiated angiogenesis process (Xia et al., 1996). Indeed, VEGF stimulation has been shown to increase the levels of PKCα and βII in bovine aortic endothelial cells (Xia et al., 1996).

Activation of PKC, through Raf, leads to phosphorylation of extracellular-regulated kinase (ERK1/2), a form of mitogen-activated protein kinase (MAPK) and its subsequent translocation to the nucleus (Mukhopadhyay et al., 1998). Both VEGFR-1 and VEGFR-2 have been shown to activate the MAP kinase pathway (Sawano et al., 1997; Takahashi and Shibuya, 1997). The formation of a receptor Shc/Grb2/SOS complex is another pathway that can also activate ERK1/2 via the Ras/Raf cascade (Kroll and Waltenberger, 1997).

PKC, through Ras, also causes the activation of PI3-kinase, which in turn can activate the protein kinase B (PKB/Akt) pathway. The activation of this downstream effector of PI3-kinase seems to be important in endothelial cell protection against serum-starvation induced apoptosis (Kennedy et al., 1997b). Activation of Akt occurs through

the direct binding of the phosphoinositide products of the PI3-kinase reaction to the pleckstrin homology domain of Akt. Phosphoinositol lipids also induce phosphorylation of Akt, resulting in further activation (Franke et al., 1997). In Rat1a fibroblasts, activated Akt did not alter Bcl-2 or Bcl-X(L) expression but inhibited caspase activity. Thus, the PI3-kinase/Akt signalling pathway transduces a survival signal that ultimately blocks caspase activity (Kennedy et al., 1997b). It has been shown that VEGF-induced Akt activation and cytoprotection in endothelial cells was suppressed by wortmannin, a PI3-kinase inhibitor, indicating that Akt acts downstream of PI3-kinase in the VEGF signalling pathway. In contrast, VEGF mitogenic functions are transduced through protein kinase C, which is not inhibited by wortmannin (Fujio and Walsh, 1999). Taken together, these data suggest that the cytoprotective and mitogenic signals of VEGF are transduced by independent pathways in endothelial cells.

Through calcium release, VEGF can also stimulate the phosphorylation of the related adhesion-focal tyrosine kinase (RAFTK/Pyk2) and the focal adhesion kinase (FAK) (Abedi and Zachary, 1997; Mukhopadhyay et al., 1998). RAFTK can activate Rac, which can activate the stress-activated protein kinase 2 (SAPK-2), leading to phosphorylation of c-Jun through the activation of c-Jun NH_2-terminal kinase (JNK) (Mukhopadhyay et al., 1998). The endothelial specific transcription factor (Ets-1) can also be activated following JNK phosphorylation (Iwasaka et al., 1996; Tanaka et al., 1998). This transcription factor is known to induce the expression of matrix-degrading proteins, such as gelatinase and collagenase, which enables growing vessels to migrate through the intersitium.

Calcium appears to be implicated in microvascular permeability and vasodilatation by activating the endothelial specific nitric oxide synthase (eNOS). eNOS is a functionally important member of the nitric oxide synthase family which includes inducible nitric oxide synthase (iNOS) and the neuronal nitric oxide synthase (nNOS) (Nathan and Xie, 1994). NO is an important mediator of endothelial function that influences vascular tone, platelet aggregation, endothelial cell permeability and vascular cell proliferation (Kroll and Waltenberger, 1998). It has been shown that stimulation of endothelial cells with VEGF rapidly results in the upregulation of eNOS expression and NO release (Hood et al., 1998; Kroll and Waltenberger, 1998). Not only does nitric oxide induce vasodilatation, it can also activate guanylyl cyclase (GC) to cause the production of cyclic GMP (cGMP). cGMP can in turn activate protein kinase G (PKG), which has been shown to be an important regulator of microvascular permeability (Huang and Yuan, 1997; Vaandrager and de Jonge, 1996; Wu et al., 1996). PKG can also act as an activator of Raf, thus contributing to activation of the MAPK pathway (Hood and Granger, 1998).

Some studies have also shown that VEGF can activate the p38 MAP kinase pathway (Rousseau et al., 1997). p38 is activated by phosphorylation on both threonine and tyrosine. Three p38 activators have been characterized: SEK1 (which is also an activator of JNK1) MKK3 and MKK6, a p38-specific MAP kinase kinase (Lin et al., 1995). The known downstream targets of p38 are a seryl/threonyl kinase, MAPK-activated protein kinase (MAPKAPK) 2, and the transcription factor ATF-2 (Gupta et al., 1995; Rouse et al., 1994). However, the molecular mechanisms connecting tyrosine kinase activation to p38 activation remain to be ascertained. It would seem though, that

the p38 pathway stimulation by VEGF is an important component of the signalling network which transduces the migratory signals generated by VEGF, suggesting that it may play an important role in regulating angiogenesis (Rousseau et al., 1997). MAPKAPK 2 activation by p38 could be responsible, at least in part, for the transduction of this migratory signal, since it has been shown to play a role in actin regulation through two of its known substrates, LSP-1 and HSP27, which are actin-binding proteins (Huang and Yuan, 1997; Miron et al., 1991). The level of expression of HSP27 is particularly high in endothelial cells. Thus, it could be a potential downstream effector of p38 in the regulation of VEGF-induced actin rearrangement and the resulting increased cell migration (Rousseau et al., 1997).

Since endothelial cells express both VEGFR-1 and VEGFR-2, it is difficult to elucidate the specific responses of each receptor following VEGF stimulation. To counter this problem, cells which do not usually express either receptor have been transfected with either VEGFR-1 or VEGFR-2.

VEGFR-1

VEGF stimulation results in weak tyrosine phosphorylation that does not generate a mitogenic signal in NIH 3T3 cells transfected with VEGFR-1 (Seetharam et al., 1995). This lack of a mitogenic response following of VEGFR-1 was associated with its inability to stimulate the MAP kinase pathway, even though PLCγ and Ras GAP were phosphorylated (Seetharam et al., 1995). These findings agree with other studies showing that PlGF, which binds with high affinity to VEGFR-1 but not to VEGFR-2, lacks direct mitogenic or permeability-enhancing properties, or the ability to effectively stimulate

tyrosine phosphorylation in endothelial cells (Park et al., 1994). However, it has been shown that high concentrations of PlGF, sufficient to saturate the binding sites on VEGFR-1, are able to potentiate the effects of VEGF, *in vivo* and *in vitro* (Park et al., 1994). This, and the fact that VEGFR-1 knockout mouse embryos display an excessive number of endothelial cells has led to the suggestion that VEGFR-1 is not a signalling receptor, but a ligand binding molecule, able to regulate in a negative way the activity of VEGF on endothelial cells by sequestering this ligand and rendering it less available to VEGFR-2 (Park et al., 1994). To support this hypothesis, it has been demonstrated that a targeted mutation resulting in a VEGFR-1 mutant lacking a tyrosine kinase split domain of this receptor but able to bind VEGF does not result in impaired embryonic development and angiogenesis in mice, while deletion of the receptor results in embryonic lethality (Hiratsuka et al., 1998). Furthermore, endothelial cells isolated from these animals displayed normal mitogenicity in response to VEGF (Hiratsuka et al., 1998). Moreover, VEGF mutants that bind selectively to VEGFR-2 are fully active endothelial cell mitogens (Keyt et al., 1996). However, other studies show that the VEGFR-1 is indeed able to be phosphorylated by VEGF and can subsequently interact with many signal transducing proteins, such as PI3-kinase, and generate a mitogenic signal in some transfected cells (Cunningham et al., 1995; Waltenberger et al., 1994). At least some biological responses, such as the migration of monocytes in response to VEGF or PlGF have been shown to be mediated by the VEGFR-1 (Barleon et al., 1996). This macrophage migration induced by VEGF or PlGF was suppressed in mice which have a non-functional tyrosine kinase domain VEGFR-1 (Hiratsuka et al., 1998). VEGFR-1 can also mediate the expression of tissue factor in endothelial cells (Clauss et al., 1996).

Finally, the ability of VEGF to inhibit the maturation of dendritic cells has been associated with the activation of VEGFR-1 (Oyama et al., 1998).

In addition to the full length receptor, the VEGFR-1 gene can also generate a soluble form containing only 6 Ig domains through alternative splicing. The soluble form of VEGFR-1 also displays strong binding to VEGF, and thereby can act as an antiangiogenic factor (Yamaguchi et al., 2002).

VEGFR-2

VEGFR-2 appears to trigger most of the effects of VEGF in endothelial cells. When activated, VEGFR-2 can induce migration of endothelial cells, extracellular matrix degradation and a mitogenic response on endothelial cells (Waltenberger et al., 1994). Furthermore, VEGFR-2 activation has been shown to be required for the antiapoptotic effects of VEGF for endothelial cells under serum-starved conditions (Gerber et al., 1998a; Gerber et al., 1998b). This antiapoptotic effect appears to be mediated by the PI3-kinase/Akt pathway (Gerber et al., 1998b). VEGFR-2 also appears to be responsible for the release of NO and the increased expression of eNOS following stimulation of endothelial cells with VEGF (Kroll and Waltenberger, 1998). Moreover, some studies have suggested that VEGFR-2 is the receptor responsible for the permeability of endothelial cells (Joukov et al., 1998; Murohara et al., 1998). However, this is contradicted by other studies that show that mutant forms of VEGF that lack the ability to bind VEGFR-2 still retain the ability to induce vascular permeability (Stacker et al., 1999).

VEGFR-2 can be autophosphorylated on at least four tyrosine residues located within the cytoplasmic domains of the protein (Dougher-Vermazen et al., 1994). Two of these residues, tyrosine 1054 and 1059, are located in the activation loop of the tyrosine kinase domains. The other two tyrosine phosphorylation sites at amino acid position 951 and 996 are located in a poorly-conserved region, known as the tyrosine kinase insert loop, that is characteristic of type III receptor tyrosine kinases (Fantl et al., 1993). Phosphorylation of the corresponding tyrosines in PDGF β-receptor provides docking sites for downstream signal transduction proteins (Fantl et al., 1993). VEGF stimulation of KDR-expressing cells is known to result in the phosphorylation of GAP, members of the Src family of protein kinases, and PLCγ as well as of p42 MAP kinase (Takahashi and Shibuya, 1997; Waltenberger et al., 1994). It seems that the activation of these proteins through VEGFR-2 could account for most of the effects of VEGF in endothelial cells.

Like VEGFR-1, VEGFR-2 gene can also produce a soluble form of VEGFR-2 (sVEGFR-2) by alternative splicing, which can bind VEGF (Ebos et al., 2004). However, the biological relevance of sVEGFR-2 remains unknown.

1.4 VEGF and hematopoiesis

1.4.1 Expression and induction of VEGF and its receptors in normal hematopoiesis

VEGF is a critical factor in the development of the hematopoietic system. Its role in developmental hematopoiesis has been demonstrated by the heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene in mice. Absence of VEGF results in impaired blood island formation and angiogenesis (Carmeliet et al., 1996;

Ferrara et al., 1996). Disruption of two of its receptors, VEGFR-1 and VEGFR-2 also results in embryonic lethality (Fong et al., 1995; Shalaby et al., 1995). The role of VEGFR-1 during hematopoietic development is unclear because mice lacking the tyrosine kinase domain of VEGFR-1 have no obvious developmental hematopoietic defects (Hiratsuka et al., 1998), although VEGF-induced monocyte migration is strongly suppressed. In contrast, VEGFR-2-deficient embryos die at midgestation (E9.5) because of the absence of blood islands (Shalaby et al., 1995) and of hematopoietic and endothelial progenitors. When differentiated in vitro, VEGFR-2-/- embryonic stem cells retain the capacity to produce hematopoietic cells, suggesting that VEGFR-2 is not involved in hematopoietic commitment per se (Hidaka et al., 1999; Schuh et al., 1999), but might be important for the survival and migration of early mesodermally derived precursors into a microenvironment that is permissive for hematopoiesis.

The prenatal lethality of VEGF and its receptors observed in knockout embryos has made it difficult to study the role of these proteins in hematopoiesis in adult settings. Even though the role of VEGF and its receptors in adult hematopoiesis still remains unclear, it has been observed in recent years that these proteins are expressed in a variety of normal and malignant cells of the hematopoietic lineage.

A variety of hematopoietic cells secrete VEGF. Möhle et al. have demonstrated that megakaryocytes and platelets can secrete different isoforms of VEGF ($VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$) following stimulation with thrombin or cytokines such as IL-3 and thrombopoietin (Tpo) (Mohle et al., 1997). Moreover, $CD34^+$ hematopoietic progenitors can also secrete VEGF when stimulated with hematopoietic cytokines (SCF, IL-3, GM-CSF, G-CSF) (Bautz et al., 2000). Primitive hematopoietic stem cells have also

been reported to secrete VEGF, and this cytokine has been shown to be essential for the ability of stem cells to repopulate lethally irradiated animals (Gerber et al., 2002).

VEGF receptors are also present on subsets of hematopoietic cells. VEGFR-2 is expressed on 0.1 to 0.5% of $CD34^+$ cells in human postnatal hematopoietic tissues, and it has been argued that the pluripotent hematopoietic stem cells are restricted to the CD34+VEGFR-2+ cell fraction (Ziegler et al., 1999). However, in adult mice, the expression levels of VEGFR-2 are low or undetectable in $Lin^-cKit^+Sca\text{-}1^+CD34^{low/-}$ cells as well as Hoechst 33342^- cells (side population), which have long-term reconstitution activity, and neither $CD34^{low/-}VEGFR\text{-}2^+$ nor $CD34^+VEGFR\text{-}2^+$ have long-term reconstitution activity in mice (Haruta et al., 2001). In addition, it has been reported that megakaryocytes and platelets express the VEGFR-2 transcript (Katoh et al., 1995), which might help those cells survive following stress such as radiation.

In addition to being present on endothelial cells, VEGFR-1 is also expressed on inflammatory cells such as monocytes and macrophages. In fact, the majority of human peripheral blood monocytes express VEGFR-1 as a cell surface marker (Sawano et al., 2001). Using a VEGFR-1 blocking antibody, it has been demonstrated that VEGFR-1 is responsible for VEGF-induced migration of monocytes, which may play a role in inflammation. VEGFR-1 is also present on megakaryocytes, where it can help promote maturation and polyploidization (Casella et al., 2003). Finally, VEGFR-1 was found to be expressed on a significant proportion of human $CD34^+$ and mouse $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ bone marrow-repopulating stem cells (Hattori et al., 2002; Heissig et al., 2002).

1.4.2 Role of VEGF and its receptors in hematopoietic cells

The bone marrow microenvironment plays a major role in the proliferation, maintenance and differentiation of hematopoietic stem cells. The bone marrow stroma is a rich source of cytokines, hormones and growth factors, which can have profound effects on hematopoietic homeostasis. Such cytokines include tyrosine kinase ligands (SCF, flt-3 ligand), colony stimulating factors (G-CSF, M-CSF, GM-CSF), interleukins, members of the transforming growth factor (TGF) family and angiogenic factors (bFGF, VEGF). Until recently, the effects of VEGF in the bone marrow microenvironment were thought to be limited to modulating neoangiogenesis in hematopoietic malignancies (Fiedler et al., 2001). It is now clear that VEGF has an important role in adult hematopoiesis. Gerber *et al.* have shown that VEGF is critical in regulating hematopoietic stem cell survival during bone marrow repopulation experiments of lethally irradiated recipient mice (Gerber et al., 2002). In these experiments, VEGF-deficient cells failed to repopulate lethally irradiated animals, despite the coadministration of large numbers of wild-type cells. Moreover, VEGF-deficient hematopoietic stem cells failed to repopulate wild-type animals. According to the authors, these findings indicate that a wild type bone marrow microenvironment is not sufficient to promote the survival of VEGF-deficient stem cells and has led to the hypothesis that VEGF can stimulate hematopoietic stem cells through an autocrine private loop. The effects on hematopoietic cells are not limited to the survival of hematopoietic stem cells, but can also direct the expansion and differentiation of more mature hematopoietic progenitors. Long term infusion experiments with VEGF in mice resulted in accumulation of B cells and immature Gr-1^+ myeloid cells, and dramatically

inhibited dendritic cell development (Gabrilovich et al., 1998). Finally, VEGF can also modulate hematopoiesis indirectly by promoting the release of hematopoietic cytokines by bone marrow endothelial cells. Endothelial cells stimulated with VEGF have been shown to upregulate expression of cytokines such as IL-6 (Dankbar et al., 2000) and GM-CSF (Fiedler et al., 1997; Zhang et al., 2004), which can in turn affect the survival, expansion and differentiation of hematopoietic progenitors.

The VEGF receptors, which are expressed on subsets of hematopoietic cells, also play significant roles in hematopoiesis. VEGFR-1 regulates gene expression and VEGF-induced migration of monocytes, which may be critical in physiological processes such as wound healing (Shibuya, 2001). VEGFR-1 can also convey signals for the recruitment of hematopoietic stem cells and reconstitution of hematopoiesis. PlGF, a VEGFR-1-specific ligand, mediates the early phase bone marrow recovery of irradiated recipients through the mobilization of preexisting VEGFR-1^+ bone marrow repopulating cells (Hattori et al., 2002). During the late phase of bone marrow recovery, PlGF promotes hematopoiesis primarily by inducing matrix metalloproteinase 9 (MMP-9), which can release SCF from the extracellular matrix, resulting in enhanced cell motility, cycling and differentiation of VEGFR-1^+ long-term repopulating cells (Heissig et al., 2002).

VEGFR-2 is expressed on a small proportion of hematopoietic cells, and has been argued to be a marker of pluripotent hematopoietic stem cells in human (Ziegler et al., 1999). Although it plays a critical role in embryonic hematopoiesis, its role in post-natal physiological hematopoiesis remains unclear. VEGFR-2^+ bone marrow cells fail to repopulate lethally irradiated mice (Haruta et al., 2001). Moreover, mice myeloablated with 5-fluorouracil treated with an anti-VEGFR-2 antibody showed only a transient delay

in the recovery of lymphoid and erythroid cells, which might have been caused by the interference of lineage-specific cytokines such as GM-CSF and IL-6 (Hattori et al., 2002). It has therefore been argued that mouse bone marrow VEGFR-2^+ cells may mark a population of endothelial progenitors (Lyden et al., 2001; Peichev et al., 2000), rather than primitive hematopoietic stem cells.

1.4.3 VEGF and its receptors in hematological malignancies

Several reports have demonstrated that there is increased angiogenesis in the bone marrow of patients with various hematological malignancies such as acute myelogenous leukaemia (AML) and multiple myeloma (Litwin et al., 2002; Padro et al., 2000; Pruneri et al., 1999). In addition, emerging data suggests that VEGF and its receptors are expressed in a variety of cell lines derived from hematological malignancies (Bellamy et al., 1999). Table III lists the hematopoietic malignancies in which VEGF was detected.

Table III Expression of VEGF and its receptors in hematopoietic malignancies

Hematopoietic malignancies	VEGF/VEGFR expression
- Myeloproliferative disorders	VEGF
- Myelodysplastic syndromes	VEGF, VEGFR-1
- Chronic myelomonocytic leukemia	VEGF, VEGFR-1, VEGFR-2
- Leukemia (acute and chronic lymphocytic, acute and chronic myelogenous)	VEGF, VEGFR-1, VEGFR-2 (AML); VEGF, VEGFR-1 (ALL); VEGF, VEGFR-2 (CLL); VEGF (CML)
- Non-Hogkin's lymphoma	VEGF
- Multiple myeloma	VEGF, VEGFR-1, VEGFR-2

The detection of VEGFR-1 and VEGFR-2 expression in a significant proportion of these cell lines suggests that VEGF might stimulate tumour cells through an autocrine mechanism in hematopoietic malignancies (Bellamy et al., 1999; Bellamy et al., 2001; Santos and Dias, 2004). Alternatively, VEGF can also potentiate signals in hematopoietic

malignancies through the paracrine induction of other cytokines and hematopoietic growth factors, which include G-CSF, M-CSF, GM-CSF, SCF and IL-6 (Bellamy et al., 2001; Fiedler et al., 1997). Taken together, these results suggest that anti-angiogenic therapies that interfere with the VEGF/VEGFR pathway may represent novel approaches to effect treatment for certain hematological malignancies. In fact, inhibition of leukaemia growth has been achieved both *in vivo* and *in vitro* in animal models, using a variety of anti-angiogenic compounds that include anti-VEGF antibodies (Bellamy et al., 2001), anti-VEGFR-2 antibodies (Dias et al., 2000; Zhang et al., 2004) and receptor tyrosine kinase inhibitors (Mesters et al., 2001).

Blockade of VEGF signalling in hematological malignancies has been shown to result in inhibition of hematopoietic cytokine production by bone marrow stroma derived from malignant bone marrow (Bellamy et al., 2001), promotion of differentiation of immature progenitors, inhibition of MMP production and induction of apoptosis in receptor-expressing cells (List, 2001). Moreover, VEGF inhibition can block the formation of leukemic colonies in vitro (Bellamy et al., 2001).

Inhibition of VEGFR-1 and VEGFR-2 signalling with small molecular tyrosine kinase inhibitors such as SU5416 and SU6668 has also shown inhibitory effects on the growth of human leukemic blasts, independently of their effects on angiogenesis (Lin et al., 2002; Mesters et al., 2001). However, it must be noted that these inhibitors can also block signalling of other structurally related tyrosine kinase receptors such as c-Kit and PDGFR. Other studies, which used VEGFR-2 blocking antibodies, showed that inhibition of VEGFR-2 signalling inhibited the proliferation of human leukemic cells, both *in vivo* and *in vitro* (Santos and Dias, 2004; Zhang et al., 2004; Zhu et al., 2003). Taken together,

these results suggest that inhibition of VEGF or signalling through VEGFR-1 and/or VEGFR-2 may be effective in the treatment of hematological malignancies, since this would only interfere with tumour cell proliferation, but would also block secretion of paracrine factors elaborated by the bone marrow stroma.

1.5 Rationale and thesis hypotheses

Recent evidence clearly underlined the prominent role of VEGF in a variety of physiological and pathological processes. Numerous studies have shown the critical role played by this cytokine in processes such as wound healing, hematopoiesis, tumour angiogenesis, vasculogenesis and tumourigenesis. Due to the numerous receptors that can interact with VEGF on the surface of cells and thus the difficulty of studying each receptor in isolation, few studies have put in focus the respective role of each VEGF receptor. Due to the critical importance of VEGFR-2 in embryonic hematopoiesis and vasculogenesis, and the relatively small amount of information available on its role in adult hematopoiesis, we chose to study the effects modulated by this receptor in adult hematopoietic settings.

VEGFR-2 (-/-) embryos do not form endothelial or hematopoietic cells, and this has been hypothesized to be due to a lack of migration, proliferation and survival of early mesodermal cells. The first part of this thesis focuses on studying specific signalling pathways and biological responses, such as migration, proliferation and induction of classical endothelial markers, triggered by VEGFR-2 in primary fibroblasts transduced with the full length VEGFR-2 cDNA. The use of fibroblasts transduced with VEGFR-2

as a model allowed us to study VEGFR-2 signalling in isolation, since fibroblasts do not express other VEGF receptors, such as VEGFR-1.

The second part of this thesis involves studying VEGFR-2 and its effects in adult hematopoietic cells. Since VEGFR-2 is endogenously expressed on subsets of normal and malignant hematopoietic cells, the appreciation of some of the effects triggered by this receptor may prove useful for a better understanding of the mechanisms underlying normal and malignant hematopoiesis. To achieve this, we used a VEGFR-2 construct that could be specifically activated with a chemical inducer of dimerization. This approach allowed us to specifically study the biological effects of VEGFR-2 activation in hematopoietic progenitors in a controlled fashion, by making it possible to induce VEGFR-2 signalling specifically without activating endogenous VEGF receptors present on hematopoietic cells. We studied some of the biological effects triggered by VEGFR-2 in hematopoietic progenitors, including cell survival and proliferation, and differentiation of progenitors. We also investigated specific signalling pathways induced by VEGFR-2 in hematopoietic cells, and how they can affect some biological effects such as cell survival.

In a continuation of the second result section of the thesis, the third result section continued to focus on the effects of VEGFR-2 activation in hematopoietic cells, but in an *in vivo* setting. The transplantation of mice with bone marrow transduced with the inducible VEGFR-2 construct allowed us to study a situation in which a relatively large proportion of bone marrow and blood cells express VEGFR-2, which has been shown to occur in certain hematological disorders (Padro et al., 2002; Schuch et al., 2002;

Verstovsek et al., 2002). Therefore, this study allowed us to understand some of the mechanisms by which VEGFR-2 can contribute to normal and malignant hematopoiesis.

Recent studies have demonstrated the contribution of cells derived from the bone marrow to the formation of new blood vessels in tumours. The relative contribution of bone marrow-derived endothelial progenitors to tumour vasculature and the mechanisms by which they are mobilized from the bone marrow and differentiate into mature endothelial cells are still under scrutiny. In the last result chapter, we looked at the contribution of bone marrow-derived cells to tumours implanted in the dorsal area of mice transplanted with bone marrow expressing GFP. Since different experimental settings could potentially affect the proportion of bone marrow-derived cells integrated into tumour vasculature, we studied the implication of variables such as the type of bone marrow used for the transplantation (untransduced bone marrow from GFP transgenic mice vs. bone marrow cultured *ex vivo* for GFP transduction) or tumour type in the relative contribution of bone marrow cells to the formation of tumour blood vessels. Since VEGF has been reported to be critical for the mobilization of endothelial progenitors through activation of VEGFR-2 (Hattori et al., 2001), we examined whether VEGF overexpression by tumour cells could affect the proportion of endothelial progenitors that incorporate in the tumour vasculature. Moreover, the relative contribution of endothelial progenitors to tumour blood vessels was examined following VEGFR-2 activation in hematopoietic cells. Based on these observations, we were able to propose a possible role for VEGFR-2 in hematopoietic cells during normal and pathological processes.

Chapter 2

Materials and Methods

2.1 Cell culture

HMEC-1 endothelial cells (Center for Disease Control and Prevention, Atlanta, GA) were cultured in MCDB 131 medium (Gibco, Burlington, ON, Canada) supplemented with 10% FBS, 10 μg/ml recombinant human Epidermal growth factor (EGF) (Sigma) and 100 U each of penicillin and streptomycin/ml. Phoenix-AMPHO cells (G. Nolan), NIH 3T3 and GP+E86 cells (Markowitz et al., 1988) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 2mM glutamine and 100 U each of penicillin and streptomycin/ml. B6RV2 cells and B6RV2 cells transduced with the human VEGF cDNA (B6RV2-VEGF) were cultured in RPMI supplemented with 10% FBS and 100U/ml penicillin/streptomycin. Lewis lung carcinoma cells (LLC) were cultured in DMEM supplemented with 1.5 g/L sodium bicarbonate, 10%FBS, 2mM glutamine and 100 U/ml of penicillin and streptomycin. Human foreskin fibroblasts (HFF) were isolated from freshly circumcised foreskins. Briefly, foreskins were cut finely using a scalpel blade in DMEM supplemented with 5% fetal bovine serum. The minced tissue was incubated in a solution of 0.01% collagenase (Sigma) at 37°C for 2 hours. The tissue was then washed twice with DMEM containing 5% FBS, resuspended in DMEM supplemented with 20% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and plated in a 60 mm tissue culture dish. After 48 hours, the medium was removed and cells were washed twice with PBS to remove debris and excess tissue. The cells were fed with DMEM supplemented with 10% FBS until they reached confluence.

