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Vascular Endothelial Growth Factors and the Bmx Tyrosine Kinase in the Regulation of Angiogenesis and Lymphangiogenesis

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Academic Dissertation

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ABBREVIATIONS

Ang	Angiopoietin
ATP	Adenosine triphosphate
Bmx	Bone marrow tyrosine kinase gene in chromosome X
Btk	Bruton's tyrosine kinase
E8.5	Embryonic day (8.5)
EC	Endothelial cell
EPC	Endothelial progenitor cell
Etk	Epithelial and endothelial tyrosine kinase (Bmx)
FGF	Fibroblast growth factor
HIF	Hypoxia inducible factor
HPC	Hematopoietic progenitor cell
HSPG	Heparan sulfate proteoglycan
Itk	Interleukin-2-inducible T cell kinase
KO	Knockout
mRNA	Messenger ribonucleic acid
NRTK	Non-receptor tyrosine kinase
P2	Postnatal day (2)
PC	Pericyte
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PlGF	Placenta growth factor
PTKs	Protein tyrosine kinases
RA	Rheumatoid arthritis
RTK	Receptor tyrosine kinase
SMC	Smooth muscle cell
STAT	Signal transducer and activator of transcription
sVEGFR-1	Soluble VEGFR-1
Tec	Tyrosine kinase expressed in hepatocellular carcinoma
TG	Transgenic
TGF-□	Transforming growth factor alpha
Tie	Tyrosine kinase with Ig and EGF homology domains
TNF-□	Tumor necrosis factor alpha
Txk	T cell X chromosome kinase
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WT	Wildtype

LIST OF ORIGINAL PUBLICATIONS

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III Paavonen, K., Puolakkainen, P., Jussila, L., Jahkola, T., Alitalo, K. VEGFR-3 in lymphangiogenesis in wound healing. ***American Journal of Pathology***: 156: 1499-1504, 2000

IV Paavonen, K., Mandelin, J., Partanen, T., Jussila, L., Li, T.-F., Ristimäki, A., Alitalo, K., Konttinen, Y. T. Vascular Endothelial Growth Factors C and D and their VEGFR-2 and 3 Receptors in Blood and Lymphatic Vessels in Healthy and Arthritic Synovium. ***Journal of Rheumatology***: 29: 39-45, 2002

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* Equal contribution

ABSTRACT

Neoplastic cells are characterized by distinct alterations in the genome that are associated with characteristic changes in cell physiology: self-sufficiency in growth signals, insensitivity to anti-growth signals, resistance to programmed cell death, limitless replicative ability, the ability to invade tissue and metastasize and the capability of inducing sustained angiogenesis through the secretion of angiogenic molecules. Vascular Endothelial Growth Factor (VEGF) is the principal angiogenic molecule involved in both physiologic and pathologic angiogenesis. VEGF belongs to the VEGF gene family comprising of VEGF, VEGF-B, VEGF-C, VEGF-D and Placenta Growth Factor (PlGF). VEGF gene family members bind and phosphorylate receptor tyrosine kinases on endothelial cells. This signal is relayed to the cell nucleus through intracellular signal mediators such as the Bmx tyrosine kinase leading to changes in gene expression.

In this study, newly cloned members of the VEGF family were analysed. Human VEGF-B and VEGF-C genes were localized to the chromosomal loci 11q13 and 4q34, respectively. VEGF-B and VEGF-C were not significantly amplified in the breast cancer cell lines studied and they did not localize to known cancer amplicons in the human genome. The mRNA regulation of VEGF-B and VEGF-C by serum, growth factors, oncoproteins and hypoxia was analysed and found to differ significantly from the VEGF mRNA regulation. For example, unlike VEGF, neither VEGF-B nor VEGF-C was upregulated by hypoxia. Serum, growth factors and oncoproteins upregulated VEGF-C mRNA, whereas VEGF-B mRNA was not regulated by any of these stimuli. The receptor for VEGF-C and VEGF-D, VEGFR-3, was found to be expressed in lymphatic endothelial cells during the transient in-growth of lymphatic vessels into the wound bed during dermal wound healing. Wound healing angiogenesis and lymphangiogenesis were concurrent processes with different kinetics. VEGFR-3 was upregulated on blood vascular endothelium during chronic inflammation and VEGF-C was strongly expressed in the inflamed synovial membranes in rheumatoid arthritis. VEGFR-3 was expressed on lymphatic endothelium and also on the endothelium of synovial capillaries and venules. These studies indicate that VEGF-C is induced by various proinflammatory cytokines and growth factors and that VEGF-C and its receptor VEGFR-3 could be involved in the pathogenesis of inflammatory processes.