2.2 Plasmid construct

The intracellular domain of VEGFR-2, which exhibits tyrosine kinase activity, was fused to a modified FKBP12 domain that can dimerize in response to an analog of FK1012,

AP20187 (Ariad Pharmaceuticals, Inc, Cambridge, MA). The construct used in this thesis contained a myristoylation sequence, two modified FKBP12 domains (F36v), the signalling domain of VEGFR-2, and a C-terminal hemagglutinin HA epitope tag. A chimeric fusion protein containing an amino-terminal myristoylation signal, two copies of a mutated FKBP12, followed by a carboxy terminal HA epitope tag was released from the PC4M-Fv2E vector (Ariad) using EcoRI and BamHI, and inserted into the pEGFP-C1 plasmid (Clontech, Mississauga, ON). An *SpeI* linkered fragment encoding the intracellular domain of human VEGFR-2 was PCR-amplified from the full length cDNA (gift of C. Patterson, Carolina Cardiovascular Biology Center, Chapel Hill, North Carolina) using the following primer pairs: 5'-GACTAGTAAGCGGGCCAATGGAGGG-3' and 5'-GACTAGTAACAGGAGGAGAGCTCAGTG-3'. The amplicon was digested with *SpeI*, gel purified, and subcloned into *SpeI*-digested pBluescript. After sequence confirmation, the fragment was released from pBluescript by *SpeI* digestion, gel purified, and subcloned into the *SpeI* site of the pEGFPC1-FKBP12 plasmid. The FKBP-VEGFR-2 fragment was released using *HindIII-XbaI* digestion, overhanging ends filled in with Klenow fragment of DNA polymerase I, and cloned into the HpaI site of a previously described MSCV-IRES-GFP vector based on an original vector kindly provided by R. Hawley (American Red Cross, Rockville, Maryland) (Antonchuk et al., 2001).

2-3 Gene transfer

HMEC-1, HFF, GP+E86 and B6RV2 cells were retrovirally transduced using amphotropic packaged virus obtained by harvesting the supernatant of Phoenix-AMPHO cells transfected with vector plasmids 48 hours prior to supernatant collection. Ecotropic

packaged virus was generated using the following procedure: Phoenix-AMPHO cells were transfected with the vector plasmids using Fugene (Roche, Laval, QC, Canada) according to the instructions of the manufacturer. Medium was changed after 24 hours, and transfected cells were cultured for another 24 hours in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Supernatant was then harvested, filtered, and used for repeated infections of GP+E86 ecotropic packaging cells (Markowitz et al., 1988) in the presence of 8 μg/ml polybrene (Sigma, Oakville, ON, Canada). After sorting for GFP expression, transduced GP+E86 cells were plated at limiting dilution. Individual clones were tested, and the highest titer clone was selected by titration of supernatants on NIH 3T3 cells.

2-4 HFF proliferation assay

Cells (5000/well) were seeded in 96-well plates and, 18 hours later, were washed in serum-free medium and incubated overnight in serum-free medium containing 0.1% BSA to render them quiescent. Cells were then incubated with increasing concentrations of recombinant human VEGF (0-50 ng/ml) for 48 hours in serum-free medium and cell number estimated by MTT assay. Briefly, medium was removed and replaced with medium containing 1 mg/ml MTT and incubated for 4 h. The medium was then aspirated, and the formazan product was solubilized with dimethyl sulfoxide (DMSO). Absorbance at 630 nm was subtracted (to reduce background absorbance) from absorbance at 570 nm for each well.

2-5 Migration assays

Cell migration was assessed using a modified Boyden chamber assay as described (Kuzuya and Kinsella, 1994). Polycarbonate filters (Transwell®) were coated with 1

mg/ml collagen (type I) for 18 hours at room temperature. Serum-free medium supplemented with 0.1% BSA and recombinant human VEGF (20 ng/ml) or FGF-2 (5 ng/ml) was added to the lower chamber, and the upper chamber was seeded with 50,000 cells in serum-free medium containing 0.1% BSA. After 8 hours of incubation (37°C, 5% CO_2), filters were removed, fixed with glutaraldehyde and stained with crystal violet. After adherent cells were removed from the upper side of the filter with a cotton swab, cells that had migrated and adhered to the underside of the filter were quantified by counting three random high power fields (40X). We observed that 6 high power fields cover the whole area of the filter.

2-6 *In vitro* MAP Kinase assay

Confluent cells were starved for 18 hours in serum-free medium containing 0.1 % BSA, after which they were stimulated with 20 ng/ml recombinant human VEGF for various times (0-30 min). For Erk1/2 immunoprecipitation, cell lysates (500 μg protein) in NEFT buffer (1M NaCl, 5mM EDTA, 50 mM NaF, 50 mM Tris-HCl pH7.4) supplemented with 6% NP-40 were incubated with an anti-Erk1/2 antibody overnight at 4°C, followed by a 1 hour incubation with protein A-sepharose. After washes, immunoprecipitates were assayed for Erk1/2 MAP kinase activity by incubating with 1 μg/ml myelin basic protein (MBP) and 5 μCi of [γ-^{32}P] ATP for 20 min at 30°C. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes, which were exposed to X-Ray film at -80°C. Filters were reprobed with anti-Erk1/2 to confirm equal sample loading.

2-7 Isolation of murine bone marrow cells

Bone marrow cells were extracted from the femurs and tibias of C3Pep mice (Ly5.1/Ly5.2, cross between C3H/HeJ and Pep3b) treated 4 days previously with 150

mg/kg 5-fluorouracil (Pharmacia & Upjohn, Mississauga, ON) and cultured for 48 hours in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with a serum substitute (BIT (BSA, Insulin, Transferrin) (Stem Cell technologies ltd., Vancouver, BC, Canada)), 10^{-4} M 2-mercaptoethanol, 40 μg/ml low-density lipoproteins (LDL, Sigma), 1 ng/ml recombinant human flt3-ligand, 300 ng/ml recombinant mouse stem cell factor (SCF) and 20 ng/ml recombinant human interleukin-11 (Stem Cell Technologies). For transduction, bone marrow cells were harvested and infected by either cocultivation with irradiated (1500 cGy, X-ray) GP+E86 viral producer cells or by the addition of virus-containing supernatant from the GP+E86 producer cells in fibronectin-coated dishes. Both infection protocols involved 48 hr growth on tissue culture plates with the above cytokine combination and with the addition of 5 μg/ml protamine sulfate (Sigma). Following infection, bone marrow cells were plated in the same medium for another 2 days. Cells were then sorted for GFP expression (FACS 440, Becton Dickinson).

2-8 Animals

2-8-1 Bone marrow transplant

Previously transduced and sorted bone marrow cells (1 x 10^6 GFP^+ cells/animal) were injected into the tail vein of lethally irradiated (900 cGy, using a ^{137}Cs source) B6C3 mice (Ly5.2, cross between C3H/HeJ and C57Bl/6J) within 24 hours of irradiation. Mice were housed in microisolator units and provided with sterilized food, water, and bedding. Irradiated animals were additionally provided with acidified water (pH 3.0) and 100 mg/L ciprofloxacin. Transplanted animals were allowed to reconstitute their bone marrow for 4 to 8 weeks before any experiments were performed. Peripheral blood and bone marrow cells were harvested from the mice to check for GFP engraftment. For bone

marrow collection, mice were anaesthetised using isoflurane gas (Associated Veterinary Purchasing, Abbotsford, BC) supplied by a vaporizer (Ohio medical products, Madison, WI), and a 22 gauge needle was inserted into the knee joint. Bone marrow was aspirated into a syringe containing phenol-free medium supplemented with 2% FBS. Blood was also collected by making a small incision on the tail using a sterile scalpel blade. Blood was harvested into a capillary tube coated with heparin. Red blood cells were lysed by hypotonic shock using red blood cell lysis buffer (0.8% ammonium chloride, 0.1 mM EDTA). Both bone marrow cells and blood mononuclear cells were then processed for flow cytometry analysis. Mice were then treated with AP20187 or vehicle for 10 days, after which they were sacrificed by CO_2 inhalation. Peripheral blood was obtained by heart puncture, while bone marrow was obtained by flushing the four long bone of the limbs with IMDM supplemented with 2% FBS.

2-8-2 Single cell transplants

Hoechst staining of bone marrow cells for side-population cell analysis was done as previously described (Goodell et al., 1997). Briefly, 2 x 10^6 bone marrow cells/ml from GFP^+CD45.1 C57BL/6 mice were incubated at 37°C, in the presence of 5 μg/ml of Hoechst 33342 (Sigma). After 90 min, cells were washed and antibody staining (c-Kit-phycoerythrin, Lineage markers (CD3 (Clone no. 145-2C11), B220 (RA3-6B2), Gr-1 (RB6-8C5), Mac-1 (M1/70) and ter119 (Ter119)), Sca-1-allophycocyanin) was done in cold Hank's Balanced Salt Solution (HBSS). Detection of lineage markers was assessed using streptavidin-phycoerythrin–Texas red from Caltag Laboratories. Cells were double-sorted on a FACSVantage SE (BD Biosciences), at 1 cell/well, into 96-well plates containing 50 μl of PBS. Individual wells were screened by light and fluorescence

microscopy with GFP and Hoechst filters. In a typical experiment, >90% of the wells were found to contain single cells, and the remaining wells were empty. Wells containing two cells were found with an approximate frequency of 1 in 200. Only wells containing a single cell were used for transplantation. To guarantee the survival of the recipients during the lag phase between the injection of the donor cell and the development of sufficient functional single cell–derived peripheral blood cells, stem cell–depleted bone marrow from GFP^- congenic mice was coinjected with the single hematopoietic stem cells. Stem cell–depleted bone marrow was obtained by removal of Sca-1^+ cells using Sca-1–phycoerythrin (clone D7, PharMingen), followed by anti-phycoerythrin magnetic beads (Miltenyi Biotec) and an automated magnetic cell sorter (autoMACS, Miltenyi Biotec). One million of these helper cells were added to each well containing a single hematopoietic stem cell, and the total volume of the well was injected into lethally irradiated (950 cGy) CD45.2 C57BL/6 mice.

2-8-3 CFU-Spleen (CFU-S_{12}) assay

Transduced GFP^+ bone marrow cells were cultured in IMDM supplemented with 10% FBS with or without 100 nM AP20187 for 7 days. 25,000 cells were injected into the tail vein of lethally irradiated B6C3 mice. 12 days later, mice were sacrificed and spleens were harvested, fixed in Telleyesniczky's fixative (ethanol 70%:glacial acetic acid:formalin / 20:1:1) and hematopoietic colonies counted.

2-9 AP20187 formulation

AP20187 was a gift from Ariad Pharmaceuticals. Lyophilized AP20187 was dissolved in 100% ethanol at a concentration of 62.5 mg/ml and stored at −20°C. For *in vitro* use, the ethanol stock was diluted in complete culture medium to the desired concentration

immediately before use. The final concentration of ethanol in the culture medium was below 0.5%. For *in vivo* use, peritoneal injections were prepared from the 62.5 mg/ml ethanol stock diluted to 2.5 mg/ml in an injection solution consisting of 4% ethanol, 10% PEG-400, and 1.7% Tween 20 in water. All injections were administered to mice within 30 min of dilution into the injection solution. The volume of injection solution was adjusted according to mouse body weight to deliver 10 mg AP20187 per kg mouse. The average injection volume was 100 µl per mouse. For *in vivo* experiments, mice were injected with AP20187 daily for 10 consecutive days.

2-10 Antibody staining for Fluorescence Activating Cell Sorting (FACS) analysis

After red blood cells lysis, cells from bone marrow and peripheral blood were washed and resuspended in PBS containing 4% goat serum (Sigma) followed by monoclonal primary antibody staining for 1 hour at room temperature. Antibodies used for analysis were anti-Mac-1/CD11b (monocytes), anti-Gr-1 (granulocytes), anti-B220 (B lymphocytes), anti-CD5 (T lymphocytes), anti-Ter119 (erythroid progenitors), anti-CD144/VE-cadherin and anti-VEGFR-2 (BD Pharmingen). Isotype rat IgG was used for negative control. Cells were then washed in PBS containing 4% goat serum and incubated for 30 minutes with phycoerythrin (PE)-conjugated secondary antibody. After subsequent washes, cells were resuspended in PBS for FACS analysis. Samples were run on an EPICS ELITE-ESP flow cytometer (Beckman Coulter), and data were analyzed with FCS Express, version 2 (De Novo softwares, Thornhill, ON).

2-11 Preparation of cDNA and reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from murine bone marrow using Qiagen RNeasay Quick spin columns (QIAGEN) as described by the manufacturer. The purified total RNA prep was used as a template to generate first strand cDNA synthesis using random hexamer (Invitrogen) priming and reverse transcriptase (SuperScript II; Invitrogen). PCR was performed using the following primer pairs:

Table IV Murine primers and PCR conditions used in this thesis

Target	Sense	Anti-sense	Tm(°C)	Cycles	Size (bp)
M-CSF	agctgcttcaccaaggactatgag	ctctgtcaacggcctgtctgttat	55	35	737
Tpo	tgtggactttagcctgggagaatg	ttgactctgaatccctgaagcctg	55	35	491
SCF	ctgcgggaatcctgtgactgataa	cgggacctaatgttgaagagagca	55	35	430
Flt3-ligand	gacacctgactgttacttcagcca	acgaatcgcagacattctggtagg	55	35	290
IL-6	gttctctgggaaatcgtgga	tgtactccaggtagctatgg	53	35	207
GM-CSF	cttggaagcatgtagaggccatca	cttgtgtttcacagtccgtttccg	55	35	254
VEGF	gctttactgctgtacctccaccat	atctctcctatgtgctggctttgg	55	35	319
BMP-2	atcaactagaagccgtggaggaac	catggttagtggagttcaggtggt	55	35	686
BMP-4	cagaaatggttcctggacacctca	cacaatccaatcattccagcccac	55	32	415
Jagged-1	aatggagactccttcacctgt	cgtccattcaggcactgg	53	35	383
Delta-1	tggttctctcagagttagcagag	agacccgaagtgcctttgta	55	35	409
Delta-4	gcattgtttacattgcatcctg	gtagctcctgcttaatgccaaa	55	30	473
GAPDH	gcatggccttccgtgt	gggccgagttgggatagg	53	22	256

PCR products were detected by electrophoresis on 2.0% agarose gels.

2-12 ELISA

Murine GM-CSF levels were determined by standard sandwich ELISA according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN, limit of detection: 1 pg/ml). For detection of human $VEGF_{165}$ in mouse serum, the following protocol was used: a 96-well plate (Costar) was coated with an anti-VEGF capture antibody (R&D Systems, Minneapolis, MN) overnight. Wells were blocked for 1 hour with blocking

buffer (PBS containing 1% BSA, 5% sucrose and 0.05% sodium azide) and then were incubated with serum samples or VEGF standards for 2 hours at room temperature. Wells were washed 3 times with PBS containing 0.05% Tween, and a biotinylated anti-VEGF detection antibody (R&D Systems) was added and incubated at room temperature for 2 hours. After washing, the plate was incubated with a streptavidin-Horseradish peroxidase (HRP) conjugate (Vector Laboratories, Burlingame, CA) and incubated at room temperature for 20 min. Plates were washed and incubated with a substrate solution (0.1 mg/ml 3,5,3',5'-tetramethylbenzidine (TMB) and 0.05% H_2O_2 in sodium acetate buffer pH 5.5). The colorimetric reaction was stopped by addition of 50 µl stop solution (1M H_2SO_4). Absorbance at 450 nm was read on a microplate reader with wavelength correction set at 540 nm. Good linear correlation was observed with standards in the range between 7.8 and 1000 pg/ml.

2-13 Bone marrow cells viability assays

Sorted bone marrow cells were plated in IMDM supplemented with 10% FBS with or without the addition of 100 nM AP20187. Cells were harvested at various times and counted on a hemacytometer.

2-14 Hematopoietic colonies assay

For *in vitro* assays, transduced GFP^+ bone marrow cells were grown *in vitro* in IMDM supplemented with 10% FBS, with or without 100 nM AP20187, for 7 and 14 days. At these time points, hematopoietic clonogenic progenitor frequencies were determined by plating 20,000 bone marrow cells in methylcellulose medium containing 50 ng/ml SCF, 10 ng/ml IL-3, 10 ng/ml IL-6 and 3 U/ml erythropoietin (Methocult GF M3434, Stem Cell Technologies). For quantitation of *in vivo* murine progenitors, GFP^+ and GFP^- bone

marrow cells were sorted for GFP expression and plated in methylcellulose medium. Resultant colonies were scored after 10 days of incubation.

2-15 Immunofluorescence and microscopy

For BrdU staining, sorted GFP^+ bone marrow cells were cultured in IMDM supplemented with 10% FBS for 2 days, then treated for 2 hours with 10 μM BrdU with or without 100 nM AP20187. Cytospin preparations of bone marrow cells were fixed with 4% paraformaldehyde for 5 minutes, washed with PBS, and permeabilized with ice cold methanol for 1 min. Slides were then incubated for 20 min at 37°C with 2N HCl to denature DNA. Slides were blocked in PBS containing 5% Goat serum and 0.1% Triton X-100 for ten minutes, followed by 1 hour incubation with primary antibody (Anti-BrdU conjugated with AlexaFluor 594 (Molecular Probes, Eugene, OR), 1:50 dilution in PBS containing 5% goat serum and 0.1% Triton X-100). After washing, nuclear DNA was stained with DAPI (1 μg/ml), and slides were mounted in anti-fading solution. For activated caspase 3 staining, cytospin preparations of hematopoietic progenitors grown in culture for 14 days in IMDM containing 10% FBS were stained using the same protocol as above (DNA denaturation step was omitted) and the following antibodies were used: anti-activated caspase 3 (BD Pharmingen, San Diego, CA) and goat anti-rabbit Ig conjugated with Texas Red (Molecular Probes).). Cells were visualized through a 40x Neofluor objective (numerical objective 0.75) using a Zeiss Axioplan II Imaging inverted microscope (Carl Zeiss, Toronto, Canada), and images were captured with a 1350EX cooled charge-coupled device (CCD) digital camera (QImaging, Burnaby, BC, Canada) using Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada).

2-16 Immunoblotting

Proteins from total cellular extracts were separated by SDS-PAGE and assessed by immunoblotting. Antibodies against phosphorylated VEGFR-2 and total and phosphorylated Akt and Erk MAP kinase were obtained from Cell Signalling Technology (Mississauga, ON). Anti-HA antibody was obtained from BABCO (Richmond, CA). The kinase inhibitors LY294002 and U0126 were obtained from Calbiochem (La Jolla, CA).

2-17 Endothelial progenitor assays

Human mononuclear cells were obtained from umbilical cord blood. Cord blood samples, which were collected during standard or caesarean delivery of full-term infants, were harvested in 200 ml plastic bottles containing 40 ml of IMDM containing 800 U/ml heparin. Ammonium chloride lysis was performed to remove red blood cells. Mononuclear cells were either selected for the surface marker CD133 using anti CD133-coupled magnetic micro-beads (Miltenyi Biotech) as indicated by the company or lineage depleted (Lin-) using lineage panel antibodies (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, Glycophorin A; Stem cell technologies, Vancouver, Canada) and separated using a magnetic separation device, StemSep (Stem Cell technologies) or further used without selection. For endothelial progenitor assays, cells were plated onto tissue culture dishes in MCDB131 medium supplemented with 20% FBS, endothelial cell growth supplement (ECGS, Sigma), heparin, 5 ng/ml human bFGF and 30 ng/ml human VEGF. For 3 consecutive days, non-adherent cells were replated onto a new tissue culture dish in order to remove any contaminating endothelial cells. After 3 days, non-adherent cells were harvested, counted and resuspended in fresh medium and plated onto tissue culture dishes coated with 0.2% gelatin. Half of the medium was replaced twice

weekly. Endothelial colonies were scored after 4 weeks, as identified by staining with the P1H12 antibody (Chemicon).

2-18 Tumour tissue immunohistochemical staining

Tumour tissues were fixed overnight at -20°C in 2% paraformaldheyde / 30% glycerol. After washing with PBS, tumours were embedded in OCT compound and sectioned with a cryostat. Sections (8 μm) were stained with antibodies against VE-cadherin, CD31 and CD11b (BD pharmingen). Secondary antibodies were either Alexa 594- or Alexa 633-conjugated (Molecular Probes).

2-19 lacZ and hPLAP staining

Tumour tissues were fixed in 4% paraformaldehyde for 4 h, and cryopreserved by incubating overnight in 15% sucrose in PBS at 4°C. The samples were washed in PBS before being embedded in OCT compound. Sections were fixed in 0.2% glutaraldehyde for 10 min, washed in 100 mM sodium phosphate (pH7.3) and washing buffer solution (2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% NP-40 in 100mM sodium phosphate pH 7.3) and stained in fresh X-gal solution (0.5 mg/ml X-gal, 5mM potassium ferrocyanide, 5mM potassium ferricyanide in washing buffer solution) at 37°C overnight. For alkaline phosphatase staining, slides were washed three times in PBS for 5 min, and endogenous alkaline phosphatase was inactivated by incubating slides at 70°C in PBS for 30 min. Slides were rinsed in PBS, washed in alkaline phosphatase buffer (100 mM Tris-HCl pH9.5, 100 mM NaCl, 10 mM $MgCl_2$) and incubated in NBT/BCIP stain (Sigma) for 15 to 40 min at room temperature, in the dark. Slides were washed in PBS and either stained for VE-cadherin or dehydrated through an ethanol series and mounted with coverslips.

2-20 Statistical Analysis

Results were analyzed by analysis of variance (ANOVA) to ascertain differences between groups, followed by a Tukey test for multiple comparisons. $P<0.05$ was considered significant.

Chapter 3

Biological effects and signalling pathways induced by VEGFR-2 in isolation

3.1 Introduction

The effects of VEGF on a variety of cell types have been studied in recent years. VEGF stimulates angiogenesis by induction of endothelial cell proliferation and by prevention of endothelial cell death, i.e., anti-apoptotic effects (Ruhrberg, 2003). It was shown that VEGF stimulation leads to the activation of signalling pathways such as the PI3-kinase/Akt-signalling pathway and the Erk 1/2 MAP kinase pathway, which mediate VEGF effects (Ferrara, 1999b). Due to the fact that numerous receptors can bind VEGF (VEGFR-1, VEGFR-2, VEGFR-3, neuropilin-1 and neuropilin-2) and that most cell types responsive to VEGF express more than one receptor, the role of each receptor in transmitting VEGF signals and biological effects remains unclear. Due to the critical importance of VEGFR-2 in the generation of the endothelial and hematopoietic lineages, and the increasing evidence of its role in adult vasculogenesis, angiogenesis and hematopoiesis, we chose to study the biological effects triggered by VEGFR-2 in isolation.

In the first part of this thesis, we studied the specific signalling pathways and some of the biological effects triggered by VEGFR-2 activation. We used primary fibroblasts transduced with VEGFR-2 to analyze the role of this receptor in endothelial differentiation, proliferation and migration. Primary fibroblasts were used as a model since they do not express endogenous VEGF receptors, which makes studying VEGFR-2 signalling without the interference of other VEGF receptors possible. Furthermore, fibroblasts have been reported to be able to transdifferentiate into endothelial cells *in vivo* (Kon and Fujiwara, 1994). Others have shown that adenoviral-mediated MyoD gene transfer into cultured human fibroblasts induces myogenic conversion (Lattanzi et al.,

1998). Since VEGFR-2 is critical in the establishment of the endothelial lineage, we also studied whether signals transmitted through VEGFR-2 are sufficient to promote an endothelial phenotype in fibroblasts.

3.2 Results

3.2.1 VEGFR-2 transmits a mitogenic signal in response to VEGF stimulation in primary fibroblasts.

To determine the biological responses induced by VEGFR-2 in the absence of other VEGF receptors, we generated a cell line of primary human foreskin fibroblasts expressing VEGFR-2 (HFF-VEGFR-2) by retroviral transduction. Expression of VEGFR-2 in transduced HFF was verified by Western blot (Figure 6). Phase contrast micrographs of the cell lines are seen in Figure 6B.

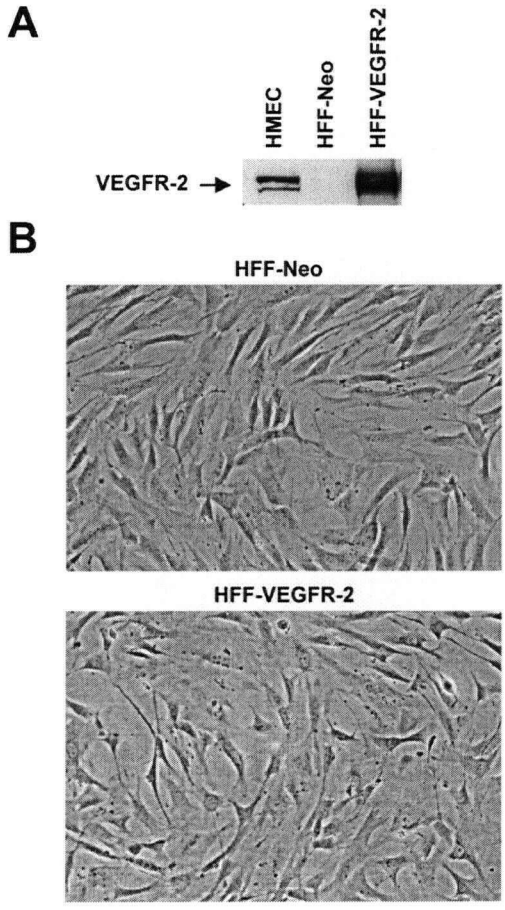

Figure 6 Generation of a primary human foreskin fibroblast cell line expressing VEGFR-2. (A) Human foreskin fibroblasts (HFF) were transduced with either the empty LNCX retroviral vector or LNCX containing the VEGFR-2 cDNA. Level of protein expression was determined by Western blot of cellular extracts from HMEC, HFF-Neo and HFF-VEGFR-2 cells. (B) Phase contrast micrographs showing the morphology of monolayers of HFF-Neo and HFF-VEGFR-2 cells incubated with 20 ng/ml VEGF for four days.

In all experiments, HFF transduced with the empty vector pLNCX (HFF-Neo) were used as a negative control. Several studies indicate that VEGFR-2, rather than VEGFR-1 stimulation is responsible for the mitogenic effect of VEGF in endothelial cells (Meyer et al., 1999; Takahashi and Shibuya, 1997). To assess this, we examined the proliferative effect of VEGF in HFF-VEGFR-2 cells. HFF-VEGFR-2 cells were found to increase in number, when stimulated with increasing concentrations of VEGF for 48 hours (Figure 7A). By contrast, HFF-Neo cells did not show a proliferative response when stimulated

with VEGF for the same amount of time. These results were confirmed by BrdU incorporation assays (Figure 7B).

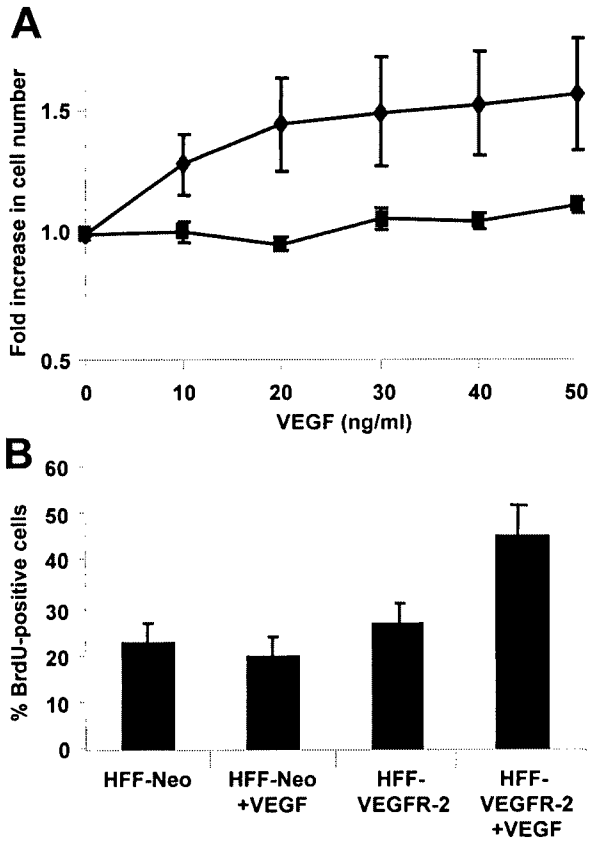

Figure 7 VEGF induces a proliferative response in HFF-VEGFR-2 cells but not in HFF-Neo cells. The proliferative effect of VEGF on HFF-Neo and HFF-VEGFR-2 was measured by either MTT assay (A) or BrdU incorporation assay (B). (A) HFF-Neo (■) and HFF-VEGFR-2 cells (♦) (5000 cells/well) were incubated in serum-free medium in the presence of VEGF for 48 hours. Relative cell number in each well was estimated by measuring absorbance at 570 nm following incubation with MTT. (B) Quiescent cells were incubated with BrdU with or without VEGF for 48 hours, then fixed and stained as described. The extent of DNA synthesis was evaluated by counting the number of cells that stained positive for BrdU. Data represent the mean of four independent experiments ± SEM.

3.2.2 VEGF is chemotactic for HFF-VEGFR-2 but not HFF-Neo cells

Several reports have shown that VEGF can induce endothelial chemotaxis (Albini et al., 1996; Meyer et al., 1999). Using a modified Boyden chamber assay, we studied the

chemotactic response of HMEC, HFF-Neo and HFF-VEGFR-2 cells to VEGF. VEGF (20 ng/ml) induced a significant increase of HMEC and HFF-VEGFR-2 migration relative to the control (DMEM + 0.1%BSA), causing a 3.5 and a 5-fold increase, respectively, 8 hrs post-treatment (Figure 8). Again, VEGF did not induce chemotaxis in HFF-Neo cells. FGF-2, used as a positive control, was chemotactic for all three cell types.

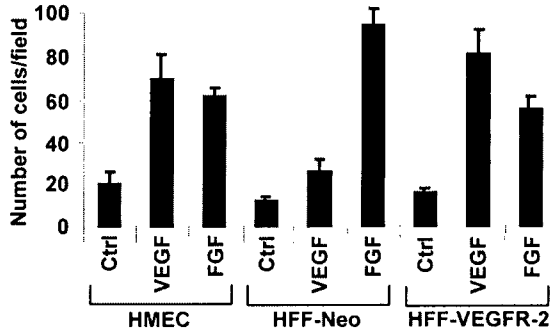

Figure 8 VEGF simulates migration of HMEC and HFF-VEGFR-2 cells, but not HFF-Neo cells. Serum free-medium with or without VEGF or FGF-2 was placed in the lower chamber of a 24-well Transwell. A total of 5×10^5 cells/well were seeded in the upper chamber, and cells were allowed to migrate for 8 h through the collagen-coated polycarbonate membrane. Cells that had migrated were quantified by counting three random high power fields/well. Data represent the mean of three independent experiments ± SEM.

3.2.3 VEGF activates Erk1/2 and p38 MAP Kinases in HFF-VEGFR-2, but not in HFF-Neo cells

We next examined whether the MAP kinase cascade, a convergent pathway in the mitogenic action of many growth factors, including VEGF, can be mediated through the activation of VEGFR-2 alone (Kroll and Waltenberger, 1997; Mukhopadhyay et al., 1998). Serum-starved cells were stimulated with VEGF (20 ng/ml) for 0 to 30 min, and Erk was immunoprecipitated from cell lysates. Using an anti-phospho-Erk antibody, we observed that Erk phosphorylation increased in response to VEGF stimulation in HFF-

VEGFR-2 cells, but not in HFF-Neo cells (Figure 9A). Maximum phosphorylation of Erk peaked at 15 min in HFF-VEGFR-2 cells. The kinetics of Erk phosphorylation was mirrored by Erk activity as assayed by *in vitro* phosphorylation of MBP (Figure 9B). We observed an increase in Erk activation after 10 minutes in HFF-VEGFR-2 cells. As with the phosphorylation, activation of Erk peaked at 15 minutes in HFF-VEGFR-2 cells. No Erk activation was detected in HFF-Neo cells following VEGF stimulation.

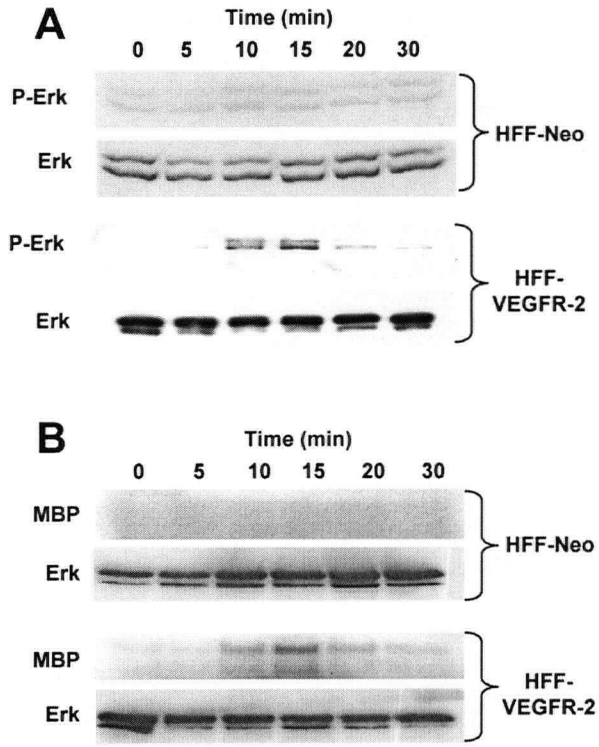

Figure 9 VEGF induces phosphorylation and activation of Erk1/2 MAP kinase in HFF-VEGFR-2 but not HFF-Neo cells. Quiescent cells were incubated with 20 ng/ml VEGF for 0 to 30 minutes as indicated. (A) Phosphorylation of Erk1/2 was determined by Western blotting using an antibody specific to phosphorylated Erk1/2 (upper panel, P-Erk-1). Membranes were reprobed with an anti-Erk antibody as a loading control. (B) Activity of Erk1/2 was determined by an *in vitro* kinase assay using MBP as a substrate. Expression of Erk1/2 was determined by Western blotting. Data show one experiment of four independent experiments.

We also examined the effect of VEGF on p38 phosphorylation for transduced fibroblasts by Western blot using an anti-phospho-p38 antibody. We found that VEGF

stimulation resulted in p38 phosphorylation in HFF-VEGFR-2 cells, but had no effect in HFF-Neo cells (Figure 10). Maximum phosphorylation of p38 was detected after 15 minutes in HFF-VEGFR-2 cells. Again, VEGF had no effect in mock-transduced cells.

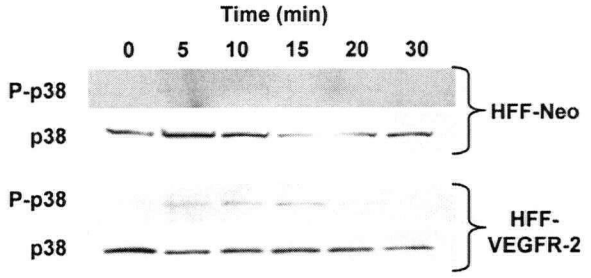

Figure 10 VEGF induces phosphorylation of p38 MAP kinase in HFF-VEGFR-2 but not HFF-Neo cells. Quiescent cells were incubated with 20 ng/ml VEGF for 0 to 30 minutes as indicated. Phosphorylation of p38 was determined by Western blotting using an antibody specific to phosphorylated p38 (P-p38). Data represent one experiment of three independent experiments.

3.2.4 VEGF does not induce expression of endothelial markers in primary fibroblasts transduced with VEGFR-2

A previous study has suggested that fibroblasts can transdifferentiate into endothelial cells (Kon and Fujiwara, 1994). Others have shown that VEGFR-2-expressing cells isolated from chick gastrula can differentiate into either hematopoietic or endothelial lineages and that the presence of VEGF in the culture medium is absolutely required for endothelial differentiation (Eichmann et al., 1997). To assess whether VEGFR-2 activation is sufficient for induction of expression of endothelial markers in fibroblasts, HFF-VEGFR-2 and HFF-Neo cells were incubated in the presence of 10 ng/ml VEGF for 4 days, after which expression of various endothelial markers was assayed by Western blot. We were not able to detect expression of endothelial markers such as VEGFR-1, Tie-1, Tie-2 and eNOS, as shown by Western blot (Figure 11). Expression of eNOS (Kroll and Waltenberger, 1998) and Tie-1 (McCarthy et al., 1998)

have been shown to be increased following VEGF stimulation of endothelial cells. However, we did not observe any increase in expression of these markers in the presence of VEGF, even in HMEC. As seen in Figure 6A, we did not observe any morphologic changes in HFF-VEGFR-2 relative to HFF-Neo cells.

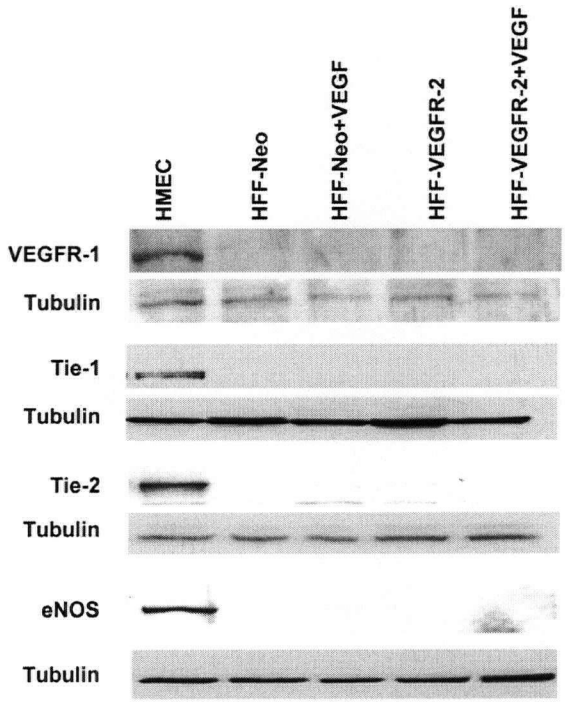

Figure 11 VEGF does not induce expression of endothelial markers in HFF-VEGFR-2 cells. Cells were incubated with VEGF for four days before cell extracts were collected and processed for western blotting. Endothelial markers examined included VEGFR-1, Tie-1, Tie-2 and eNOS. Membranes were reprobed with an anti-tubulin antibody as a loading control. Data represent one experiment repeated at least three times with similar results.

Thus, even though VEGF/VEGFR-2 interactions appear to be required for endothelial development from mesodermal precursors, these results suggest that VEGFR-2 alone is not sufficient to induce expression of endothelial markers in primary fibroblasts.

3.3 Discussion

The vascular system forms through a combination of vasculogenesis and angiogenesis. In vasculogenesis, vessels form *de novo* via the assembly of endothelial precursors called angioblasts, whereas in angiogenesis new vessels arise by migration and proliferation of endothelial cells from preexisting vessels (Risau and Flamme, 1995). VEGFR-2 is required for the embryonic production of cells of the hemangioblastic lineage, and for vascular development in the embryo and adult (Eichmann et al., 1997; Risau and Flamme, 1995; Shalaby et al., 1997). Of the endothelial receptor tyrosine kinases identified so far, VEGFR-2 is the only receptor whose function is absolutely required for the determination of the endothelial lineage (Shalaby et al., 1997; Shalaby et al., 1995). Whether it acts as a switch that triggers endothelial differentiation or is only required for the migration and survival of early mesodermal precursors remains to be shown.

Because it has been difficult to express VEGFR-2 in heterologous cells, to study the specific signalling pathways and biological effects triggered by VEGFR-2, we used a retroviral vector to transduce primary human foreskin fibroblasts with VEGFR-2. Two major biological activities of VEGF are to induce vascular endothelial cell proliferation, and to stimulate cell migration (Barleon et al., 1996; Connolly et al., 1989; Ferrara and Henzel, 1989; Rousseau et al., 1997). However, the mechanisms underlying these diverse processes are still not well characterized. We examined whether VEGFR-2 was able to elicit a proliferative response in the absence of other VEGF receptors. MTT and BrdU incorporation assays showed that VEGF was indeed able to cause the proliferation of HFF-VEGFR-2 cells whereas it had no effect on HFF-Neo cells. These results indicate

that the VEGF-induced proliferative response can be transmitted solely through the activation of VEGFR-2. This confirms the finding of another study in which NIH 3T3 cells transduced with VEGFR-2 showed a proliferative response in the presence of VEGF (Takahashi and Shibuya, 1997). A separate study showed that VEGF did not cause proliferation of VEGFR-1-transduced NIH 3T3 cells (Seetharam et al., 1995). Thus, it is likely that VEGFR-2 is the main receptor responsible for transducing VEGF-induced mitogenic signals.

Erk1/2 MAP kinase is a central signalling molecule that is activated by many growth factor receptors (Waskiewicz and Cooper, 1995). Erk activation has also been associated with cell proliferation. It is possible that the mitogenic effect of VEGFR-2 may be mediated through Erk activation. We therefore investigated whether activation of VEGFR-2 by VEGF could activate the Erk1/2 MAP kinase pathway in the absence of other VEGF receptors. We found that VEGF had the capacity to induce Erk1/2 phosphorylation and activation in HFF-VEGFR-2 cells, indicating that VEGFR-2 can activate this pathway in isolation.

Endothelial cell migration plays a critical role in angiogenesis. Several cytokines, such as VEGF and angiopoietin-1 have been shown to act as chemoattractants for endothelial cells (Neufeld et al., 1999; Witzenbichler et al., 1998). Furthermore, it has been hypothesized that VEGF may be critical for embryonic hematopoiesis and vasculogenesis by promoting the migration of early precursors (Traver and Zon, 2002). Using a modified Boyden chamber assay, we also demonstrated that VEGF can cause directed migration of HFF-VEGFR-2 cells, indicating that VEGFR-2 is indeed implicated in cell migration. Other studies have shown that VEGFR-1 can also direct cell migration

of monocytes (Barleon et al., 1996; Sawano et al., 2001). It is not known whether VEGFR-1 activates the same pathways as VEGFR-2 to induce cell migration. However, since HFF-VEGFR-2 showed a similar chemotactic response to VEGF as HMEC, it is likely that activation of both receptors is not required to induce cell migration.

We then examined the p38 signal transduction pathway elicited by VEGFR-2 in response to VEGF stimulation. It has already been shown that VEGF stimulation of endothelial cells can result in phosphorylation of p38 MAP kinase (Rousseau et al., 1997). Activation of p38 results in activation of MAP kinase activated protein kinase-2/3 and phosphorylation of the F-actin polymerization modulator, heat shock protein 27 (HSP27) (Hedges et al., 1999; Kato et al., 1999). The p38 pathway conveys the VEGF signal to microfilaments inducing rearrangements of the actin cytoskeleton that regulate cell migration (Hedges et al., 1999; Kato et al., 1999; Rousseau et al., 1997). By modulating cell migration, p38 may thus be an important regulator of angiogenesis. Here we find that the p38 pathway, as with Erk1/2 activation, can be activated by VEGFR-2 alone. Therefore, cell migration induced by VEGF in HFF-VEGFR-2 could be a consequence of the activation of the p38 pathway.

It has been reported *in vivo* that fibroblasts have the potential to differentiate into endothelial cells, and may play a role in angiogenesis, as progenitors of endothelial cells in newly formed blood vessels (Kon and Fujiwara, 1994). However, the mechanisms by which this occurs is unknown. Moreover, fibroblasts transduced with the MyoD gene have been reported to transdifferentiate into muscle cells (Weintraub et al., 1989). Thus, it seems that, under the proper conditions, fibroblasts may have a plasticity that allows them to transdifferentiate into other cell types. Since VEGFR-2 is critical in endothelial

development, it is possible that activation of this receptor by VEGF could trigger induction of endothelial markers in primary fibroblasts. However, when incubated with VEGF for several days, HFF-VEGFR-2 cells did not display an endothelial phenotype, as shown by the lack of expression of endothelial markers such as VEGFR-1, tie-1, tie-2 and eNOS. HFF-VEGFR-2 also did not adopt an endothelial cobblestone morphology. This contrasts with the findings of Eichmann and colleagues, who have demonstrated that in uncommitted precursors VEGF/VEGFR-2 interactions will upregulate endothelial markers (Eichmann et al., 1997). Although HFF-VEGFR-2 cells were found to be signalling competent, other components of endothelial signalling pathways may be required for differentiation.

In addition to VEGFR-2, other membrane receptors, intracellular signalling molecules or transcription factors are also likely necessary for endothelial differentiation. Alternatively, it is also possible that VEGFR-2 is not required for endothelial differentiation, but that its role is limited to drive the survival, proliferation and /or migration of early mesodermal precursors (Habeck et al., 2002).

Chapter 4

Effects of VEGFR-2 activation in murine bone marrow cells *in vitro*

4.1 Introduction

VEGF and its receptors have been shown to play an important role in adult hematopoiesis. VEGFR-2 has been found to be expressed on a subset of hematopoietic stem cells that can differentiate into hematopoietic or vascular endothelial cells depending on the culture conditions (Gehling et al., 2000; Peichev et al., 2000; Ziegler et al., 1999). Because of the increasing amount of data that supports a role of VEGFR-2 signalling in adult hematopoietic cells, both normal and malignant, we decided to study the effects of VEGFR-2 signalling in isolation in murine hematopoietic cells. However, since subsets of hematopoietic cells express VEGF receptors, we chose not to use hematopoietic cells transduced with the full length VEGFR-2, as stimulation of those cells with VEGF may activate the endogenous VEGF receptors present on some of those cells, which in turn may mask or interfere with VEGFR-2 signalling. Moreover, it has been shown that neuropilin-1 is a receptor for VEGF and acts as a co-receptor that enhances the function of VEGF through VEGFR-2 (Zachary and Gliki, 2001). Furthermore, VEGFR-2 has been shown to heterodimerize with VEGFR-1 (Kendall et al., 1996). We therefore chose to rely on a strategy that allowed us to specifically activate VEGFR-2 with a chemical compound. The strategy we used allows us to study the unique signalling properties of VEGFR-2, without any interference from other endogenous VEGF receptors, allowing us to exclude the effects of neuropilin, or heterodimerization with VEGFR-1.

The strategy we used to study VEGFR-2 signalling has recently been used to study the unique signalling effects of some hematopoietic receptors (flt-3, MPL, granulocyte-colony stimulating factor receptor, c-kit) (Jin et al., 1998a; Jin et al., 2000;

Otto et al., 2001b). The biological effects triggered by those receptors have been studied by fusing the signalling domain of these receptors to an FK506 binding protein (FKBP) that can be specifically activated using synthetic FKBP ligands (Blau, 1999; Blau et al., 1997; Jin et al., 1998a; Jin et al., 1998b; Jin et al., 2000; Otto et al., 2001b; Richard et al., 2000). This system has permitted the demonstration that the self-renewal and differentiation of hematopoietic progenitors can be influenced through distinct, receptor-initiated signalling pathways (Zeng et al., 2001).

In this chapter, we used this inducible dimerization strategy to specifically study the biological effects of VEGFR-2 signalling on hematopoietic progenitors. To specifically study the unique signalling effects of VEGFR-2, we fused the cytoplasmic domain of this receptor, which contains the split tyrosine kinase domain, to a mutated FKBP12 domain that harbours a phenylalanine to valine mutation at amino acid 36. Although other studies have shown the signalling effects of VEGFR-2 by using VEGFR-2 specific ligands, such as VEGF-E (Meyer et al., 1999), the use of a non-toxic chemical inducer of dimerization, AP20187 (Ariad Pharmaceuticals), allowed us to study with high specificity VEGFR-2 signalling pathways and biological effects in a cell autonomous manner. This strategy also allowed us to rule out any potential signalling effects that could be triggered by neuropilin-1, which acts as a co-receptor for VEGF, enhancing its binding to VEGFR-2 (Zachary and Gliki, 2001).

4.2 Results

4.2.1 Activation of VEGFR-2 delays loss of murine hematopoietic progenitors

We cloned the intracellular domain of VEGFR-2 and fused it to a modified FKBP domain that can be specifically dimerized with a chemical inducer, AP20187 (Figure

12A). When transduced into HMEC-1 cells or murine bone marrow cells, this construct gave a 100 kDa protein (Figure 12B), which mainly localized to the cytoplasmic membrane when unstimulated (Figure 12C). Stimulation of HMEC-1 cells with 10 nM AP20187 for 0 to 30 minutes, resulted in progressive translocation of the fused VEGFR-2 construct from the cytoplasmic membrane to the cytoplasm. Phosphorylation of the construct was observed as early as 30 seconds after stimulation with 10 nM AP20187 in HMEC cells, and remained over a period of at least 30 minutes (Figure 12D).

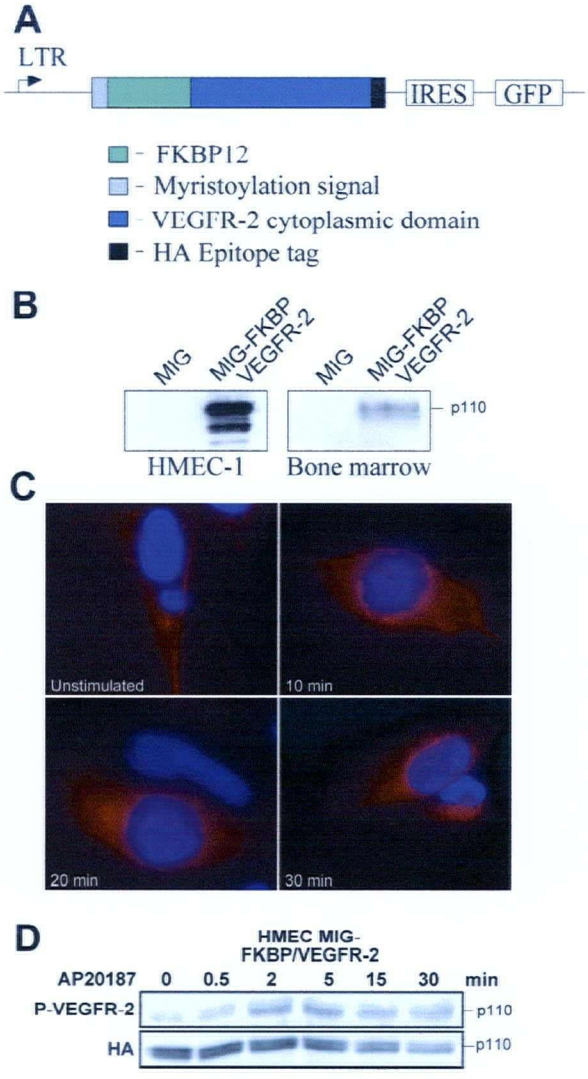

Figure 12 MIG-FKBP/VEGFR-2 fusion construct. (**A**) The construct includes the intracellular domain of VEGFR-2 fused to a modified FKBP12 domain. A hemagglutinin (HA) epitope tag was included at the C-terminus and a myristoylation sequence at the N-terminus. (**B**) Immunoblotting of HMEC-1 and bone marrow cells demonstrates expression of a 110 kDa protein, consistent with the predicted size of the VEGFR-2 construct. (**C**) Immunofluorescent staining of HMEC-1 cells reveals that the fusion protein localizes mainly to the cytoplasmic membrane when unstimulated. Stimulation with 10 nM AP20187 resulted over a period of 30 minutes resulted in partial translocation of the fusion protein to the cytoplasm. (**D**) HMEC-1 cells were incubated with 10 nM AP20187 for 0 to 30 minutes. Phosphorylation of FKBP/VEGFR-2 fusion protein was determined by immunoblotting using an antibody specific to phosphorylated VEGFR-2 (Tyr 951). The membrane was reprobed with anti-HA antibody as a loading control. The fusion protein was detected in HMEC-1 cells using an anti-HA monoclonal antibody for both immunoblotting and immunofluorescence.

To investigate the effect of VEGFR-2 in hematopoietic cells, bone marrow from mice treated with 5-fluorouracil to activate bone marrow precursor cells was harvested and transduced with the VEGFR-2 fusion construct. As a control, the empty MSCV-IRES-GFP (MIG) vector was used. After sorting, transduced GFP^+ cells were plated in IMDM medium supplemented with 10% FBS with or without 100 nM AP20187, and cells were counted at days 5, 7 and 14. For all experiments, only transduced GFP^+ cells were used. We found that the number of viable cells decreased rapidly in the absence of cytokines. However, in marrow cells in which the VEGFR-2 construct was dimerized by the addition of AP20187, we observed a smaller decrease in cell number (Figure 13A). After 2 weeks in culture, cell numbers in bone marrow control cultures were 2.5 fold lower than the ones in which VEGFR-2 was dimerized. This effect was not observed in VEGFR-2 transduced cells that did not receive AP20187, indicating that dimerization of VEGFR-2 is required for maintaining hematopoietic cell numbers. We next tested whether dimerization of VEGFR-2 had an additive effect on medium supplemented with hematopoietic cytokines that provide optimal growth conditions (Thorsteinsdottir et al., 1999). Transduced bone marrow cells were cultured in medium containing cytokines that are known to induce hematopoietic cell proliferation (IL-3, IL-6, SCF), with or without 100 nM AP20187 (Figure 13B). With these growth conditions, we did not observe any significant change when VEGFR-2 was dimerized in comparison to the control cells, suggesting that VEGFR-2 does not signal a proliferative or anti-apoptotic effect that is synergistic with these hematopoietic cytokines.

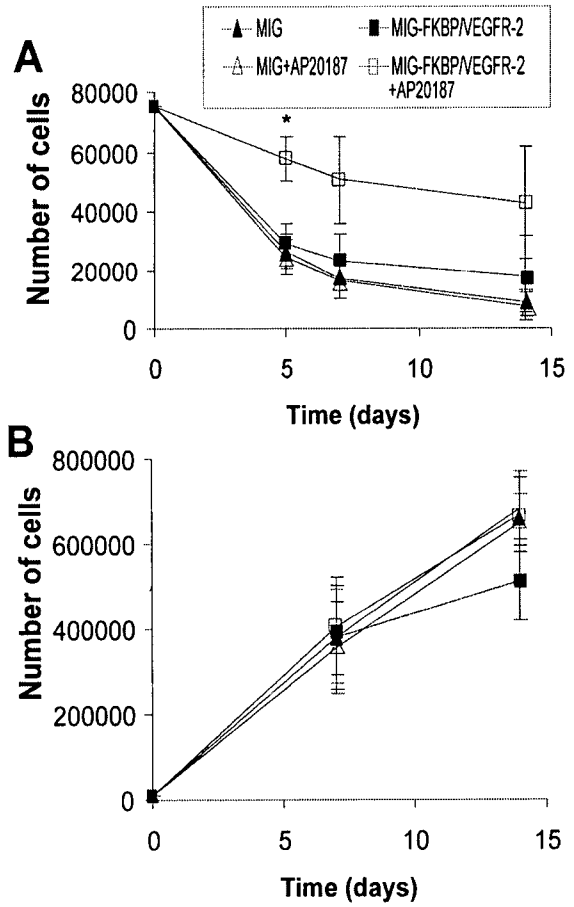

Figure 13 VEGFR-2 maintains hematopoietic cell number. MIG or MIG-FKBP/VEGFR-2-transduced bone marrow cells were incubated in IMDM supplemented with 10% FBS with or without 100 nM AP20187 in the absence (**A**) or the presence (**B**) of hematopoietic cytokines (IL-3, IL-6, SCF) for 0 to 14 days. Cells were harvested at specific time points as indicated and cell number was determined. Data represent the mean ± SEM of three independent experiments. * $P<0.05$.

To test whether VEGFR-2 can preserve the viability and activity of hematopoietic progenitors in the absence of hematopoietic cytokines, VEGFR-2 and control cells were cultured in cytokine-free medium for 7 and 14 days with or without addition of dimerizer, after which cells were plated in methylcellulose medium to assay for hematopoietic

progenitors. We found that dimerization of VEGFR-2 maintained hematopoietic progenitor potential in liquid culture to a certain extent. Over a 2-week period in culture, we observed an 8-fold decrease in the number of progenitors in control bone marrow cultures. In contrast, when the FKBP-VEGFR-2 construct was dimerized with AP20187, we observed a 3-fold increase in the maintenance of progenitors over control cultures, consistent with the findings in Figure 13 (Figure 14A). Although VEGFR-2 dimerization maintained the hematopoietic progenitor population for a period of two weeks in the absence of other cytokines, we did not observe a significant change in the proportion of different hematopoietic progenitors as measured by the CFC assay (Figure 14B). This result suggests that VEGFR-2 promotes hematopoietic cell survival and/or proliferation, but does not affect differentiation of hematopoietic progenitors.

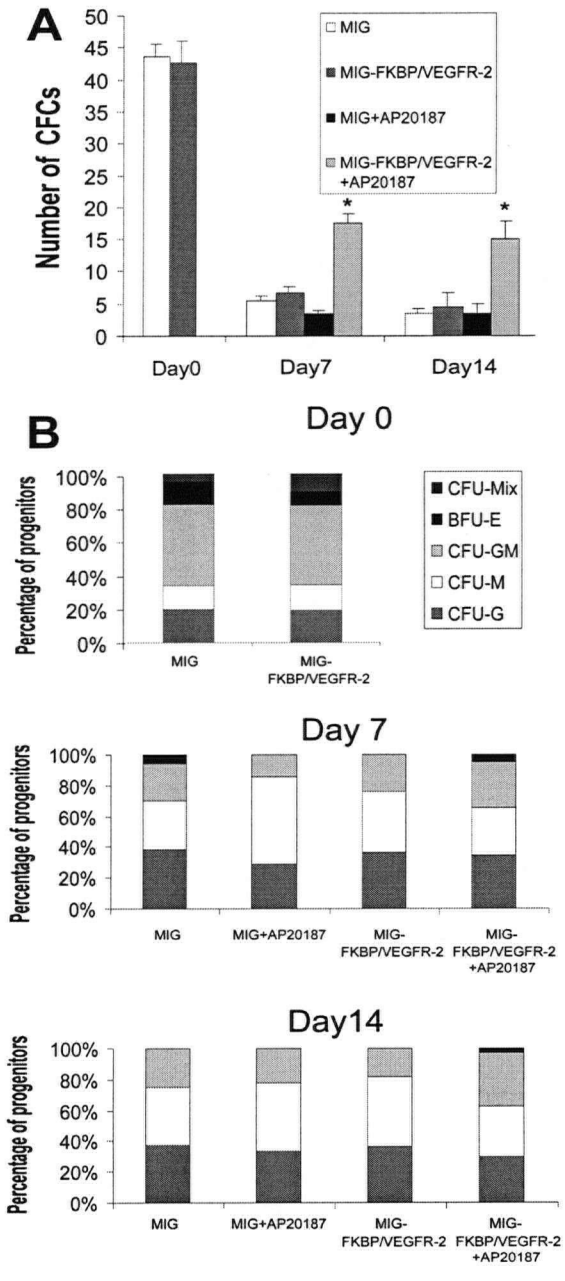

Figure 14 VEGFR-2 delays the loss of CFCs. Hematopoietic progenitors transduced with MIG- or MIG-FKBP/VEGFR-2 were grown in IMDM containing 10% FBS for 7 or 14 days, and then plated in complete methylcellulose medium to test for progenitor activity as measured by CFC number after 10 days (**A**). Dimerization of VEGFR-2 did not affect the proportion of different progenitors over time as measured by scoring for the type of colonies formed in the CFC assay (**B**). Data represent the mean ± SEM of three independent experiments. * $P<0.05$.

To confirm that VEGFR-2 can independently maintain the multipotent hematopoietic progenitor population, we utilized the CFU-Spleen (CFU-S_{12}) assay following liquid culture of bone marrow cells for 7 days in cytokine-free medium. Colonies were enumerated in each of the spleens harvested 12 days following injection of bone marrow cells (Figure 15A). As seen in Figure 15B, VEGFR-2 dimerization resulted in a 5-fold increase in the proportion of CFU-S_{12} cells, compared to bone control marrow cultures. These results suggest that VEGFR-2 can maintain the activity and viability of primitive hematopoietic progenitors in the absence of other exogenous cytokines.

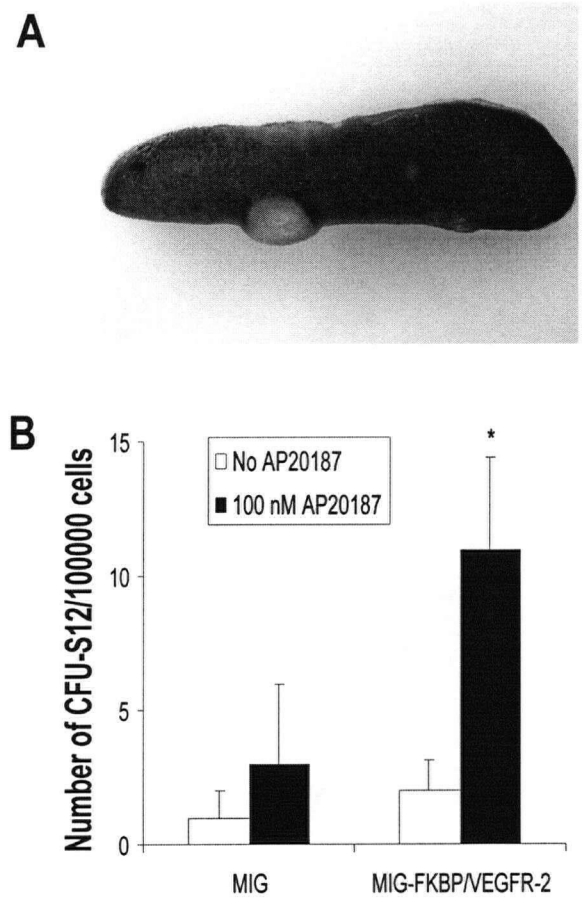

Figure 15 VEGFR-2 dimerization maintains the number of multipotential bone marrow progenitors. Hematopoietic primitive myeloid progenitors cultured for 7 days in IMDM + 10%FBS, with or without AP20187, were injected into lethally irradiated B6C3 mice, and spleen colonies were counted after 12 days (**A, B**). Data represent the mean ± SEM of three independent experiments. * $P<0.05$.

4.2.2 VEGFR-2 does not increase S-phase entry in hematopoietic precursors

It is known that, in endothelial cells, VEGF can induce cell proliferation. It has been suggested that this effect is mainly mediated through VEGFR-2 (Ferrara, 1999a). Moreover, we have demonstrated in the previous chapter that VEGFR-2 can induce proliferation of primary fibroblasts. We tested whether dimerization of VEGFR-2 also resulted in bone marrow cell proliferation, which could account in part for the delay in

the loss of hematopoietic progenitors that we observed. Bone marrow cells were grown in cytokine-free medium for 2 days, then exposed to BrdU with or without AP20187 for 2 hours. Cytospin preparations of cells were then labelled with an anti-BrdU antibody (Figure 16A). We found that dimerization of VEGFR-2 did not result in a greater proportion of cells which incorporated BrdU, indicating that VEGFR-2 signalling alone may not be sufficient to induce proliferation of hematopoietic progenitors (Figure 16B).

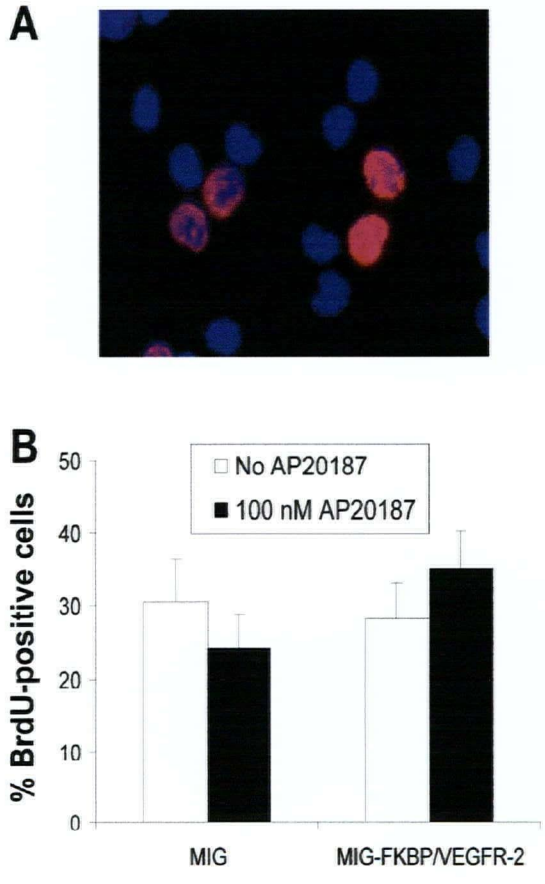

Figure 16 VEGFR-2 dimerization does not increase BrdU uptake in hematopoietic progenitors. MIG- or MIG-FKBP/VEGFR-2-transduced bone marrow cells cultured for 2 days in IMDM supplemented with 10% FBS were treated for 2 hours with 10 nM BrdU with or without 100 nM AP20187 and cytospin preparations were stained with an anti-BrdU antibody conjugated with Alexa 594. DAPI staining was used to identify nuclei (**A**). The number of $BrdU^+$ cells were quantitated and expressed as a percent of total nuclei counted (**B**). 200-300 cells were counted per cytospin preparation. Data represent the mean ± SEM of three independent experiments.

4.2.3 VEGFR-2 activation reduces the number of apoptotic cells in hematopoietic precursors

It has been shown that VEGF can induce anti-apoptotic signalling through PI3-kinase in endothelial cells subjected to serum deprivation (Gerber et al., 1998b). Since we observed a delay in loss of progenitors when VEGFR-2 is dimerized, we postulated that this effect was caused by an inhibition of apoptosis since VEGFR-2 dimerization alone did not affect proliferation of hematopoietic progenitors. It has been shown that caspase 3 is present in hematopoietic precursor cells and is activated during apoptosis (Nicholson and Thornberry, 1997; Zermati et al., 2001). To test whether VEGFR-2 inhibits hematopoietic cell apoptosis, transduced bone marrow cells were subjected to cytokine deprivation, and incubated with or without AP20187 for 14 days. At this point, cytospins were made and stained for the activated form of caspase-3 (Figure 17A). We found that the proportion of apoptotic cells was 2-fold lower in bone marrow cells in which VEGFR-2 was dimerized compared to bone marrow control cultures (Figure 17B). Hence, inhibition of apoptosis through VEGFR-2 signalling would explain in part the maintenance of hematopoietic progenitors observed.

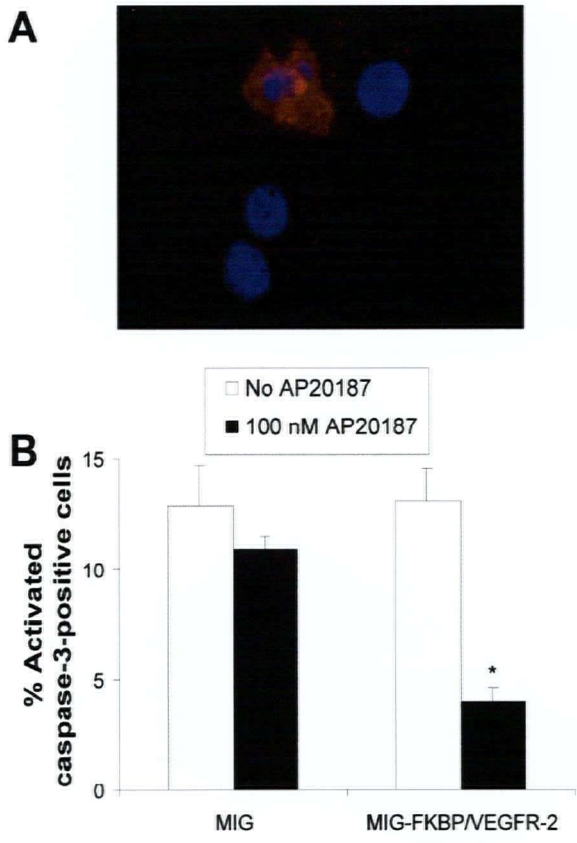

Figure 17 VEGFR-2 dimerization inhibits apoptosis of cytokine-starved hematopoietic progenitors. Cytospin preparations of MIG- or MIG-FKBP/VEGFR-2-transduced bone marrow cells cultured for 14 days in IMDM containing 10% FBS with or without 100 nM AP20187 were stained with an anti-activated caspase 3 antibody. Nuclei were counter-stained with DAPI (**A**). Cells with activated caspase 3 were quantitated and expressed as a percent of total cells counted (**B**). 200-300 cells were counted per cytospin preparation. Data represent the mean ± SEM of four independent experiments. * $P<0.05$.

4.2.4 VEGFR-2 activates the PI3-kinase and Erk MAP kinase pathways

Since VEGFR-2 dimerization reduces the proportion of apoptotic cells, we examined signalling pathways known to be induced by VEGF in endothelial cells. In particular, the PI3-kinase/Akt and the MAP kinase pathways are both implicated in VEGF signalling and have potential roles in cell survival (Gerber et al., 1998b; Thakker et al., 1999). To determine the kinetics of activation of Akt and Erk1/2 by VEGFR-2,

endothelial cells transduced with MIG or MIG-FKBP/VEGFR-2 were starved overnight in medium supplemented with 5%FBS, and then treated with AP20187 for 0 to 60 minutes. Membranes were reprobed with total Akt or Erk as a loading control. Following dimerization of VEGFR-2, we found that both Akt and Erk1/2 were activated. Akt phosphorylation peaked between 10 and 20 minutes (Figure 18A), whereas maximum Erk 1/2 phosphorylation was observed between 20 and 30 minutes (Figure 18B). Activation of Akt was biphasic, with a second peak of phosphorylation after 60 minutes (Figure 18A). This biphasic activation of Akt in response to VEGFR-2 dimerization was observed in three independent experiments. We next checked whether the Akt and Erk 1/2 pathways were also induced in murine bone marrow cells. Transduced GFP^+ bone marrow cells were incubated for 2 days in cytokine-free medium, then stimulated for 20 minutes with 100 nM AP20187. As with endothelial cells, we also observed activation of Akt (Figure 18C) and Erk1/2 (Figure 18D) in bone marrow following VEGFR-2 dimerization. Either of these kinases could account, at least in part, for the survival that we observed in response to VEGFR-2 dimerization, since induction of these signalling pathways by other hematopoietic cytokines, such as SCF and erythropoietin has been implicated in hematopoietic cell survival (Kinoshita et al., 1997; Liu et al., 1997; Sui et al., 2000).

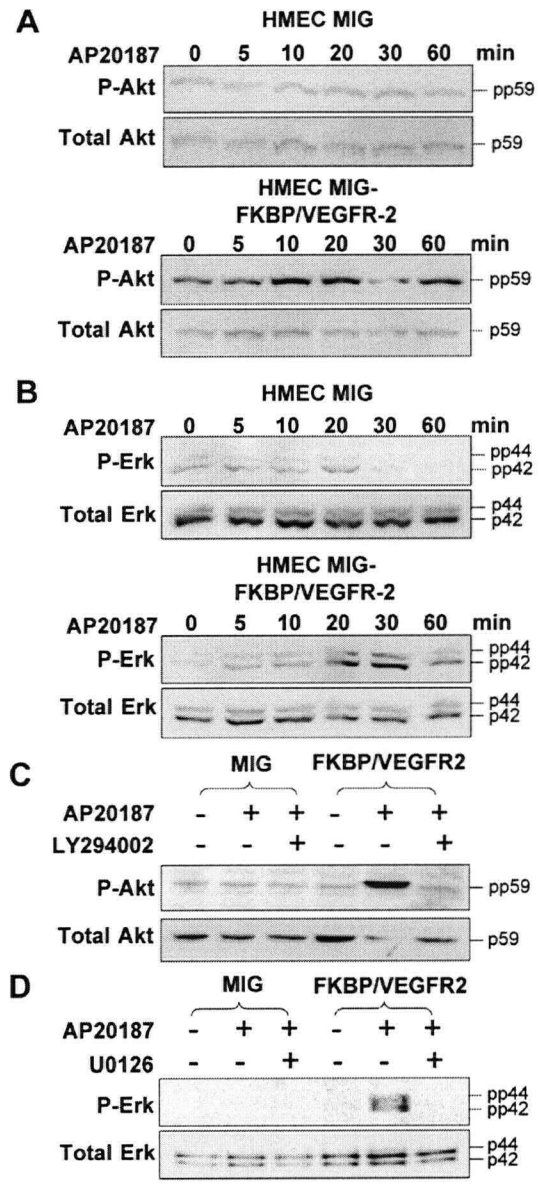

Figure 18 VEGFR-2 dimerization activates Akt and Erk1/2 MAP kinases in hematopoietic progenitors. Quiescent HMEC-1 cells were incubated with 10 nM AP20187 for 0 to 60 minutes as indicated (**A, B**). Cytokine-starved MIG- or MIG-FKBP/VEGFR-2-transduced bone marrow progenitors were treated for 20 minutes with 100 nM AP20187 (**C, D**). Phosphorylation of Akt (**A, C**) or Erk1/2 (**B, D**) was determined by immunoblotting using antibodies specific to phosphorylated Akt or Erk1/2. Lanes: 1,4: untreated; lanes 2,5: 100 nM AP20187 for 20 min; lanes 3,6: pre-treatment with 20 μM LY294002 (Akt) or 10 μM U0126 (Erk) for 90 min, then treated with 100 nM AP20187 for 20 min. Membranes were reprobed with anti-Akt or anti-Erk1/2 antibodies as loading controls. Data represent one experiment of three independent experiments showing similar findings.

To specifically study the above signalling pathways in mediating hematopoietic progenitor survival, we used specific inhibitors of each signalling pathway. LY294002 is an inhibitor of PI3-kinase, which activates Akt, while U0126 has been shown to block Erk 1/2 MAP kinase phosphorylation by specific inhibition of MEK (Davies et al., 2000). To determine whether VEGFR-2 mediated survival was mediated through Akt and/or Erk1/2, transduced GFP^+ bone marrow cells were incubated with or without 100 nM AP20187 in the presence of the PI3-kinase inhibitor LY294002, or the MEK inhibitor U0126 at concentrations that blocked each of these kinases (Figure 18C, D). Cell number was monitored over a period of 14 days. We found that inhibition of PI3-kinase blocked the anti-apoptotic effect of VEGFR-2 dimerization induced by cytokine deprivation, indicating the essential role of this pathway in VEGFR-2-mediated survival in bone marrow cells (Figure 19A). Blockade of PI3-kinase also inhibited the survival of hematopoietic progenitors induced by VEGFR-2 dimerization (Figure 19B), further demonstrating the critical role of this pathway in survival of hematopoietic progenitors. In contrast, blockade of the MAP kinase pathway with U0126 did not inhibit VEGFR-2 induced cell survival (Figure 19C). Interestingly, despite minimal effect on cell survival, inhibition of Erk1/2 partially inhibited hematopoietic progenitor activity mediated by VEGFR-2 (Figure 19D). This discrepancy suggests that hematopoietic progenitors are more dependent on the Erk1/2 MAP kinase pathway for survival than more mature/differentiated cells such as macrophages, which constitute the majority of cells present after 14 days in liquid culture (data not shown).

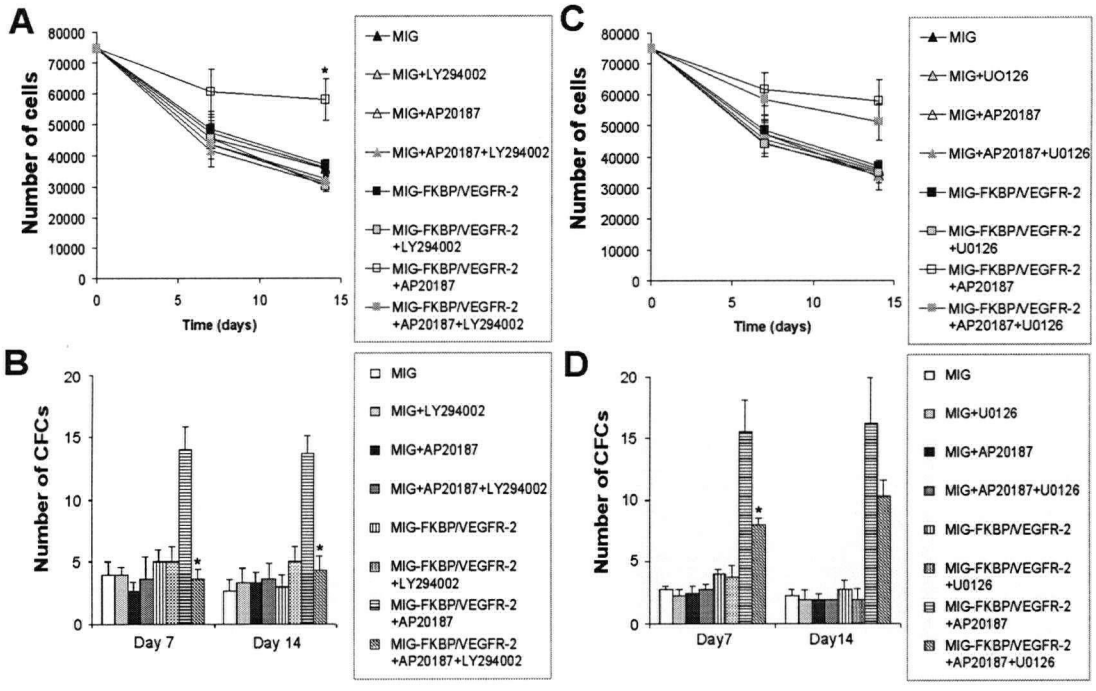

Figure 19 Effect of PI3-kinase or MEK inhibition on hematopoietic progenitor survival. MIG- or MIG-FKBP/VEGFR-2-transduced hematopoietic progenitors were cultured in IMDM supplemented with 10% FBS with or without 100 nM AP20187 in the presence or in the absence of 20 μM of the PI 3-kinase inhibitor, LY294002 (**A, B**), or the MEK inhibitor, U0126 (**C, D**), for 0 to 14 days. Cells were harvested at specific time points and cell number was determined (**A, C**). Cells were collected after 7 and 14 days and plated in methylcellulose medium to assay for hematopoietic progenitor activity (**B, D**). Data represent the mean ± SEM of three independent experiments. * $P<0.05$.

4.3 Discussion

Although many studies postulate that VEGF mediates most of its effects through VEGFR-2, it is difficult to make a clear statement about this because of the complexity of the VEGF-VEGFR system. To specifically study the effects of VEGFR-2 signalling, we used a strategy that allowed the specific activation of the VEGFR-2 signalling domain by using a chemical inducer of dimerization. A similar strategy has previously shown that

the thrombopoietin (Tpo) receptor c-mpl can induce long term proliferation of murine hematopoietic progenitors, whereas the effects of flt-3 and G-CSFR are much more modest (Zeng et al., 2001). In this study, the VEGFR-2 construct was localized to the cytoplasmic membrane in unstimulated HMEC-1 cells, and translocated into the cytosol with concomitant phosphorylation following stimulation with AP20187, a phenomenon observed with many endogenous hematopoietic receptors. Interestingly, however, Otto *et al.* (Otto et al., 2001a) have shown that membrane localization of the thrombopoietin receptor, mpl, is not required for the full range of c-mpl function in hematopoietic cells.

We were interested in the role of VEGFR-2 in hematopoietic progenitors since little is known about the effects of this receptor on hematopoiesis. In murine bone marrow, our results indicate that VEGFR-2 can maintain hematopoietic progenitor potential following dimerization, since cells fail to survive in the absence of AP20187. It is interesting to note that when hematopoietic progenitors were cultured in the presence of hematopoietic cytokines (IL-3, IL-6, SCF), there was no effect of VEGFR-2 dimerization on cell number. This would suggest that VEGFR-2 does not induce a proliferative signal in hematopoietic progenitors that is distinct from IL-3, IL-6 and SCF. Although Erk1/2 MAP kinase was activated, VEGFR-2 dimerization failed to induce proliferation of bone marrow progenitors, indicating that signals provided by other factors may be necessary for the proliferation of hematopoietic cells. It is likely then that the delay in cell loss observed through VEGFR-2 dimerization reflects an effect on cell survival. This was verified when we found that VEGFR-2 signalling decreased the fraction of apoptotic cells, when hematopoietic progenitors were cultured in the absence of exogenous cytokines.

The importance of cell survival in the maintenance of hematopoietic progenitors was further demonstrated by blocking the PI3-kinase pathway. This signalling pathway, through Akt, has been shown to play a crucial role in cytokine-mediated survival (Cantley, 2002; Gerber et al., 1998b), as well as in the self-renewal of primary multipotential hematopoietic progenitors (Zhao et al., 2002). Blockade of PI3-kinase completely abolished the maintenance of hematopoietic progenitors mediated by VEGFR-2, indicating that this pathway is critical for VEGFR-2 mediated survival in bone marrow progenitor cells. In contrast, we did not observe a significant effect on cell survival mediated by VEGFR-2 when the Erk1/2 MAP kinase pathway was blocked using the MEK inhibitor, U0126. This would imply that, although the Erk1/2 MAP kinase pathway has been shown to play a role in apoptosis prevention (Franklin and McCubrey, 2000), the PI3-kinase pathway through Akt is the main regulator of VEGFR-2-induced survival signalling in hematopoietic progenitors. However, it is noteworthy that inhibition of Erk1/2 activation did reduce the number of hematopoietic progenitors as measured by the CFC assay. This effect may be explained by the fact that most cells remaining after 14 days in culture in the absence of exogenous cytokines are differentiated, and studies have shown that the cytokine-induced survival of differentiated cells, such as macrophages, is mediated mainly by the PI3-kinase pathway and not the Erk1/2 MAP kinase pathway (Jaworowski et al., 1999; Xaus et al., 2001). Thus, this may indicate that hematopoietic progenitors are more dependent on the Erk1/2 MAP kinase pathway for VEGFR-2-induced survival than more mature cells.

Although VEGFR-2 signalling promoted survival of hematopoietic progenitors and maintained their progenitor potential, it did not seem to affect the differentiation of

those progenitors. In contrast, other hematopoietic cytokine receptors, such as mpl, induce a dramatic expansion of multipotential progenitors and megakaryocytes (Richard et al., 2000; Zeng et al., 2001). A recent study has demonstrated that a combination of signals, JAK2 plus either c-kit or flt-3 together can support extensive hematopoietic progenitor cell self-renewal even though neither of these receptors can sustain the growth of bone marrow cells alone (Zhao et al., 2002). Whether VEGFR-2 requires additional signals to induce cell proliferation in hematopoietic cells remains unknown and further studies would be needed to assess this issue.

The strategy used in the present study allowed us to demonstrate that VEGFR-2 can activate the PI3-kinase and Erk1/2 pathways, without interaction with other VEGF receptors such as the neuropilins or VEGFR-1, in hematopoietic progenitors. These results show that VEGFR-2 can induce maintenance of hematopoietic progenitors in the absence of exogenous hematopoietic cytokines. This may help to explain, at least in part, the critical role of VEGFR-2 not only in embryonic hematopoiesis, but also in adult hematopoiesis, both normal and malignant.

Chapter 5

Activation of VEGFR-2 in bone marrow cells leads to accumulation of myeloid cells *in vivo*: role of GM-CSF

5.1 Introduction

VEGF may play an important role in the development of haemopoietic cells, but available data do not provide a definitive answer to this question. For example, although murine embryos with inactivating VEGFR-2 mutations remain 'bloodless' it is unclear if this is due to a primary stem cell defect or defective vascularization and blood island formation (Shalaby et al., 1997). In addition, it has been reported that VEGF and SCF co-stimulate the development of common precursors for primitive and definitive murine haemopoiesis (Kennedy et al., 1997a) and that VEGF is essential for the reconstitution of hematopoiesis in lethally irradiated animals (Gerber et al., 2002).

VEGFR-2 is expressed on a significant proportion of normal and malignant hematopoietic cells. Moreover, it has been shown that pluripotent hematopoietic stem cells are restricted to the $CD34^{+}VEGFR\text{-}2^{+}$ cell fraction in human cells (Ziegler et al., 1999). However, it has also been reported that neither $VEGFR\text{-}2^{+}CD34^{low/-}$ cells nor $VEGFR\text{-}2^{+}CD34^{+}$ cells have long-term reconstitution capacity in mice (Haruta et al., 2001).

Hematopoietic malignancies often result from impaired control of early hematopoietic stem cell survival, proliferation and self-renewal. VEGF is expressed in cell lines derived from various hematological malignancies (Bellamy et al., 1999). RNA for both VEGF receptors have been detected in some but not all hematopoietic malignancies (Bellamy et al., 2001; Fiedler et al., 1997; Padro et al., 2002). In addition, several reports have documented inhibitory effects by various classes of small molecule inhibitors targeting VEGFR-1 and VEGFR-2 on the growth of human myeloid leukaemia cell lines and in acute myeloid leukaemia blasts independently of their effects on

angiogenesis (Mesters et al., 2001). Moreover, Dias et al. have shown that neutralisation of human VEGFR-2 with specific monoclonal antibodies prolonged survival in mice xenotransplanted with human VEGFR-2^+ leukemic cell lines (Dias et al., 2001). Therefore, understanding the effects of VEGF and its receptors on hematopoietic cells will prove essential for the development of new therapies targeting the growth of hematopoietic malignancies.

Most groups studying the effects of VEGF signalling in normal and malignant hematopoietic cells have used strategies to block VEGF signalling by using either blocking antibodies or small molecule kinase inhibitors. In the present study, we have used the opposite approach by activating VEGFR-2 in normal bone marrow cells, and investigating its effects on hematopoiesis. By using the VEGFR-2 fusion protein mentioned in the previous chapter, we studied the hematopoietic effects that arise when VEGFR-2 is expressed and activated in a relatively large proportion of hematopoietic cells *in vivo*. AP20187 is well tolerated *in vivo*, which allows its use in studying specific signalling pathways, and evaluate its potential use in therapeutic strategies. We examined bone marrow progenitors and the proportion of mature hematopoietic cells in the blood and bone marrow of mice following VEGFR-2 activation. Furthermore, we also looked at the expression of some hematopoietic growth factors triggered by VEGFR-2 and their potential role in mediating VEGFR-2 effects.

5.2 Results

5.2.1 Activation of VEGFR-2 induces expansion of bone marrow myeloid cells

Although VEGFR-2 plays a critical role in the formation of the hematopoietic system, its role in adult hematopoiesis remains unclear (Gerber and Ferrara, 2003). VEGF and its receptors have been reported to be expressed in a variety of hematopoietic malignancies, and such expression is usually associated with poor prognosis (Verstovsek et al., 2003). We have demonstrated in the previous chapter that VEGFR-2 dimerization in murine bone marrow results in hematopoietic progenitor survival *in vitro*.

To investigate the role of VEGFR-2 activation in *in vivo*, murine bone marrow was transduced with the MIG-FKBP-VEGFR-2 construct, which can be dimerized with the chemical inducer of dimerization AP20187. The empty vector MIG was used as control. We have previously demonstrated that the MIG-FKBP-VEGFR-2 construct is signalling-competent when stimulated with AP20187 *in vitro*, and can induce biological responses in murine bone marrow cells. Transduced bone marrow was used to transplant lethally irradiated B6C3 mice. Four to six weeks after transplantation, bone marrow and peripheral blood were obtained from mice to determine baseline engraftment of transduced cells (based on GFP expression). Following this, mice were injected in the peritoneal cavity with 10 mg/kg/day AP20187 or vehicle (4% EtOH, 10% PEG-400, and 1.7% Tween 20 in water) for 10 days, after which they were sacrificed. Peripheral blood and bone marrow were harvested and the proportion of GFP^+ cells was determined by flow cytometry. To confirm that the cells which express the FKBP-VEGFR-2 construct were indeed GFP^+, cells from the bone marrow were stained with LY5.1 and LY5.2 to confirm the donor/recipient origin of the bone marrow cells. We found in a typical

experiment that >90% of GFP$^-$ cells were LY5.1$^-$, and therefore recipient-derived, which therefore implies that the vast majority of GFP$^-$ cells do not express the FKBP-VEGFR-2 construct in the FKBP-VEGFR-2-transplanted animals.

We observed, on average, a 3.5-fold increase in the proportion of GFP$^+$ cells in the bone marrow of the mice in which the VEGFR-2 construct was dimerized with AP20187 (Figure 20). By contrast, no significant changes were observed in the proportion of GFP$^+$ cells in the peripheral blood of these mice. Mice in which VEGFR-2 was not dimerized (mice that were transplanted with empty vector bone marrow or received vehicle) did not show any changes in the proportion of GFP$^+$ cells in either bone marrow or peripheral blood.

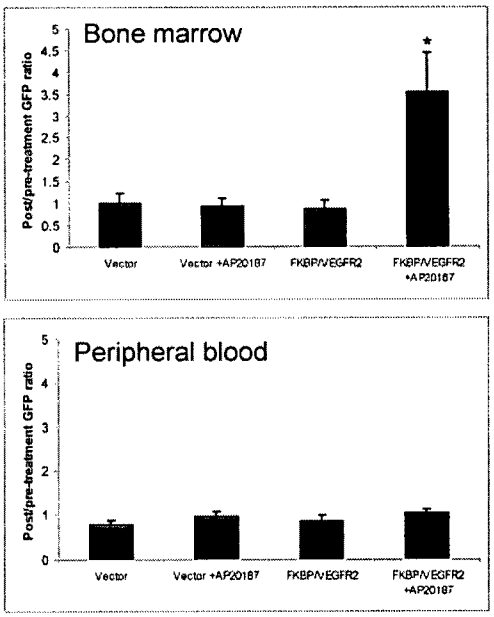

Figure 20 VEGFR-2 induces expansion of retrovirally transduced hematopoietic cells *in vivo*. Ratios of the proportion of GFP$^+$ cells post-treatment relative to pre-treatment are shown for bone marrow (top panel) and peripheral blood (lower panel). Data ± SEM shows average of 8 to 10 mice per group. * $P < 0.01$.

This result indicates that VEGFR-2 activation can elicit a marked expansion of transduced hematopoietic cells in the bone marrow, but interestingly, this expansion of

GFP^+ cells in the bone marrow does not translate into an increase in the proportion of GFP^+ cells in the peripheral blood during the time frame of the experiment.

We next determined the phenotype of cells responding to AP20187 in mice transplanted with MIG-FKBP-VEGFR-2-transduced marrow cells. AP20187 treatment did not result in significantly increased numbers of leukocytes or erythrocytes in the peripheral blood of mice transplanted with MIG-FKBP-VEGFR-2 (Table V).

Table V Peripheral blood counts

Mice	Red blood cell counts ($x10^{12}$ cells/L)	White blood cell counts ($x10^9$cells/L)
Vector	6.77 ± 1.08	5.42 ± 0.90
Vector + AP20187	7.93 ± 1.88	3.82 ± 0.46
FFBP/VEGFR-2	7.31 ± 1.24	4.77 ± 0.91
FKBP/VEGFR-2 + AP20187	9.26 ± 1.83	5.67 ± 0.34

Cells from bone marrow (Figure 21) and peripheral blood (Figure 22) of MIG or MIG-FKBP-VEGFR-2 transplanted mice injected with AP20187 or vehicle for 10 days were stained for the myeloid markers CD11b (monocyte/macrophage) and Gr-1 (granulocyte), lymphoid markers CD5 (T-cells) and B220 (B-cells) or an erythroid marker (Ter119) to determine whether VEGFR-2 activation affects the proportion of specific hematopoietic populations. VEGFR-2 dimerization induced a significant increase in the proportion of GFP^+ myeloid cells ($CD11b^+$ and $Gr\text{-}1^+$; 1.5-fold-increase on average) in the bone marrow of transplanted mice (Figure 21). It is interesting to note that when the percentage of cells of different subsets of the bone marrow (lymphoid, myeloid and erythroid) are added, the total exceeds 100% in the FKBP-VEGFR-2 transplanted mice stimulated with AP20187. This did not apply for any of the other subgroups tested. One possible explanation for this phenomenon lies in the fact that expression of some of the markers used is not absolutely restricted for the specific lineage for which it was tested.

For example, CD11b is expressed on subsets of activated B and T lymphocytes, eosinophils and Natural Killer (NK) cells in addition to being expressed in mature and immature myeloid cells (Cabanas and Sanchez-Madrid, 1999). Moreover, B220 is expressed on dendritic cell precursors (Asselin-Paturel et al., 2001; del Hoyo et al., 2002) and monocyte precursors (Dannaeus et al., 1999) in addition to B lymphocytes. This could indicate that VEGFR-2 activation can affect, to a certain extent, the differentiation and expansion of immature cells, which have been shown to co-express lymphoid and myeloid markers (Lu et al., 2002).

We also observed a moderate increase in the GFP^+ proportion of myeloid cells compared to the GFP^- population in the peripheral blood (Figure 22), but this increase was not as marked as observed in the bone marrow. These results suggest that VEGF-elicited myeloid expansion can be mediated solely through VEGFR-2 (Gabrilovich et al., 1998; Melani et al., 2003). However, VEGFR-2 activation did not significantly affect the levels of lymphoid or erythroid cells in the bone marrow or peripheral blood.

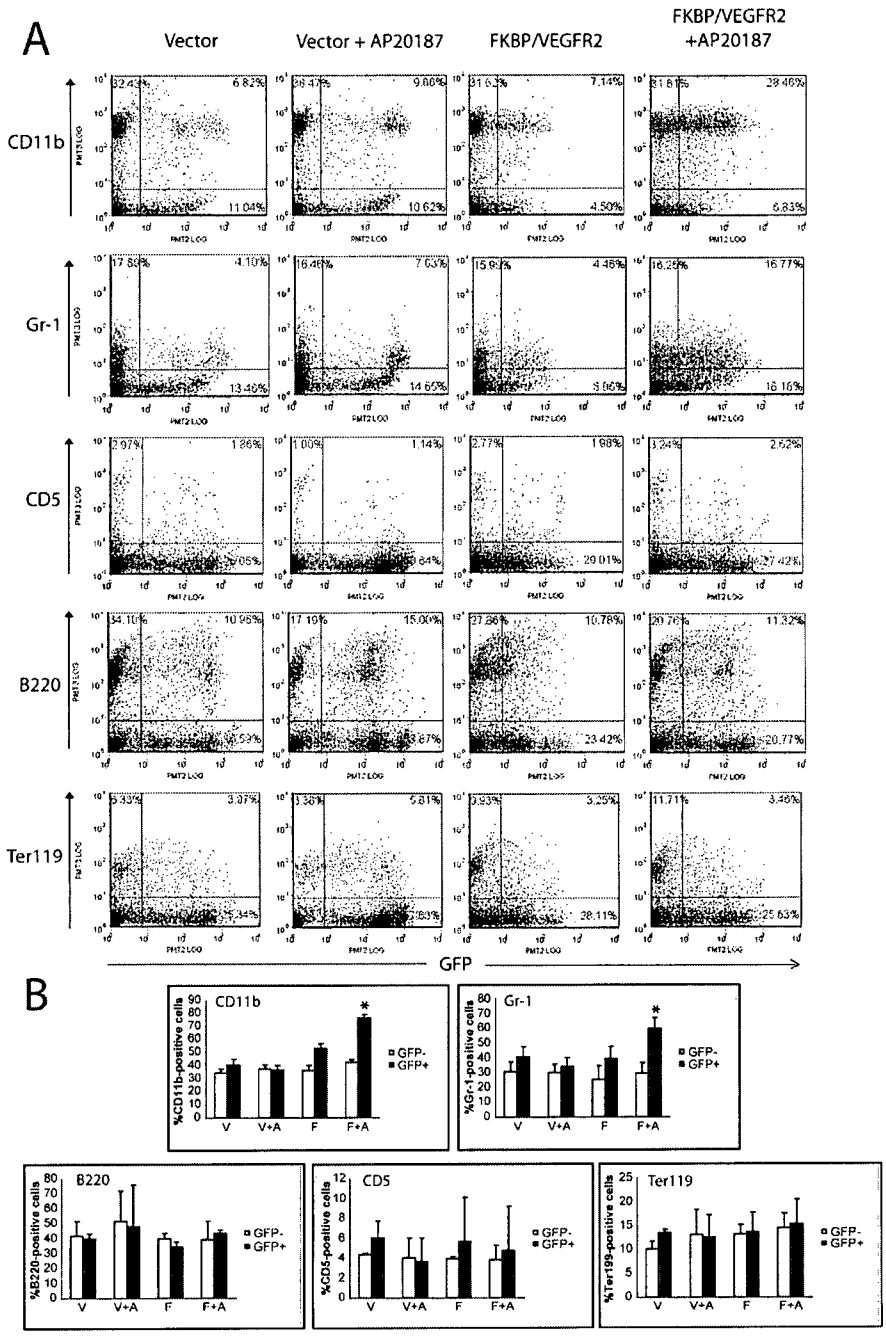

Figure 21 VEGFR-2 induces expansion of myeloid cells, but not of erythroid and lymphoid cells in the bone marrow. B6C3 mice transplanted with bone marrow transduced with either MIG or MIG-FKBP-VEGFR-2 were injected with vehicle or AP20187 for 10 days. Cells were harvested from bone marrow and labeled for specific myeloid (Gr-1, CD11b), lymphoid (CD5, B220) or erythroid markers (Ter119). (**A**) Representative flow cytometry dot plots. (**B**) Average proportion of specific markers in the GFP^+ and GFP^- bone marrow populations post AP20187 treatment. Data ± SEM represents average of at least 5 mice per group. Legend: V=Vector; F=FKBP-VEGFR/2; A=AP20187. * $P < 0.05$.

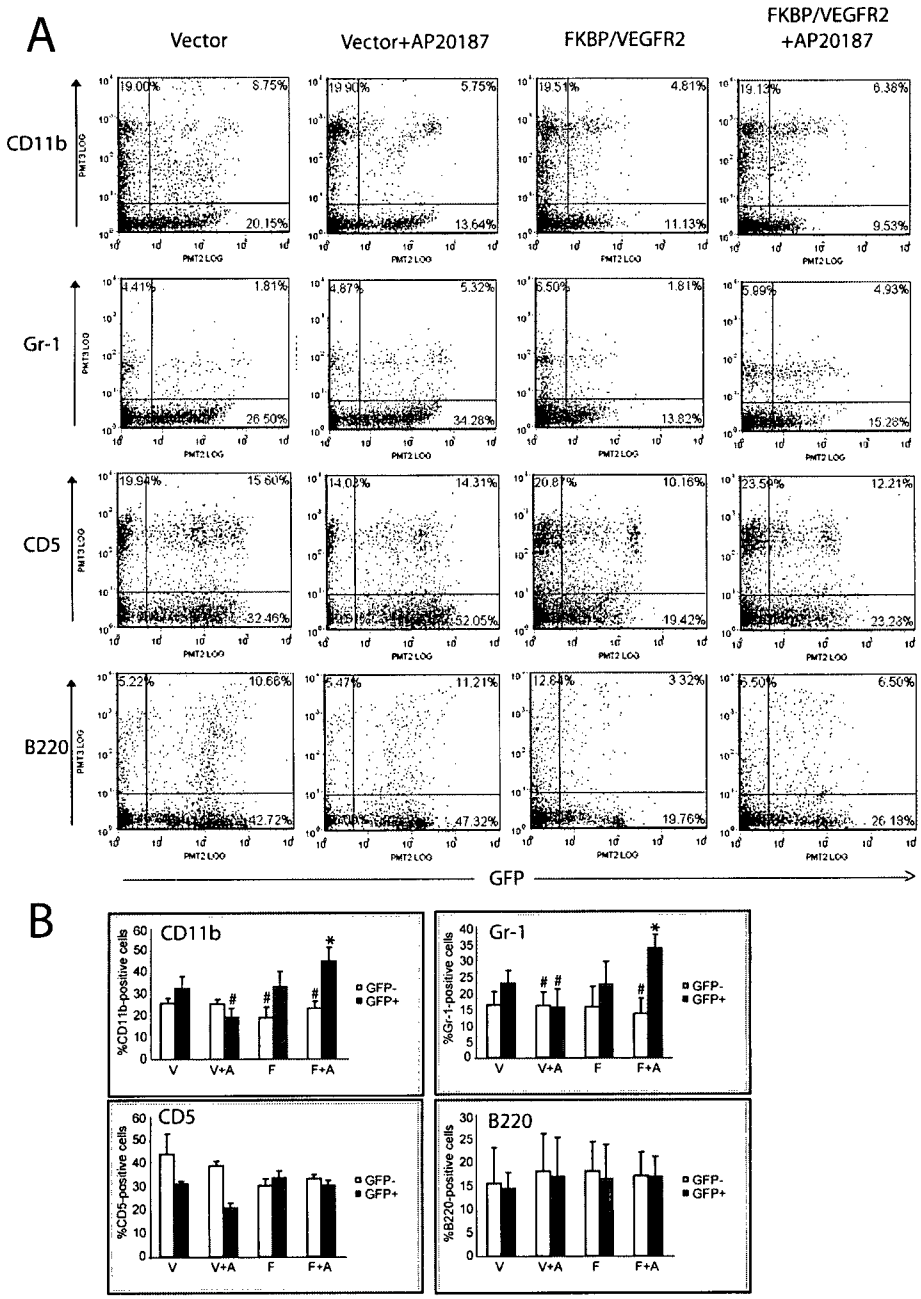

Figure 22 FKBP-VEGFR-2-transduced cells are not mobilized in the peripheral blood in response to AP20187. B6C3 mice transplanted with bone marrow transduced with either MIG or MIG-FKBP-VEGFR-2 were injected with vehicle or AP20187 for 10 days. Cells were harvested from peripheral blood and labeled for specific myeloid (Gr-1, CD11b) or lymphoid markers (CD5, B220). (A) Representative flow cytometry dot plots. (B) Average proportion of specific markers in the GFP^+ and GFP^- peripheral blood populations post AP20187 treatment. Data ± SEM represents average of at least 5 mice per group. Legend: V=Vector; F=FKBP-VEGFR/2; A=AP20187. * indicates a significant difference with # ($P < 0.05$). Groups marked with a # are not statistically different from other groups except those marked with an *.

5.2.2 VEGFR-2 increases the proportion of myeloid progenitors in bone marrow

To assess whether VEGFR-2 induces expansion of myeloid progenitors, bone marrow was harvested from AP20187 or vehicle-treated mice, and the GFP^+ and GFP^- populations were collected separately and plated in methylcellulose to quantitate myeloid progenitors. AP20187-induced VEGFR-2 dimerization produced an increase in bone marrow myeloid progenitors in both the GFP^+ and GFP^- populations (Figure 23).

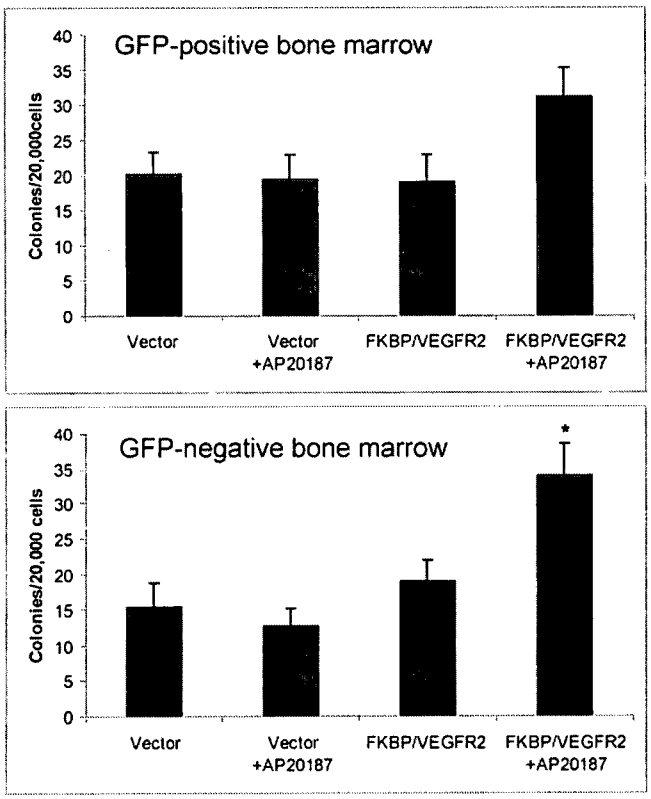

Figure 23 VEGFR-2 induces expansion of bone marrow myeloid progenitors *in vivo*. B6C3 mice transplanted with bone marrow transduced with either MIG or MIG-FKBP-VEGFR-2 were injected with vehicle or AP20187 for 10 days. Bone marrow was harvested, sorted for GFP expression, and the GFP^+ and GFP^- cells were plated in methylcellulose supplemented with cytokines (SCF, IL-3, IL-6 and Erythropoietin) for colony assays. Colonies were enumerated after 10 days. Data ± SEM represents average of 6 independent experiments. * $P < 0.05$.

The fact that we did not observe any increase in the proportion of $Gr\text{-}1^+$ or $CD11b^+$ cells in the GFP^- population, whereas we observed an increase in myeloid progenitors, which

are Gr-1$^+$ and/or CD11b$^+$, may be due to the low proportion of progenitors in the bone marrow. Therefore, an increase of progenitors would not be reflected in the proportion of CD11b$^+$ and Gr-1$^+$ cells in the bone marrow. Surprisingly, the expansion of GFP$^-$ progenitors was even greater than the one observed in the GFP$^+$ population. Since GFP$^-$ cells do not express the FKBP-VEGFR-2 construct (over 85% of GFP$^-$ cells were Ly5.1$^-$ in a typical experiment, and therefore recipient-derived; data not shown) and do not respond to AP20187, it is likely that VEGFR-2 dimerization in the GFP$^+$ cells induced expression of a factor that can positively modulate myeloid progenitor expansion in the GFP$^-$ population, indicating that VEGFR-2 activation can promote myelopoiesis in part through a paracrine mechanism.

5.2.3 VEGFR-2 activation induces GM-CSF expression and secretion

To identify the potential paracrine factor in VEGFR-2-mediated hematopoietic effects, semi-quantitative RT-PCR was performed on murine bone marrow cells to detect the effect of VEGFR-2 activation on the mRNA levels of various growth factors and cytokines known to modulate hematopoiesis. Among these, we assayed for expression of cytokines such as stem cell factor (SCF), Flt-3-ligand, interleukin-6 (IL-6), macrophage-colony stimulating factor (M-CSF), GM-CSF, thrombopoietin (Tpo) and VEGF. We also examined the expression of Notch ligands, Delta-1, Delta-4 and Jagged-1, and the bone morphogenetic proteins, BMP-2 and BMP-4, which are known to modulate myelopoiesis. After retroviral transduction, bone marrow cells were sorted for GFP expression, plated in cytokine free-medium and stimulated with 100 nM AP20187 for 1 hour, after which RNA was harvested and processed for RT-PCR (Figure 24).

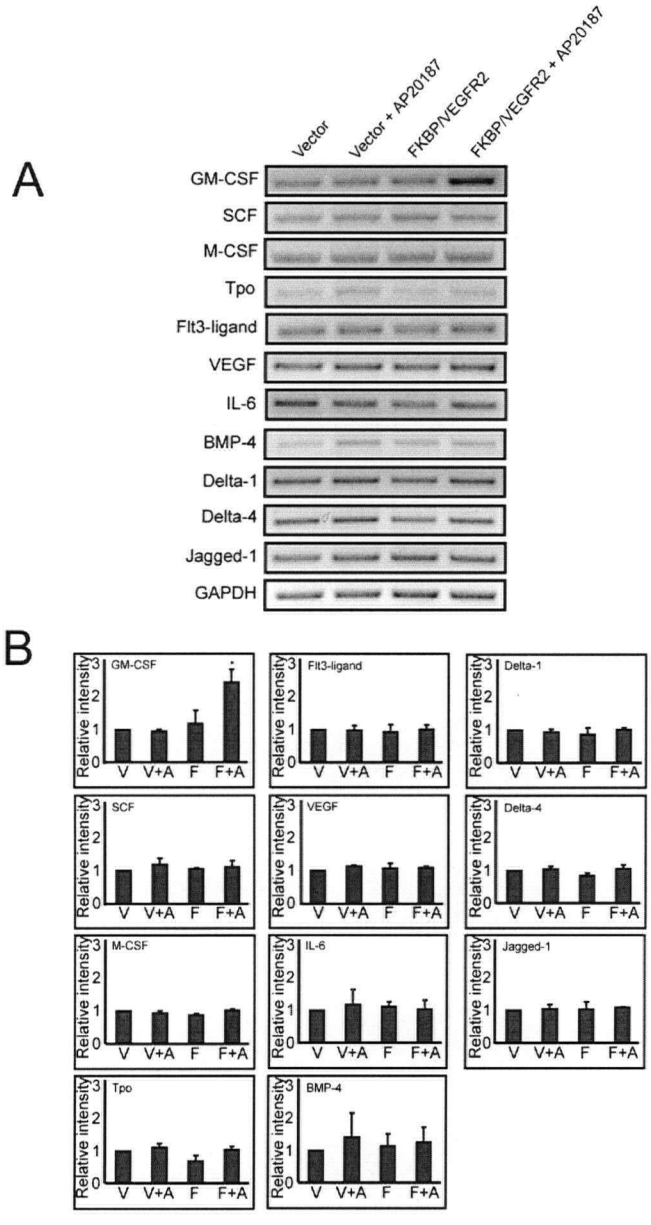

Figure 24 Effects of VEGFR-2 activation on expression of hematopoietic factors in murine bone marrow cells. (**A**) Bone marrow cells transduced with MIG or MIG-FKBP-VEGFR-2 were incubated with or without 100 nM AP20187 for 60 minutes. RNA was harvested, and RT-PCR was performed using primers specific for mouse hematopoietic factors. (**B**) Densitometric analysis of RT-PCR results, normalized to GAPDH. Data ± SEM represents average of 3 independent experiments. Legend: V=Vector; F=FKBP-VEGFR/2; A=AP20187. * $P < 0.05$.

VEGFR-2 activation did not induce any changes in the levels of expression of the Notch ligands or BMP-4. BMP-2 could not be detected in any of the bone marrow RNA

samples. However, among the cytokines tested, we found that VEGFR-2 dimerization induced a significant increase of GM-CSF at the mRNA level (2.5-fold), whereas it did not significantly affect the levels of the other cytokines tested. To determine whether GM-CSF was also up-regulated at the protein level, we collected supernatant from transduced bone marrow cells stimulated with AP20187 for 48 hours. Protein concentration determined by ELISA demonstrated a significant increase of GM-CSF protein in the medium of cultured bone marrow cells when VEGFR-2 is activated in the presence of AP20187 (Figure 25A). These results confirm that VEGF, through VEGFR-2, is able to stimulate cells in the bone marrow to up-regulate GM-CSF.

To determine whether AP20187-induced VEGFR-2 activation can increase levels of GM-CSF *in vivo*, levels of GM-CSF were assayed in serum and found to be significantly increased when VEGFR-2 was dimerized *in vivo* compared to control mice (Figure 25B).

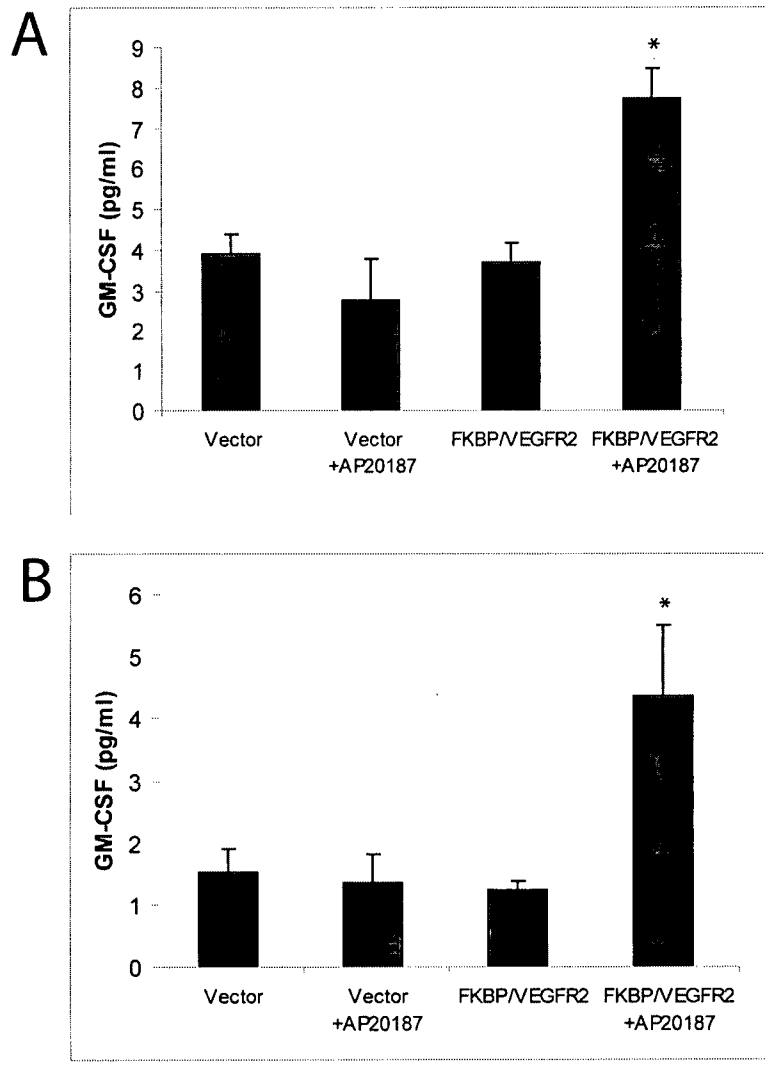

Figure 25 VEGFR-2 dimerization increases expression of GM-CSF at the protein level in murine bone marrow *in vitro* (A) and *in vivo* (B). (**A**) Bone marrow cells transduced with either MIG or MIG-FKBP-VEGFR-2 were incubated in the presence of 100 nM AP20187. Cell supernatant was harvested after 48 hours. (**B**) Serum was collected from mice transplanted with MIG or MIG-FKBP-VEGFR-2 which had been stimulated for 10 days with or without 10 mg/kg/day AP20187. Data ± SEM represents average of 3 independent experiments (**A**) or 5 mice per group (**B**). * $P < 0.05$.

To determine whether GM-CSF was primarily responsible for the VEGFR-2-induced paracrine effects on hematopoietic progenitors, we tested whether blocking the activity of GM-CSF was sufficient to abolish VEGFR-2-induced paracrine activity on hematopoietic progenitors. Bone marrow cells transduced with either MIG or MIG-

FKBP-VEGFR-2 were co-cultured with untransduced primary bone marrow at a 1:1 ratio, with or without AP20187, in the presence or absence of a blocking GM-CSF antibody (1 μg/ml) and the following cytokine combination: Flt3-ligand (1 ng/ml), SCF (10 ng/ml) and IL-11 (1 ng/ml). After 10 days, cells were sorted based on GFP expression. The proportion of GFP^+ cells in the cultures after 10 days revealed that VEGFR-2 activation could expand transduced cells in the presence of the above cytokines as there was a 1.32±0.20-fold increase in the proportion of GFP^+ cells relative to the GFP^- cells in the FKBP-VEGFR-2+AP20187 cultures (data not shown). It is interesting to note that the addition of the GM-CSF blocking antibody could partially block VEGFR-2-mediated expansion of transduced cells as we observed only a 1.1±0.1-fold increase in the proportion of GFP^+ cells in the FKBP-VEGFR-2+AP20187+GM-CSF blocking antibody cultures. In contrast, the GM-CSF blocking antibody in untreated cultures or cultures transduced with the empty vector did not affect the proportion of GFP^+ cells. These results suggest that VEGFR-2 has the potential to expand hematopoietic cells in the presence of Flt3-ligand, SCF and IL-11. This effect was not observed in the presence of IL-3, IL-6 and SCF (chapter 4, Figure 13B). The fact that blocking GM-CSF partially inhibited the expansion of transduced cells suggests that this cytokine may play a role in VEGFR-2-mediated cell expansion. GM-CSF up-regulation may also help explain why VEGFR-2 activation did not affect cell expansion in the presence of IL-3, IL-6 and SCF while it did in the presence of Flt3-ligand, SCF and IL-11. The redundancy between IL-3 and GM-CSF signalling, which share the common β-chain receptor signalling unit (Martinez-Moczygemba and Huston, 2003), may help explain why VEGFR-2 does not potentiate the expansion of hematopoietic cells when IL-

3 is present, while it could promote cell expansion in the absence of IL-3, through the release of GM-CSF. Activation of the β-chain receptor signalling unit by IL-3 or GM-CSF may therefore be necessary, but not sufficient since VEGFR-2 activation alone could not induce hematopoietic cell proliferation (Figure 13A), to induce hematopoietic cell proliferation.

For each culture condition, the GFP^+ and GFP^- populations were plated in methylcellulose to assay progenitor activity. VEGFR-2 dimerization resulted in an increase in the number of progenitors in both the GFP^+ and GFP^- populations of bone marrow cells in the presence of the previously mentioned cytokines, indicating that VEGFR-2 signalling is not redundant with that of the specific cytokines in the assay medium (Figure 26).

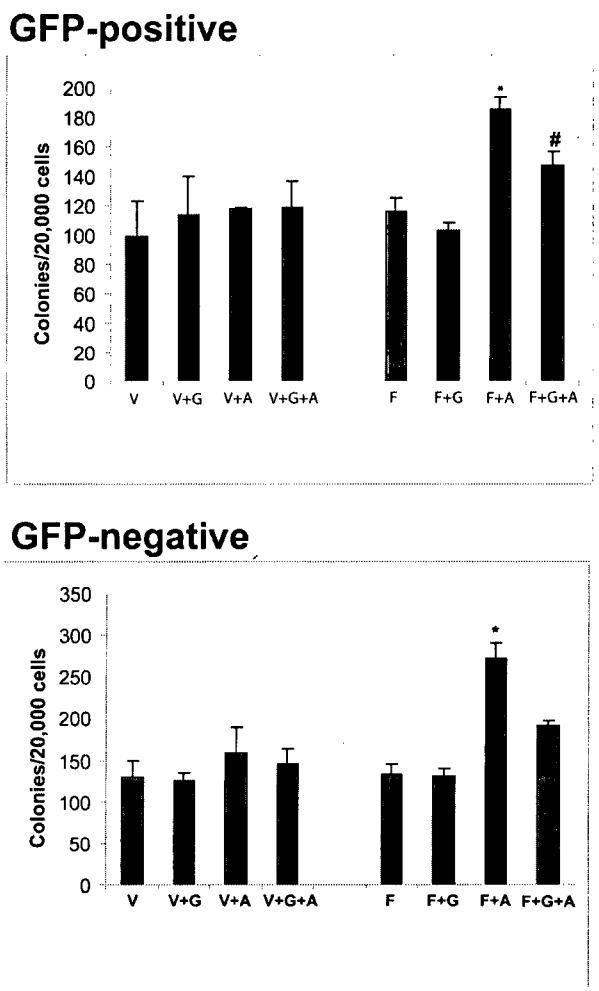

Figure 26 Blocking of GM-CSF inhibits VEGFR-2-induced expansion of myeloid progenitors. Transduced (GFP^+) and untransduced (GFP^-) bone marrow cells were co-cultured for 10 days with or without 100nM AP20187 and/or 1 μg/ml GM-CSF blocking antibody or isotype control. After 10 days, GFP^+ and GFP^- cells were separated by FACS and plated in methylcellulose medium for progenitor assays. Data ± SEM represents average of 3 independent experiments. Legend: V=Vector; F=FKBP-VEGFR/2; G=GM-CSF blocking antibody; A=AP20187. * Indicates a significant difference with other treated cultures ($P < 0.05$), except for the one marked with a # ($P = 0.17$). Group marked with # is not significantly different from other treated cultures.

However, addition of the GM-CSF blocking antibody resulted in a significant reduction ($P = 0.031$) in the number of progenitors in the GFP^- cells co-cultured with VEGFR-2-transduced cells in the presence of AP20187, indicating that GM-CSF is responsible at least in part for the increase in the number of progenitors observed when

VEGFR-2 is dimerized. Although blocking GM-CSF also decreased the number of progenitors in the GFP^+ population when VEGFR-2 was dimerized, this decrease was not as marked as the one observed in the GFP- population and was not found to be statistically significant ($P = 0.17$), which suggests that VEGFR-2 can promote expansion of myeloid progenitors through other mechanisms, in addition to up-regulating GM-CSF. The findings suggest that VEGFR-2 promotes myeloid progenitor activity to a certain extent in a cell autonomous manner, independent of GM-CSF. An alternate explanation may be that GFP^+ cells are in part stimulated by GM-CSF through an internal private autocrine loop, which has been reported (Lang et al., 1987; Young and Griffin, 1986).

5.3 Discussion

VEGF and its receptors are expressed in both normal and malignant hematopoietic cells. Although the mechanisms by which VEGF regulates hematopoiesis remain to be further elucidated, recent data have shown that VEGF can have profound effects on hematopoiesis. VEGF can inhibit dendritic cell development and increase the production of B cells and the generation of immature myeloid cells (Gabrilovich et al., 1998; Hattori et al., 2001). Moreover, it has been shown that VEGF-deficient bone marrow cells fail to repopulate lethally irradiated hosts, and also fail to form colonies *in vitro* (Gerber et al., 2002). Since subsets of hematopoietic cells express VEGFR-1 and VEGFR-2, it can be difficult to identify the respective role of each receptor, and how they can specifically mediate VEGF effects. Using a strategy that allowed us to specifically activate VEGFR-2, we have demonstrated in the previous chapter that VEGFR-2 was able to mediate hematopoietic progenitor survival *in vitro* by activating

the PI3-kinase pathway. Here, we demonstrate that VEGFR-2 activation in bone marrow cells elicits the expansion of myeloid cells *in vivo*. After a 10 day-regimen of AP20187, there was a net increase in the proportion of $CD11b^+$ and $Gr\text{-}1^+$ cells in the GFP^+ population of the bone marrow of FKBP-VEGFR-2-transduced mice. The GFP^- population of these mice did not display changes in the proportion of $CD11b^+$ and $Gr\text{-}1^+$ cells. However, when we examined at level of myeloid progenitors, we observed a different trend, such that the cells of the GFP^- population had a marked increase in progenitor activity when VEGFR-2 was dimerized. Proteins that could potentially act in a paracrine manner to promote myelopoiesis include ligands that activate the Notch pathway (Bigas et al., 1998; Jonsson et al., 2001; Schroeder et al., 2003; Ye et al., 2004), the bone morphogenetic proteins (Zon, 2001), and the hematopoietic cytokines SCF, Flt3-ligand, IL-6, M-CSF and GM-CSF (Barreda et al., 2004). Of all the hematopoietic factors examined, we found that VEGFR-2 only upregulated GM-CSF in bone marrow cells. Dimerization of the FKBP-VEGFR-2 construct in murine endothelial cells also upregulated GM-CSF (data not shown), suggesting that the hematopoietic effects of VEGF *in vivo*, may include stromal regulation of cytokines. GM-CSF has pleiotropic and widespread effects on hematopoietic cells, and exhibits overlapping activities on hematopoietic progenitors with other cytokines including M-CSF, G-CSF, IL-3, IL-6 and SCF (Barreda et al., 2004). Our data suggest that VEGFR-2-induced upregulation of GM-CSF is a significant paracrine mechanism in the proliferation of myeloid progenitors. It is worthwhile mentioning that VEGFR-2-induced up-regulation of GM-CSF may also occur through an indirect mechanism. GM-CSF is normally secreted by subsets of T lymphocytes, fibroblasts, vascular endothelial cells and mast cells (Gasson, 1991).

Cytokines such as IL-12 (Hou et al., 2003), VEGF (Zhang et al., 2004), TNF-α and IL-1β (Burg et al., 2002) have also been shown to up-regulate the expression of GM-CSF in T lymphocytes (IL-12) and endothelial cells of the bone marrow stroma (VEGF, TNF-α and IL-1β) . Therefore, if VEGFR-2 activation increases production of those cytokines it is possible that cytokine-stimulated hematopoietic cells (T lymphocytes) or bone marrow stroma cells (endothelial cells) may account at least in part for the increased levels of GM-CSF. Although we confirmed that VEGFR-2 activation did not up-regulate VEGF, further testing will be required to determine whether VEGFR-2 can up-regulate other cytokines that may be implicated in the indirect expression of GM-CSF by bone marrow stroma cells.

The paracrine activity of GM-CSF on hematopoietic progenitors cannot, however, explain all the effects that VEGFR-2 has on hematopoiesis. The observation that blockade of GM-CSF did not inhibit expansion of progenitors expressing VEGFR-2 significantly suggest that VEGFR-2 also has a direct effect on the expansion of myeloid progenitors, which could be due in part to VEGFR-2-dependent increased survival. It could also indicate that GM-CSF can stimulate VEGFR-2$^+$ progenitors via an internal autocrine loop. Such an autocrine loop has previously been described for GM-CSF (Lang et al., 1987; Young and Griffin, 1986). Furthermore, it is also possible that VEGFR-2 can up-regulate molecules we did not test for. Such molecules may include other hematopoietic cytokines (IL-1β, IL-3, IL-5, IL-11) (Krishnaswamy et al., 1999; Mangi and Newland, 1999; Mordvinov and Sanderson, 2001) or signalling molecules (sonic hedgehog, wnt) (Bhardwaj et al., 2001; Yamane et al., 2001). Up-regulation of some of

these molecules could potentially account for some of the VEGFR-2-induced effects on myeloid cells.

We observed that VEGFR-2 activation increased the proportion of Gr-1^+ and $CD11b^+$ cells in the GFP^+ population of the bone marrow but not in the GFP^- population, whereas the increase in progenitors was more marked in the GFP^- population than the GFP^+ population. It therefore appears that, in the GFP^+ population, VEGFR-2 signalling in combination with GM-CSF drives the rapid expansion and differentiation of myeloid progenitors, whereas in the GFP^- population, the progenitors show increased self-renewal accompanied by a more modest cell expansion. Since we have previously shown that VEGFR-2 by itself cannot induce proliferation of hematopoietic progenitors, it is likely that VEGF acts synergistically with other hematopoietic growth factors to promote expansion and differentiation of hematopoietic progenitors. Since VEGFR-2 activation upregulates GM-CSF, VEGFR-2 autocrine signalling combined with GM-CSF could drive the rapid expansion of myeloid cells in the GFP^+ population. Although no data exists on synergistic signalling between GM-CSF and VEGF, both VEGF (Gerritsen et al., 2003; Xin et al., 2001) and GM-CSF (Lennartsson et al., 2004; Miyazawa et al., 1991; Piacibello et al., 1995) have been shown to synergize with other cytokines. GM-CSF signals through the recruitment and the activation of Janus kinase (JAK)-2 and signal transducers and activators of transcription (STAT)-3 and -5 (Lehtonen et al., 2002; Valdembri et al., 2002). The JAK/STAT pathway is involved in embryonic stem cell self-renewal and has been hypothesized to be an important hallmark of self-renewal capabilities in general (Bruno et al., 2004; Ramalho-Santos et al., 2002). GM-CSF can act synergistically with SCF, whose receptor c-Kit is part of the same receptor family as

VEGFR-2, to promote the growth and differentiation of primitive hematopoietic cells (Lund-Johansen et al., 1999). It is therefore possible that the signals provided by VEGFR-2, which activates the MAP kinase and PI3-kinase pathways, complement those generated by GM-CSF to drive the rapid expansion and differentiation of myeloid progenitors. This could account, at least in part, for the differences observed in the proportion of bone marrow Gr-1$^+$ and CD11b$^+$ cells observed between the GFP$^+$ population and the GFP$^-$ population.

Increased accumulation of immature myeloid cells and CD11b$^+$ macrophages in the bone marrow, lymphoid organs and spleens of mice implanted with tumours that secrete VEGF has previously been reported (Melani et al., 2003; Young et al., 1987). More recently, increased production of a more defined population of Gr-1$^+$/CD11b$^+$ immature myeloid cells has been described in several mouse tumour models (Bronte et al., 2000; Kusmartsev et al., 2000). Increased production of these cells might be triggered by different soluble tumour-derived factors such as VEGF, GM-CSF, M-CSF, IL-6 and IL-10 (Kusmartsev and Gabrilovich, 2002). Treatment of mice with VEGF resulted in dramatic accumulation of Gr-1$^+$ cells in peripheral lymphoid organs (Gabrilovich et al., 1998). Similarly, in a preliminary study, we observed that VEGFR-2 induced a marked increase in the proportion of Gr-1$^+$ and CD11b$^+$ cells in the bone marrow and spleen (data not shown) of transplanted mice. These effects are likely to be the result of VEGFR-2 signalling in hematopoietic cells and the subsequent increase in production of GM-CSF. The contribution of GM-CSF might be essential in this process, since blocking GM-CSF signalling inhibited the expansion of myeloid progenitors in cells not expressing VEGFR-2.

It has been demonstrated that VEGFR-2 is expressed in AML, and that blockade of VEGFR-2 signalling can inhibit human leukaemia growth in an animal model (Dias et al., 2001; Fiedler et al., 1997). In light of the results we present here, it is possible that VEGF-induced autocrine and paracrine stimulation has the potential to induce rapid expansion of leukemic blasts expressing VEGFR-2. VEGFR-2 activation in leukemic cells could drive upregulation of GM-CSF, which could in turn synergize with VEGF signalling to drive rapid leukemic cell expansion. It will therefore be of interest to further study the effects of VEGFR-2 in both normal and malignant hematopoietic cells.

Chapter 6

Implication of bone marrow-derived cells in tumour neovasculature formation

6.1 Introduction

Until recently, tumour vasculature was thought to arise solely through angiogenesis, a mechanism by which new blood vessels form from pre-existing vessels through endothelial cell migration and proliferation (Ruoslahti, 2002). However, recent studies have provided evidence that tumour neovasculature can also arise though vasculogenesis, a process by which endothelial progenitors are recruited and differentiate *in situ* into mature endothelial cells to form new blood vessels (Reyes et al., 2002). Evidence for the existence of such endothelial progenitors has come from studies demonstrating the ability of bone marrow-derived cells to incorporate into tumour vasculature. However, the exact nature of such endothelial progenitors remains controversial. A population of endothelial precursors have been shown to exist among human peripheral blood, bone marrow and cord blood cells (Lin et al., 2000; Peichev et al., 2000). Moreover, expression of CD34, CD133 and VEGFR-2 on hematopoietic cells from bone marrow, peripheral blood or umbilical cord blood is usually associated with a population of endothelial progenitors (Peichev et al., 2000). When plated in culture in the presence of angiogenic factors such as VEGF or bFGF, endothelial progenitors become adherent and proliferate to form colonies of mature endothelial cells, which express markers such as von Willebrand factor (vWF), VE-cadherin, CD31 (PECAM-1) and can uptake acetylated LDL (Lin et al., 2000; Quirici et al., 2001). Additional studies have also reported that the $CD34^-$ monocyte/macrophage-containing mononuclear cell population can differentiate into endothelial-like cells *in vitro* (Rehman et al., 2003).

Because of the lack of definitive markers of endothelial progenitors, the *in vivo* contribution of endothelial progenitors to tumour neovascularization remains unclear.

Studies have reported that for some tumour types, about 90% of blood vessels are composed of bone marrow-derived endothelial cells (Lyden et al., 2001). However, other groups have reported that they were not able to observe any contribution of endothelial progenitors to the formation of tumour blood vessels (De Palma et al., 2003; Gothert et al., 2004b). This lack of consensus may be explained in part by different experimental settings such as the type of cells used to transplant animals (whole bone marrow vs purified primitive stem cells), tumour type, the time frame of the experiment, methods of endothelial cell identification and the propensity of different models to induce cell fusion.

In this chapter, the existence of a potential hemangioblast was examined both *in vivo* and *in vitro*. For *in vivo* experiments, models of bone marrow transplants were used to quantify the proportion of bone marrow-derived cells incorporating into tumour vasculature. Moreover, we used different transplant settings to determine whether endothelial progenitors are derived from primitive hematopoietic stem cells and arise by differentiation. We also looked at the effects of VEGF and VEGFR-2 activation in hematopoietic cells to examine whether it could promote the recruitment of bone marrow-derived cells in tumour blood vessels. Since the incorporation of endothelial progenitors in tumour vasculature was found to be such a rare event, we determined the proportion of endothelial progenitors in different subsets of mononuclear cells using a differentiation assay.

6.2 Results

6.2.1 Determination of the existence of bone marrow-derived endothelial cells

To quantify endothelial progenitor activity *in vivo*, chimeric mice reconstituted with GFP^+ bone marrow were generated. To determine the presence of bone marrow-derived endothelial cells in GFP-transplanted mice, they were implanted with tumour cells (B6RV2 lymphoma) sub-cutaneously in the dorsal area. Ten days post-implantation, mice were sacrificed and blood vessels were analyzed in tumour tissue sections by fluorescence microscopy. Endothelial cells were detected using antibodies against CD31 or VE-cadherin. Most studies examining the contribution of bone marrow-derived cells have used either CD31 or vWF (Bailey et al., 2004; Lyden et al., 2001). However, although both are strongly expressed in endothelial cells, they are also expressed on subsets of hematopoietic cells such as monocytes/macrophages (CD31), granulocytes (CD31) and platelets (vWF, CD31) (Newman, 1997; Ruggeri, 2003). Since leukocytes and platelets can be found in close association with the vascular wall, it may be difficult to differentiate vWF^+ or $CD31^+$ bone marrow-derived leukocytes from tumour endothelial cells, thereby leading to incorrect identification of bone marrow-derived endothelial cells. In order to avoid such misinterpretation, we also used VE-cadherin, which is specific to vascular endothelial cells (Vincent et al., 2004), to stain blood vessels. To account for different levels of GFP^+ cell engraftment in transplanted animals, results were normalized for the proportion of GFP^+ cells in the peripheral blood of the mice at the time of sacrifice. Between 300 and 500 blood vessels per section were counted, and 3 sections per tumours were quantified. GFP^+ bone marrow-derived cells could be detected in tumour blood vessels, although at a very low frequency. Figure 27A

displays some representative images of $CD31^+$ and VE-$Cadherin^+$ blood vessels which contained at least one bone marrow-derived cell.

Since macrophages can reside in close contact with blood vessels, and could therefore be misinterpretated as endothelial cells, tumour slides were co-stained with both the monocyte/macrophage marker CD11b and VE-cadherin to avoid such a misinterpretation (Figure 27B). Double staining for VE-cadherin and CD11b was not observed in GFP^+ bone marrow-derived cells in the vessels walls, ruling out the possibility of macrophages being misinterpreted as endothelial cells, and thereby confirming the presence of bone marrow-derived endothelial cells in B6RV2 tumours.

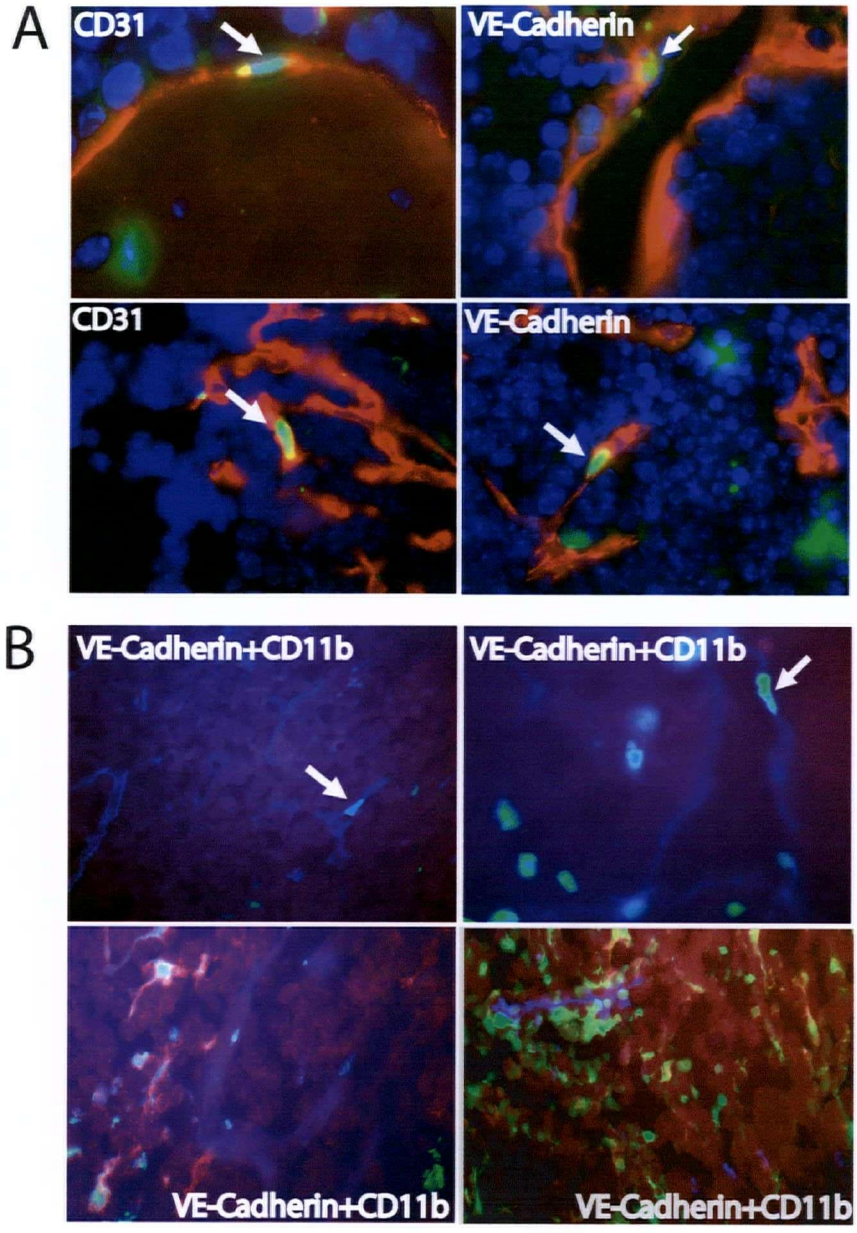

Figure 27 Bone marrow derived endothelial cells incorporate into tumour vasculature. (A) Tumour sections (~8 μm) were stained with either CD31 or VE-cadherin (red) and were examined for the presence of bone marrow-derived cells (GFP^+, green) present as part of the blood vessels. DAPI was used to counterstain nuclei (blue). (B) To confirm the phenotype of bone marrow-derived endothelial cells, B6RV2 tumour sections were stained with CD11b labeled with PE (red) and VE-cadherin-Alexa 350 (blue).

We then quantified the relative contribution of bone marrow cells to the formation of B6RV2 blood vessels. As a source of GFP^+ bone marrow cells in transplanted animals,

we either used cells obtained from GFP^+ transgenic mice (Okabe et al., 1997) that were injected into lethally irradiated recipients immediately after harvesting (Figure 28A), or by harvesting bone marrow cells from C3Pep mice that were expanded *ex vivo* (2 days) before transduction with a retroviral vector encoding GFP (MIG) (Figure 28B).

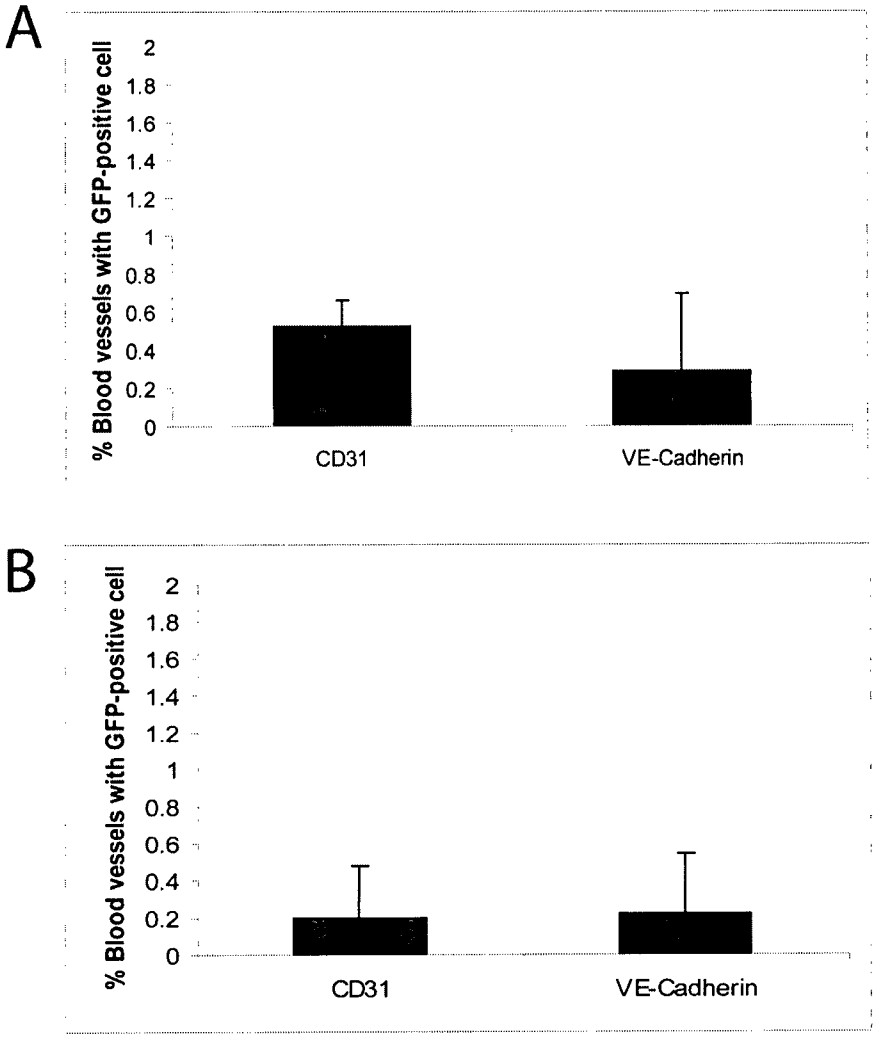

Figure 28 Bone marrow-derived endothelial cells have a relatively small contribution to the formation of blood vessels in B6RV2 lymphomas. B6RV2 tumours were implanted sub-cutaneously in the dorsal area of B6C3 mice previously transplanted with GFP^+ bone marrow from GFP transgenic donors (A) or with bone marrow transduced *ex vivo* with a GFP-encoding vector (MIG) (B). Data ± SEM represents the average of the quantification of 4 tumours per group.

This approach allowed us to determine whether *ex vivo* culture of bone marrow cells can have deleterious effects on the endothelial progenitor pool potentially present in bone marrow cells. Contribution of bone marrow cells to the formation of tumour blood vessels was minimal. In both cases, less than 1% of B6RV2 blood vessels were found to have incorporated GFP^+ bone marrow-derived endothelial cells. *Ex vivo* transduction of bone marrow cells prior to transplantation did not result in significant differences in the levels of tumour vasculature bone marrow-derived endothelial cells compared to mice transplanted with unmanipulated bone marrow cells, indicating that *in vitro* culture of bone marrow cells does not result in loss of endothelial progenitor activity within the time frame of this experiment.

6.2.2 Determination of the existence of an adult hemangioblast

Bone marrow cells have been shown to be able to fuse with other cell types such as hepatocytes (Grompe, 2003) and this has lead some authors to believe that hematopoietic cells may not be as multipotent as once thought, since cell fusion could in fact be responsible for some of the reported plasticity of stem cells (Terada et al., 2002). Analysis of DNA content of bone marrow-derived endothelial cells in liver tissue has previously shown that they can arise by differentiation of bone marrow cells, and not by cell fusion (Bailey et al., 2004). However, DNA content analysis can be misleading, as fused cells have been shown to lose cellular DNA, which could potentially lead to inaccurate interpretation of results (Nowak, 1985; Pratt et al., 1992; Zheng et al., 1995). To determine whether the bone marrow-derived endothelial cells observed in B6RV2 tumours arise by differentiation or fusion, we used a strategy that allowed us to visually

determine cell fusion in mice. The following strategy was used: bone marrow cells were harvested from Z/AP double reporter transgenic mice. These mice express a transgene consisting of the lacZ gene flanked by two loxP sites (Lobe et al., 1999). Therefore, bone marrow from these animals express the lacZ reporter gene before Cre-mediated excision occurs. Cre excision, however, removes the lacZ gene, allowing expression of the second reporter, the human alkaline phosphatase gene (hPLAP). To determine whether bone marrow-derived endothelial cells arise by fusion or differentiation, we transplanted bone marrow harvested from Z/AP mice into mice that constitutively express the Cre recombinase (pCX-NLS-Cre). Therefore, if bone marrow-derived endothelial cells result from differentiation of bone marrow cells, they will express the lacZ gene, and stain blue in the presence of X-gal reagent. However, if bone marrow cells fuse with endothelial cells from the recipient mice (PCX-NLS-Cre), the Cre recombinase will excise the lacZ gene, therefore allowing expression of the human alkaline phosphatase. Fused cells will therefore appear purple when stained for alkaline phosphatase with the NBT/BCIP reagent. B6RV2 tumours harvested from PCX-NLS-Cre mice transplanted with bone marrow harvested from Z/AP mice were cyosectioned and stained with X-gal (lacZ) and NBT/BCIP (hPLAP), followed by CD31 staining with diamino-benzidine (DAB) (Figure 29).

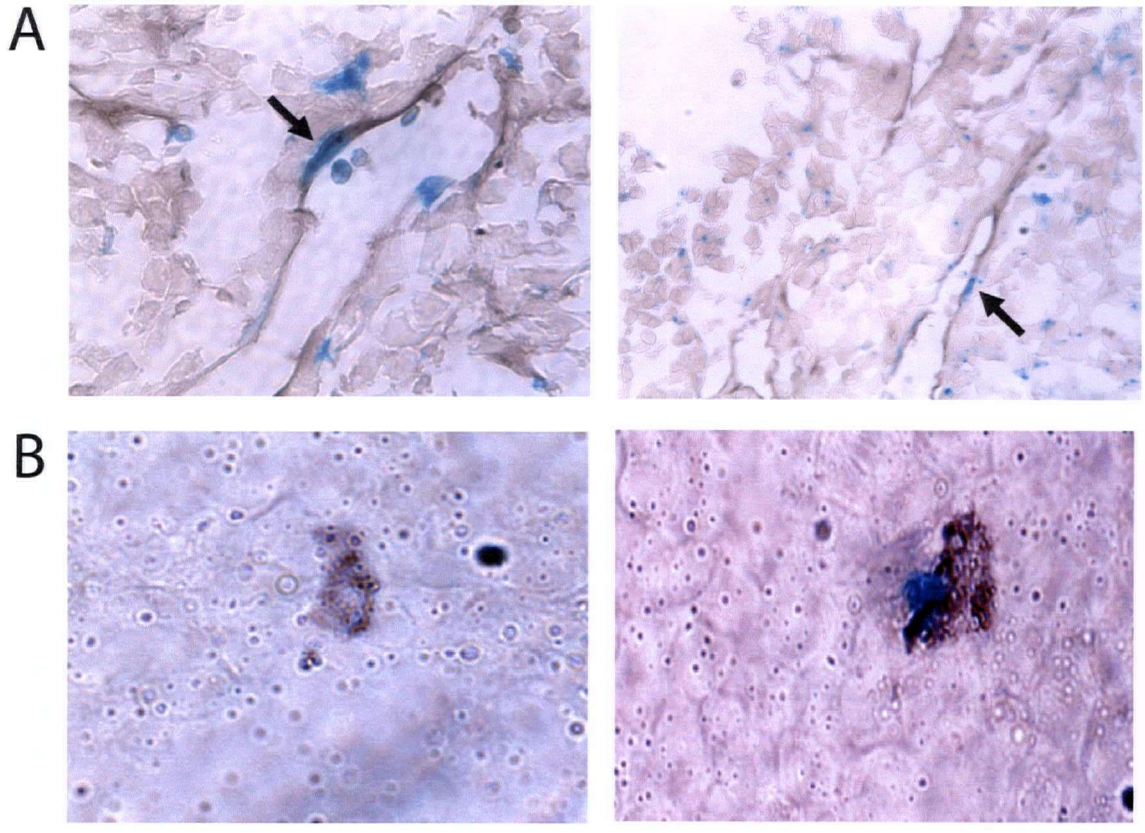

Figure 29 Bone marrow-derived endothelial cells arise by differentiation and not by cell fusion. (A) B6RV2 tumours were harvested from PCX-NLS-Cre mice transplanted with Z/AP bone marrow. Tumour sections were stained with X-gal (blue), NBT/BCIP (purple) and CD31 (brown). Arrows highlight stained bone marrow-derived endothelial cells. No NBT/BCIP$^+$ cells were detected in the tumour sections examined. (B) As control for cell fusion, spleen sections were stained with NBT/BCIP.

Since DAB produces a brown color that could potentially mask the purple color generated by NBT/BCIP, tumour sections were carefully examined after NBT/BCIP staining and before CD31 staining. In tumour sections obtained from 5 different animals, we were able to observe a rare proportion of blood vessels containing lacZ$^+$ cells (Figure 29A), indicating that differentiation of bone marrow-derived endothelial precursors contributes to the formation of tumour blood vessels. By contrast, no hPLAP$^+$ endothelial cells were observed, allowing us to rule out cell fusion or engulfment of hematopoietic cells as a mechanism implicated in the generation of bone marrow-derived endothelial

cells. Since no hPLAP$^+$ cells could be detected in tumour tissues, spleen sections from the same mice were stained with the NBT/BCIP reagent and used as a positive control (Figure 29B).

Studies have suggested that, in the embryo, a single cell known as the hemangioblast can give rise to cells of both the hematopoietic and endothelial lineages. In adult bone marrow, however, the existence of the hemangioblast remains controversial. To determine whether adult bone marrow contains a hemangioblast, mice were transplanted with a single GFP$^+$ hematopoietic stem cell as previously decribed (Corbel et al., 2003), and we examined whether it could give rise to GFP$^+$ tumour endothelial cells. To confirm that single cells injected were indeed hematopoietic stem cells, their ability to reconstitute all blood lineages was tested. At various times after transplantation, 150 μl of peripheral blood was collected from the tails of recipients and red blood cells were lysed. Nucleated cells were stained with biotin-conjugated rat antibodies to IgG2a, IgG2b, B220, Gr-1 and Mac-1, followed by phycoerythrin-conjugated streptavidin (Caltag), and analyzed by flow cytometry. Animals with >15% GFP$^+$ blood leukocytes (35%, 38% and 48% GFP$^+$ blood leukocytes) and showing contribution of GFP$^+$ cells to all blood lineages were chosen for further analysis and were implanted with either 5 x 10^6 LLC and B6RV2 tumour cells. In extensive analyses of B6RV2 and LLC tumours from single cell transplanted mice, we found low numbers of blood vessels (around 1%) from either B6RV2 or LLC tumours that incorporated bone marrow-derived cells that stained positive for either CD31 or VE-cadherin (Figure 30).

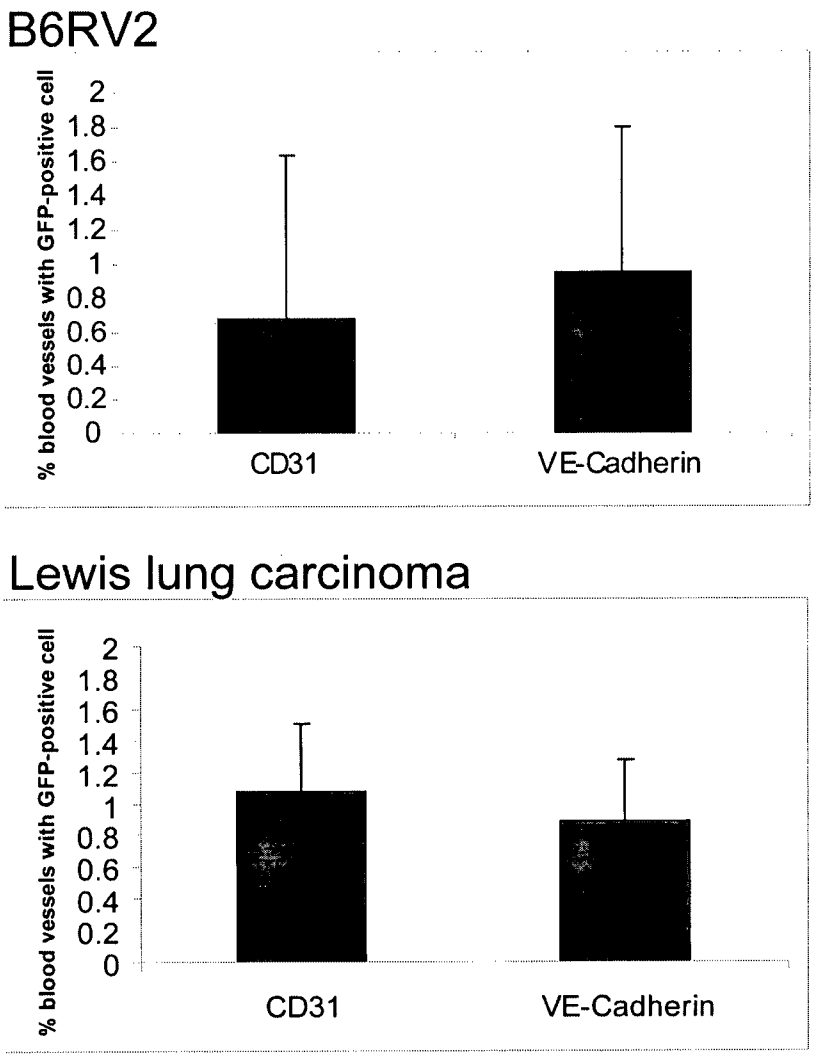

Figure 30 A single hematopoietic stem cell can give rise to endothelial progenitor cells that incorporate into tumour blood vessels. Tumours (B6RV2-upper panel; LLC-bottom panel) were implanted in the dorsal area of mice transplanted with a single GFP^+ primitive hematopoietic stem cell. Tumour sections were quantified for the presence of GFP^+ bone marrow-derived cells present into blood vessels stained with either CD31 or VE-cadherin. Data ± SEM represents the average of the quantification of 3 tumours per group.

Interestingly, the proportion of bone marrow-derived endothelial cells incorporating into tumour blood vessels was similar to that of whole bone marrow transplants. The presence of bone marrow-derived endothelial progenitors that incorporated into the vasculature of

animals transplanted with a single hematopoietic stem cell indicates that primitive hematopoietic stem cells have the potential to give rise to endothelial cells in addition to hematopoietic cells, and may therefore represent a population of adult hemangioblasts.

6.2.3 Role of VEGF and VEGFR-2 in the mobilization and differentiation of bone marrow-derived endothelial cells

VEGF is a potent inducer of both angiogenesis and vasculogenesis, and has been reported to mobilize VEGFR-2^+ endothelial progenitors from the bone marrow (Hattori et al., 2001). To determine whether increased VEGF secretion by tumour cells could lead to an increase in the contribution of bone marrow-derived endothelial progenitors to the tumour vasculature, B6RV2 cells were transduced with a retroviral vector encoding the human $VEGF_{165}$ cDNA (MSCVneo-$VEGF_{165}$). Human $VEGF_{165}$ has previously been shown to be biologically active in mice (Redaelli et al., 2004; Yang et al., 2003). Tumour cells were implanted in the dorsal area of transplanted mice, and after 10 days were harvested, cryosectioned, stained with either CD31 or VE-cadherin and quantified. Although B6RV2-VEGF tumours had an increased growth rate and displayed increased vascularity compared to B6RV2 wild-type tumours (Figure 31C), we did not observe an increase in the contribution of bone marrow cells to blood vessel formation compared to wild-type B6RV2 tumours (Figure 31A, B).

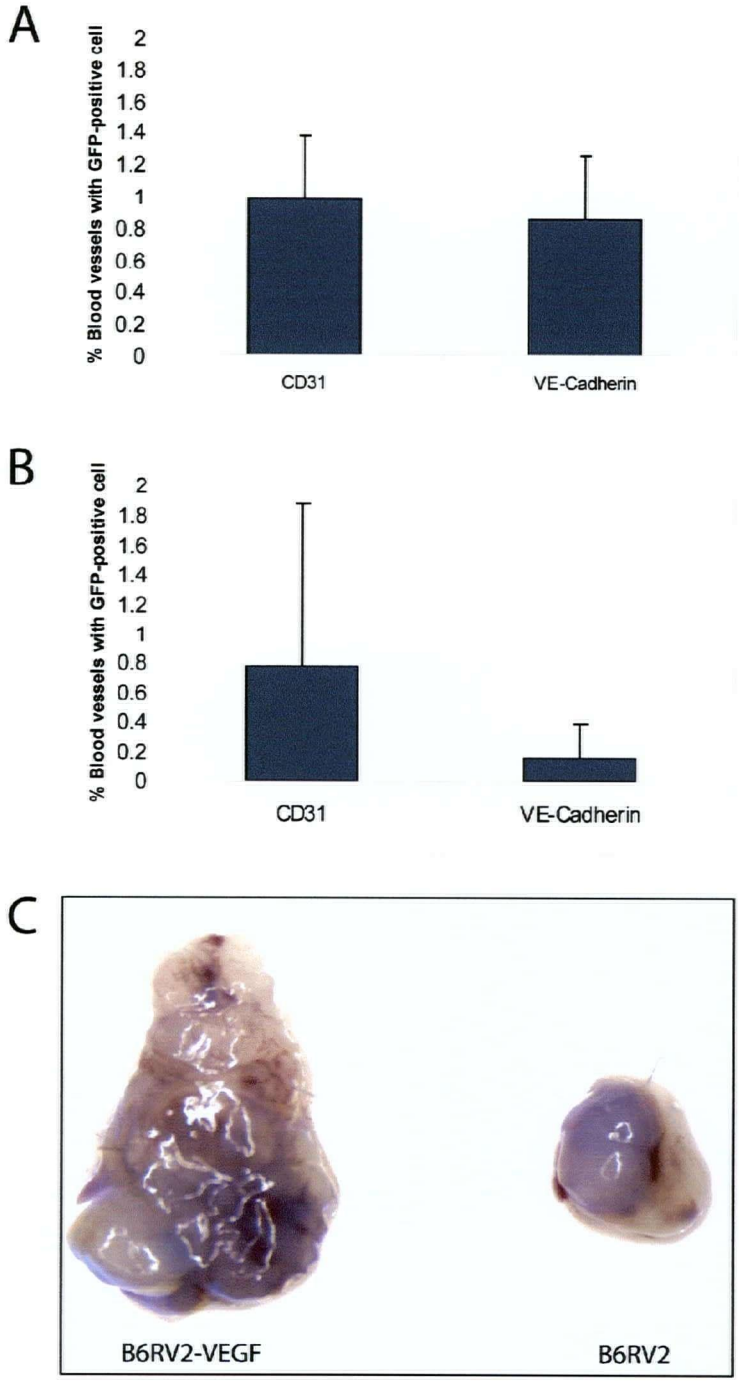

Figure 31 VEGF secretion by tumour cells does not increase the contribution of bone marrow-derived cells to the formation of tumour blood vessels. B6RV2-VEGF tumours were implanted sub-cutaneously in the dorsal area of C3Pep mice previously transplanted with GFP^+ bone marrow from GFP transgenic donors (A) or with bone marrow transduced *ex vivo* with a GFP-encoding vector (MIG) (B). (C) Photograph displaying the increase tumour mass of B6RV2-VEGF tumours (left) compared to B6RV2 tumours (right). Data ± SEM represents the average of the quantification of 4 tumours per group.

To ensure that the VEGF secreted by tumour cells was able to perfuse the bone marrow, VEGF ELISA was performed on the serum of mice implanted with B6RV2-VEGF tumours 10 days previously. Human $VEGF_{165}$ was detected in the serum of mice implanted with B6RV2-VEGF tumours (28.0 ± 4.7 pg/ml). By comparison, no detectable levels of $VEGF_{165}$ were detected in the serum of mice implanted with wild-type B6RV2 tumours. These results indicate that, in our model, increased VEGF secretion by tumour cells does not result in increased proportion of bone marrow-derived cells incorporating into tumour blood vessels, even though angiogenesis was markedly increased and tumours grew faster.

Since serum VEGF levels produced by B6RV2-VEGF tumours may not have been sufficient to mobilize bone marrow endothelial progenitors, we used the strategy described in the previous chapters to selectively activate VEGFR-2 with a chemical inducer of dimerization in bone marrow cells, and examine whether activation of this receptor was sufficient to mobilize endothelial progenitors from the bone marrow and recruit them into B6RV2 vasculature. Briefly, bone marrow cells from C3Pep mice were transduced with a MIG-FKBP/VEGFR-2 construct and used to transplant lethally irradiated B6C3 mice. Four to six weeks after transplant, mice were implanted with B6RV2 tumours and were injected daily for 10 days with 10 mg/kg AP20187 or vehicle in order to dimerize the FKBP/VEGFR-2 construct. We have shown in the previous chapter that this strategy increases myeloid progenitor activity and expands the myeloid cell population ($CD11b^+$, $Gr\text{-}1^+$) in the bone marrow and peripheral blood of transplanted mice. However, VEGFR-2 activation in hematopoietic cells did not result in increased levels of VEGFR-2^+ VE-cadherin$^+$ endothelial progenitors in the bone marrow or

peripheral blood of transplanted mice (Figure 32A, B), nor did it result in increased contribution of bone marrow-derived endothelial cells to the vasculature of B6RV2 tumours (Figure 32C). It therefore appears that the VEGF/VEGFR-2 pathway may not be sufficient for the recruitment and/or expansion of endothelial progenitor cells to the tumour vasculature.

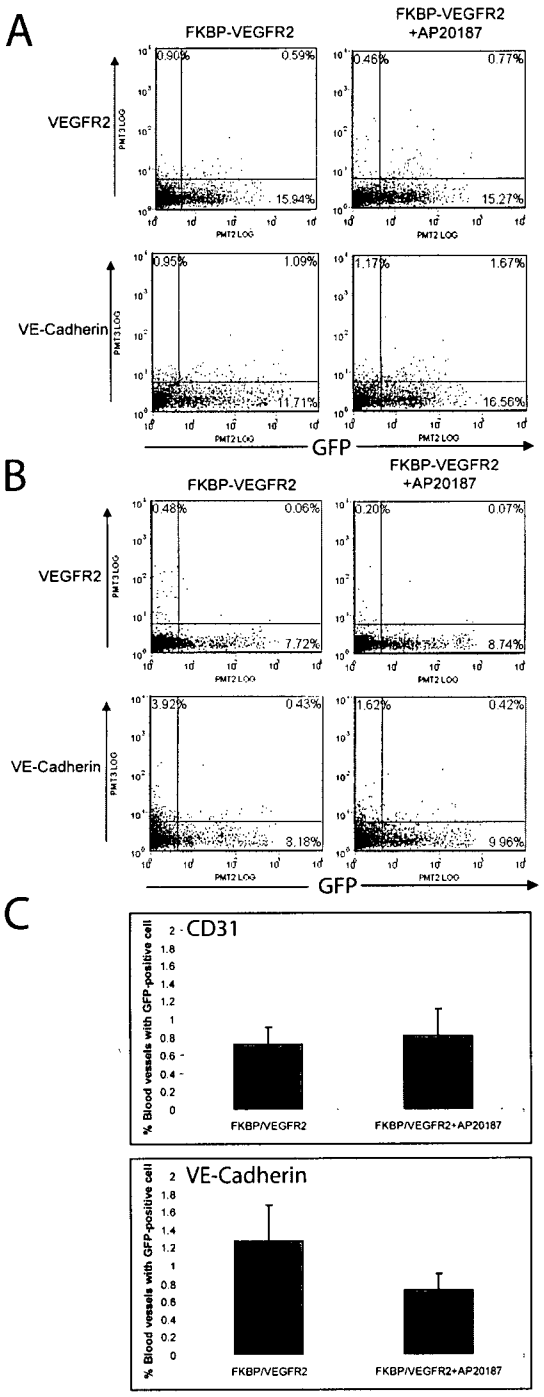

Figure 32 VEGFR-2 activation in bone marrow cells does not result in endothelial progenitor mobilization or recruitment into tumour vasculature. Mice transplanted with FKBP/VEGFR-2 were injected with or without AP20187 for 10 consecutive days. Following this, bone marrow cells (A) and peripheral blood (B) were harvested and stained with VEGFR-2 or VE-cadherin to detect endothelial progenitors. (C) B6RV2 tumour sections from the same mice were stained with CD31 or VE-cadherin to detect GFP^+ bone marrow cells incorporated into tumour vasculature. Data ± SEM represents the average of the quantification of 4 tumours per group.

6.2.4 Determination of the proportion of endothelial progenitors in human umbilical cord blood

Since incorporation of bone marrow-derived endothelial progenitors into tumour vasculature is such a rare event, it is likely that endothelial progenitors are present in exceedingly low numbers in the circulation. To determine the proportion of circulating endothelial progenitors in humans using a rich source of hematopoietic stem cells, we examined umbilical cord blood using a functional assay. $CD133^+$ cells, Lin^- cells and total mononuclear cells from umbilical cord blood were cultured for up to 6 weeks following plating of non-adherent cells over 3 days in endothelial cell medium supplemented with angiogenic cytokines (VEGF, bFGF). Serial plating of non-adherent cells ensured that we measured only transplantable endothelial progenitors, and not mature endothelial cells (Lin et al., 2000). After 3 to 4 weeks, colonies of adherent cells appeared and were quantified by counting the total number of colonies. The number of endothelial colonies was normalized to the total number of cells originally plated. Endothelial colonies adopted a cobblestone morphology and stained for endothelial markers such as vWF and the antigen marker P1H12 (Figure 33A). Analysis of endothelial colonies following expansion revealed that the cells express endothelial specific nitric oxide synthase (eNOS), Tie-1, Tie-2 and VEGFR-2 (Figure 33B, C), therefore confirming their endothelial phenotype. We observed a 16-fold enrichment of endothelial colonies in the CD133-purified cells over total mononuclear cells and a 2.7-fold increase over Lin^- cells (Figure 33D), indicating that the $CD133^+$ cells are markedly enriched for endothelial progenitors, which is consistent with the observation that $CD133^+VEGFR\text{-}2^+$ cells are associated with a population of endothelial progenitors

(Peichev et al., 2000). Nevertheless, the frequency of endothelial progenitors was less than 1 in 10^7 cells of the total mononuclear cell population, highlighting the rarity of this cell population.

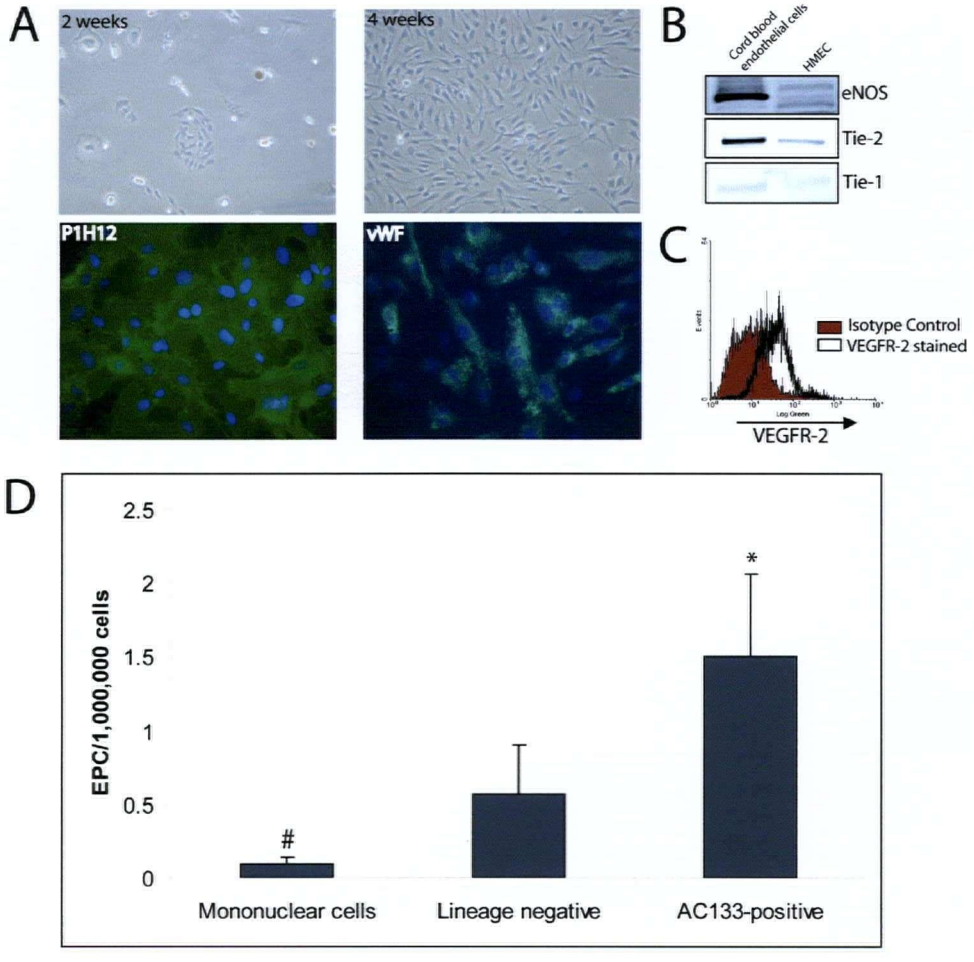

Figure 33 Rarity of endothelial progenitors in post-natal human mononuclear cells. Cells from umbilical cord blood ($CD133^+$, Lin^-, total mononuclear cells) were plated in medium supplemented with VEGF and bFGF. Endothelial colonies, which appeared 3 to 4 weeks after plating, stained positive for the endothelial markers vWF and P1H12 (immunostaining) (A) expressed eNOS, Tie-1 and Tie-2 by immunoblotting (B) and VEGFR-2 as determined by flow cytometry (C). Four weeks after plating, colonies were quantified by counting the total number of $P1H12^+$ colonies, and expressed as the total number of endothelial progenitors normalized to the original number of cells plated for each subset of mononuclear cells. Data represents the average of 7 to 12 independent experiments for each subset of mononuclear cells. * indicates a significant difference with # ($P < 0.05$).

6.3 Discussion

Classically, tumour neovascularization has been thought to be limited to angiogenesis. Prior research has demonstrated this process to be mediated through the release of angiogenic factors such as VEGF, bFGF, PDGF and NO (Folkman and Shing, 1992). However, recent advances in vascular biology have led scientists to revisit this conventional concept. *In vitro* studies have demonstrated that subsets of cells isolated from bone marrow, peripheral blood or umbilical cord blood have the potential to give rise to mature endothelial cells (Lin et al., 2000). These endothelial progenitors, or angioblasts, have also been detected *in vivo* in bone marrow transplant models (Bailey et al., 2004; Lyden et al., 2001). Studies have shown that bone marrow-derived endothelial progenitors may contribute to neovascularization during ischemic conditions or tumour angiogenesis (Isner and Asahara, 1999; Lyden et al., 2001; Murayama et al., 2002). However, the relative levels of contribution of endothelial progenitors remain unclear, as some studies have argued that it is essential to tumour neovascularization (Asahara et al., 1999; Lyden et al., 2001), while other groups failed to detect any significant contribution (De Palma et al., 2003; Gothert et al., 2004b).

The purpose of this paper was to definitively demonstrate the existence endothelial progenitors *in vivo* and examine their potential link to primitive hematopoietic stem cells. Using mice transplanted with bone marrow expressing GFP, we demonstrated that bone marrow-derived endothelial cells can contribute to the formation of new blood vessels in B6RV2 tumours. The incorporation of bone marrow-derived endothelial cells into tumour blood vessels remained a rare event however, as, on average, less than 1% of CD31- or VE-cadherin-stained blood vessels exhibited

incorporation of bone marrow-derived endothelial cells. These results contrast with those of Lyden et al., who have reported that around 90% of blood vessels in B6RV2 tumours are composed of bone marrow-derived endothelial cells (Lyden et al., 2001). This discrepancy may be due to different analytical methods such as the use of β-galactosidase instead of GFP as a marker for bone marrow-derived cells and different antibodies used to identify endothelial cells. Furthermore, the close proximity of leukocytes, such as platelets, to the tumour endothelium could have led to false identification of bone marrow-derived endothelial cells, since vWF was used to identify bone marrow-derived endothelial cells.

Recent studies have demonstrated that transplanted bone marrow cells can turn into unexpected lineages including myocytes, hepatocytes, neurons and many others. A potential problem, however, is that reports discussing such differentiation *in vivo* tend to conclude donor origin of transdifferentiated cells on the basis of the existence of donor-specific markers such as lacZ, GFP or the Y chromosome (Asari et al., 2004; Trotman et al., 2004). In recent years, studies have shown that bone marrow cells have the potential to fuse with different cell types such as hepatocytes and myocytes. It has therefore been argued that some of the transdifferentiation events reported for bone marrow cells may in fact be caused by cell fusion (Terada et al., 2002). In this study, we demonstrate definitively that bone marrow-derived endothelial cells originate through cell differentiation of a progenitor. The origin of those progenitors, their place in the hematopoietic hierarchy and their link to primitive hematopoietic stem cells remained unclear however. It has been previously demonstrated that single adult human hematopoietic stem/progenitor cells give rise to endothelial cells *in vitro* following serial

passage of these cells in long-term culture (Pelosi et al., 2002). Moreover, Bailey et al. have shown that they can detect vWF^+ and $CD31^+$ bone marrow-derived cells in the vasculature of mice transplanted with a single hematopoietic stem cell (Bailey et al., 2004). In our study, we show that tumours implanted into mice transplanted with a single hematopoietic stem cell showed rare bone marrow-derived VE-cadherin$^+$ and $CD31^+$ cells incorporating into the vasculature. The use of VE-cadherin as a specific marker for endothelial cells allowed us to demonstrate definitively that these bone marrow-derived cells were of endothelial origin, therefore confirming the existence of an adult hemangioblast *in vivo*. Moreover, bone marrow-derived endothelial cells were detected in B6RV2 tumours implanted in serially transplanted animals, confirming the long term repopulating capacity of endothelial progenitors (data not shown). Even though the percentage of endothelial progenitor incorporation was very low, these results confirm the existence of cells with hemangioblastic activity in post-natal bone marrow. It is interesting to note that hematopoietic stem cells in the single cell-transplanted animals used in this study could also participate to the regeneration of damaged skeletal myofibers (Corbel et al., 2003), therefore confirming the multilineage potential of those primitive hematopoietic stem cells.

Since the levels of incorporation of bone marrow-derived cells into tumour vasculature are low, it is likely that the differentiation of cells with hemangioblastic activity towards the endothelial lineage is a relatively rare event, which would make endothelial progenitors a rare cell population in comparison to other hematopoietic mononuclear cells. Indeed, we show here that endothelial progenitors are extremely rare among umbilical cord blood mononuclear cells, consisting of less than 1 in 10^7 cells. The

proportion of endothelial progenitors we observed here was much lower than those previously reported (Peichev et al., 2000; Quirici et al., 2001), which could be due to the fact that we determined this proportion using a functional differentiation assay to determine the proportion of endothelial progenitors in contrast to an assay that examines expression of cell surface markers. The cell surface markers CD34, CD133 and VEGFR-2, which are used to detect endothelial precursors, are also present on other cell subsets in blood and bone marrow cells and could therefore explain the higher proportion of endothelial progenitors observed in those papers. Endothelial progenitors are enriched in the $CD133^+$ population of mononuclear cells however, which also marks a population of primitive hematopoietic stem cells (Bhatia, 2001). However, whether $CD133^+$ cells constitute a population of adult hemangioblast remains to be demonstrated at the clonal level.

VEGF and its receptor VEGFR-2 have been shown to be critical in the differentiation and proliferation of endothelial progenitors *in vitro* (Fons et al., 2004). Moreover, increased levels of VEGF in mice *in vivo* have been associated with mobilization of endothelial progenitors through activation of VEGFR-2 (Hattori et al., 2001). Since endothelial progenitor incorporation into tumour vasculature is such a rare event, we were interested in determining whether VEGF was able to increase levels of incorporation of VE-$cadherin^+$ and $CD31^+$ cells into the tumour vasculature. However, when we examined the role of VEGF in the *in vivo* recruitment of bone marrow-derived endothelial progenitors, we found that overexpression of VEGF by tumour cells, even though it resulted in increased angiogenesis and tumour growth, did not cause any increase in the levels of incorporation of bone marrow-derived endothelial cells into the

tumour vasculature. These results do not correlate with those of Hattori et al. (Hattori et al., 2001), which reported rapid mobilization of VEGFR-2^+ circulating endothelial precursor cells in response to increased serum levels of VEGF. However, the VEGF serum levels that they observed in their model were much higher than the ones we measured in our system. It is therefore possible that higher VEGF serum concentrations are required in order to mobilize bone marrow-derived endothelial cells, even though those levels we measured are sufficient to promote tumour angiogenesis. Alternatively, it is possible that local VEGF secretion by tumour cells may increase endothelial progenitor recruitment and local angiogenesis to equivalent proportion, in contrast to intravenous VEGF injection, which produces increased concentration of VEGF in the serum, which may be more potent to mobilize endothelial progenitors. Furthermore, even though VEGF may be important for the mobilization of endothelial progenitors, other cytokines such as angiopoietin-1 and bFGF may be important in the subsequent differentiation of those progenitors (Young, 2004). However, activation of VEGFR-2 in bone marrow cells did not expand or mobilize VEGFR-2^+VE-cadherin$^+$ endothelial progenitors, nor did it increase the levels of incorporation of endothelial progenitors into the tumour vasculature, even though we observed that VEGFR-2 was able to expand and partially mobilize a population of bone marrow Gr-1^+ CD11b$^+$ myeloid cells. It is therefore possible that VEGFR-2 activation alone is not sufficient to induce recruitment of endothelial progenitors into the tumour vasculature. The lack of VEGFR-2 induced mobilization or expansion of endothelial progenitors could suggest a requirement for the activation of other receptors in VEGF-induced expansion and/or mobilization of endothelial progenitors. This lack of mobilization could also be a result of the

experimental settings used in this experiment, since we only examined the activation of the kinase activity of VEGFR-2. Furthermore, it is possible that cells will respond differently to AP20187, which can penetrate inside the cells, compared to VEGF which will bind the extracellular portion of the receptor. Therefore, the pharmacokinetics of AP20187 would prevent the formation of an extracellular ligand gradient, which may be necessary in order to induce cell migration. Thus, it is possible that the extracellular domain of VEGFR-2 is required in order for endothelial progenitor cells to migrate in response to a VEGF gradient. Alternatively, this may represent a requirement for VEGFR-1 signaling, or VEGFR-1/VEGFR-2 heterodimerization, in order to recruit endothelial progenitors.

Our findings demonstrate that there is an adult hemangioblast, but the differentiation of a marrow stem cell towards the endothelial lineage is a rare event, and VEGF stimulation does not enhance this process. Understanding the factors that regulate contributions from primitive hematopoietic stem cells and their circulating progenitors to new vessel formation may ultimately provide additional ways to influence the process of neovascularization, which may prove beneficial in treating conditions such as diabetic retinopathy and cancer.

Chapter 7

Conclusions and future prospectives

VEGF and its receptors, particularly VEGFR-2, have been implicated in neovascularisation processes. The fact that most tumours secrete VEGF suggests that its importance may be greatest in the context of tumour angiogenesis. Moreover, tumour endothelium is believed to express increased levels of VEGFR-2 (Takahashi et al., 1995). Experimental data also shows that VEGF acts in a paracrine manner on endothelial cells, resulting in the expansion of the tumour vasculature and the tumour mass. By contrast to the relatively large amount of information on the role of VEGF in solid tumours, little is known of its effects in "liquid tumours" such as leukemias and lymphomas. VEGF and its receptors have been shown to be present on a variety of hematopoietic malignancies. Moreover, VEGFR-2 expression in some hematological disorders has been shown to be associated with poor prognosis (Verstovsek et al., 2002). Before the significance of VEGF and VEGFR-2 expression in malignancies can be fully appreciated however, it is important to have a better understanding of the signalling pathways and the biological effects triggered by VEGFR-2 when it is activated by VEGF.

The main goal of the research presented in this thesis was to acquire a better understanding of VEGFR-2 signalling effects. However, it has proven hard to study VEGFR-2 signalling in isolation due to the numerous VEGF receptors and ligands. To overcome this problem, we relied on various strategies to study VEGFR-2 signalling, without having to worry about the possible interference of other VEGF receptors. In the first part of this study, we used retroviral transduction to express VEGFR-2 in fibroblasts, which do not express other endogenous VEGF receptors. This model has allowed us to elucidate some of the signalling pathways induced following VEGFR-2 activation. In particular, we have shown that VEGFR-2 can activate the Erk 1/2 and p38 MAP kinase

pathways, Moreover, we also demonstrated that VEGFR-2 activation resulted in cell proliferation and migration. Even though it was previously known that VEGF could induce these signalling pathways and biological effects in endothelial cells, this study allowed us to confirm that VEGFR-2 activation alone was sufficient to induce them. However, even though VEGFR-2 is critical in the establishment of the endothelial lineage, we were not able to detect expression of endothelial markers in fibroblasts following its activation. These results could be interpreted in different ways: it could mean that either VEGFR-2 does not act as a switch that can promote the appearance of an endothelial phenotype, or that fibroblasts are too differentiated to be able to adopt an endothelial phenotype, even though they have been reported to transdifferentiate to other cell types such as endothelial cells (Kon and Fujiwara, 1994) and myocytes (Lattanzi et al., 1998). It is interesting however that VEGFR-2 was able to induce cell migration in response to VEGF. It was already known that VEGFR-1 can induce cell migration in response to VEGF, particularly in monocytes (Barleon et al., 1996), but here we show that VEGFR-2 activation can also trigger migration. This VEGFR-2 migration might play an important role in endothelial migration during angiogenesis. Moreover, it could be critical in the establishment of the endothelial and hematopoietic lineages by promoting the migration of early mesodermal precursors in response to VEGF gradients during early embryonic stages.

Even though studying VEGFR-2 signalling in fibroblasts provided us with some answers concerning some of the functions of this receptor, we were interested in studying its function in compartments in which it is endogenously expressed, particularly hematopoietic cells. Other than the fact that it is expressed on subsets of normal and

malignant hematopoietic cells, little is known about the potential role of VEGFR-2 in these cells. However, studying VEGFR-2 signalling in hematopoietic cells in isolation would prove difficult, since subsets of hematopoietic cells have been shown to express VEGFR-1, and therefore stimulating VEGFR-2-transduced hematopoietic cells with VEGF would result in a certain degree of endogenous VEGFR-1 signalling "contamination". To overcome this problem, we used a VEGFR-2 construct that could be selectively activated with a chemical inducer of dimerization, which prevented interference from endogenous VEGF receptors. This VEGFR-2 construct was phosphorylated in the presence of AP20187, and could induce a signalling cascade that resulted in Erk 1/2 MAP kinase and Akt phosphorylation *in vitro*. More importantly, we were able to demonstrate that VEGFR-2 activation could induce survival of hematopoietic cells though the PI3-kinase pathway, a mechanism that may be important in some hematopoietic malignancies. In this part of the study, we only used sorted, transduced GFP^+ bone marrow cells for all experiments, and therefore, we were not able to identify a potential paracrine mechanism that could be triggered by VEGFR-2 activation. To have a better overview of the role of VEGFR-2 in hematopoietic cells, we used bone marrow transduced with the same VEGFR-2 construct to transplant mice. This approach allowed us to simulate a situation in which a relatively large proportion of hematopoietic cells express VEGFR-2, which has been shown to occur in some hematological malignancies. We observed that VEGFR-2 was able to promote the expansion of myeloid progenitors, not only among the cells that expressed the actual receptor, but also in untransduced cells, which led us to hypothesize that VEGFR-2 could promote myeloid cell expansion in a cell autonomous fashion and a paracrine fashion.

We confirmed the induction of a paracrine mechanism by showing that VEGFR-2 was able to up-regulate GM-CSF. Based on our *in vitro* and *in vivo* observations, we were able to propose a model for the effects of VEGFR-2 in hematopoietic cells (Figure 34).

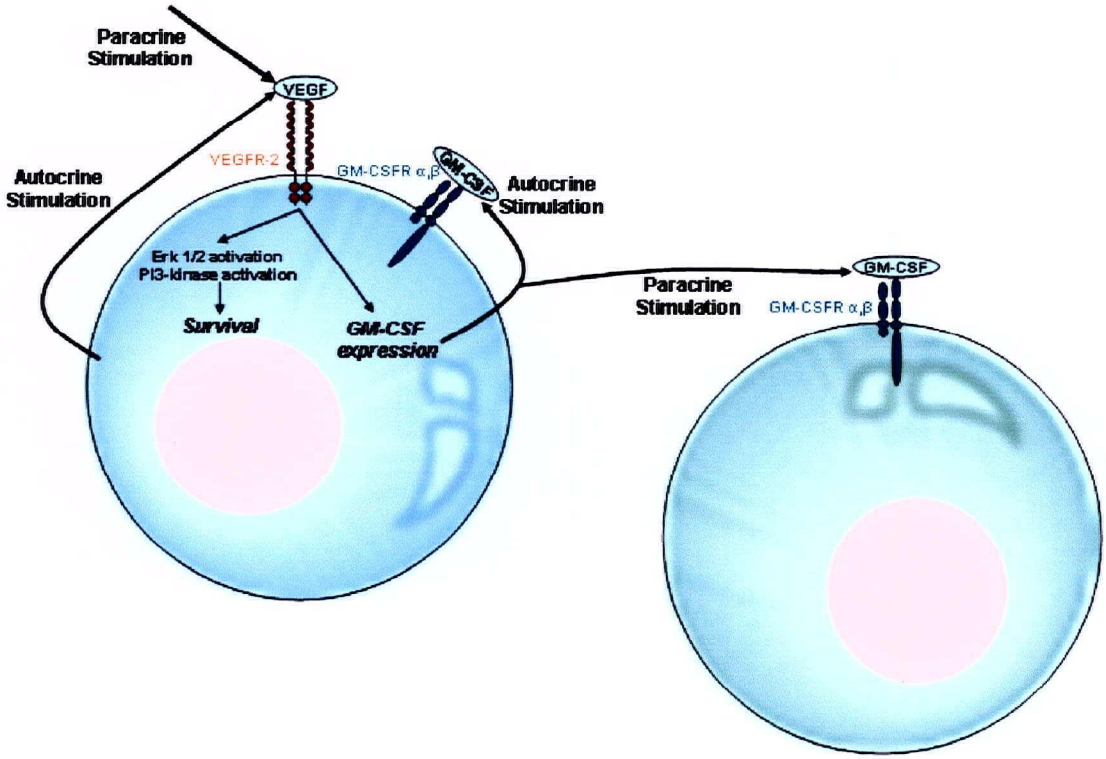

Figure 34 Hypothetical model for the possible effects induced by VEGFR-2 activation in hematopoietic cells.

According to this model, autocrine or paracrine stimulation of VEGFR-2 by VEGF would result in activation of signalling pathways such as Erk 1/2 MAP kinase and PI3-kinase, which would promote cell survival in a cell autonomous manner. Moreover, VEGFR-2 activation would induce expression of GM-CSF. The secreted GM-CSF could in turn stimulate VEGFR-2^{+}GM-CSFR^{+} cells via an autocrine loop. It is therefore possible that the signalling pathways induced by VEGFR-2 and GM-CSF would

synergise, which would result in the rapid expansion of myeloid cells expressing VEGFR-2. Moreover, the GM-CSF secreted by VEGFR-2^+ cells could in turn stimulate GM-$CSFR^+$ cells in a paracrine manner.

It is likely that this mechanism may be important in promoting the expansion and survival of malignant hematopoietic cells that express VEGFR-2, since blocking VEGFR-2 can inhibit leukemic cell growth (Dias et al., 2000). However, further studies will be required in order to determine the relative importance of VEGFR-2 signalling in physiological hematopoiesis. More specifically, due to its expression on primitive hematopoietic stem cells, it will be interesting to study the effects of VEGFR-2 on the self-renewal, proliferation and differentiation of hematopoietic stem cells.

Finally, we examined the contribution of bone marrow cells in the formation of the vascular network of tumours. Due to the conflicting reports published on this topic in the recent years, it is difficult to evaluate the specific role of bone marrow-derived cells in the formation of tumour blood vessels. Even though we were able to detect bone marrow-derived endothelial cells in the vascular network of tumours, their contribution was minimal. Furthermore, we were able to demonstrate that bone marrow-derived endothelial cells arise by differentiation, and not by fusion of bone marrow-derived cells and endothelial cells. We were able to show that primitive hematopoietic stem cells have the potential to give rise to endothelial progenitors, therefore confirming the existence of an adult hemangioblast. The contribution of bone marrow-derived endothelial cells to the tumour vascular network was so minimal however that it is unlikely that these cells play an important role in the elaboration of tumour blood vessels. We therefore examined whether VEGF could increase the contribution of bone marrow-derived endothelial cells

to tumour blood vessel formation, since it has been reported that VEGF can recruit endothelial progenitors from the bone marrow. VEGF alone however did not influence this process. To further evaluate the role of this cytokine in adult vasculogenesis, many important questions must be answered. Based on our results and previously published work, it is likely that VEGF is essential to promote the survival of endothelial progenitors. But it is unlikely that it is sufficient to promote the differentiation of these cells to mature endothelial cells. Therefore, what supplementary set of signals are required to induce the maturation of endothelial progenitors? More importantly, what is the physiological relevance of endothelial progenitors, since their *in vivo* occurrence is so rare? The answers to these questions would provide invaluable insights into the understanding of blood vessel formation and new clues for potential therapeutic strategies.

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