Bmx, a tyrosine kinase involved in VEGF signaling and expressed in arterial endothelium, was not upregulated in angiogenic vessels during angiogenesis. Bmx was however expressed in migrating epithelial keratinocytes during the re-epithelialization of dermal wounds. Epidermal overexpression of Bmx in transgenic mice stimulated keratinocyte proliferation and accelerated dermal wound healing. The Bmx transgene also induced dermal inflammation and angiogenesis with strong changes in the epidermal gene expression profiles. These results suggest that Bmx expression, that has been detected in prostate carcinomas, could be associated with important aspects of tumor progression.

REVIEW OF THE LITERATURE

Vascular development

Vasculogenesis and angiogenesis

During human embryogenesis, the vascular system appears during the third week of development when the nutritional requirements of the embryo can no longer be met solely by diffusion [1]. The angioblasts, i.e. the endothelial progenitor cells (EPC) and the hematopoietic progenitor cells (HPC) are thought to originate from a common mesoderm-derived progenitor cell, the hemangioblast. Hemangioblasts aggregate to form primitive blood islands in the yolk sac in response to growth factor signals [2]. The cells in the center of these blood islands become hematopoietic progenitor cells while the outer cells become angioblasts [2]. Angioblasts proliferate, differentiate and organize to form a primitive vascular plexus composed of endothelial tubes. VEGF and its receptors represent the first endothelial-cell specific signaling pathway during development [3]. The primitive vascular system is remodeled into a mature vascular network through a process termed angiogenesis [4]. During angiogenesis, new vessels are formed from pre-existing ones through sprouting or non-sprouting mechanisms such as splitting or bridging [3]. The novel vascular sprouts form capillary loops that develop into capillary networks. Excess vessels are pruned and vessels may fuse together. Endothelial cells (EC) acquire a basement membrane, vessels become covered by pericytes (PC) and smooth muscle cells (SMC) and undergo arterial-venous differentiation [5]. The EC at the site of angiogenesis may also be derived through recruitment of circulating EPC derived from the bone marrow [6-8]. Cells capable of differentiating into EC have also been discovered in skeletal muscle [9]. Angiogenesis is tightly controlled in the adult by a balance between angiogenic and anti-angiogenic stimuli, occurring only in special circumstances such as during the female reproductive cycle, during placental development or during wound healing [10].

Arteriogenesis

Arteriogenesis is the development of functional collateral vessels from pre-existing small arterioles in occlusive arterial disease [3,11]. Although arteriogenesis is often accompanied by angiogenesis and

vice versa, arteriogenesis differs significantly from angiogenesis at the cellular and molecular level. Angiogenesis is rarely capable of providing functional blood perfusion to acutely ischemic tissues but arteriogenesis results in the development of true collateral arteries capable of providing a 10- to 20-fold increase in tissue blood flow. Arteriogenesis is initiated by an increase in fluid shear stress, which activates the vascular endothelium. The activated endothelium begins to express monocyte chemotactic protein and cell adhesion molecules involved in the attachment and trans-endothelial migration of monocytes [12]. Monocytes are recruited to the perivascular space where they release proteases, growth factors and cytokines involved in the recruitment of EC and SMC to the new vessel. Arteriogenesis then proceeds with the remodeling phase. A new elastic lamina is synthesized by the SMC, the vessel wall thickens and the vessels increase in tortuosity.

Hematopoiesis

During early development, hematopoiesis, the development of blood cells, occurs in tight proximity to the development of the vascular system. Hematopoietic cells appear first in the extraembryonic yolk sac [13]. These inner cells of blood islands are mostly nucleated red blood cells expressing fetal hemoglobin. In humans, primitive hematopoiesis is only transient and as it declines, the so-called definitive hematopoiesis begins in the embryo proper in the fetal liver. Since different animal models have yielded contradicting data, the origin of definitive hematopoietic stem cells (HSC) is unclear [14]. The intraembryonic HSC of humans are thought to be derived primarily from clusters of hematopoietic cells attached to the ventral wall of the dorsal aorta in the para-aortic splanchnopleura [14-16]. EC and definitive HSC have common surface markers, vascular endothelial growth factor receptor -1 (VEGFR-1), VEGFR-2 and Tie-2 among others [17]. Definitive hematopoiesis occurs from mid-gestation onwards also in the spleen, moving to the bone marrow in the adult [1]. In the adult, hematopoiesis occurs solely in the bone marrow, except for certain pathologic conditions where extramedullary hematopoiesis may be present.

Lymphangiogenesis

The lymphatic system begins in the interstitium as blind-ended lymphatic capillaries that collect interstitial fluid, immune cells, proteins, other macromolecules and debris and in the gut, also lipids. Lymphatic capillaries are very permeable, thin-walled vessels with large inter-endothelial pores lacking a continuous basal lamina and pericytes [18-20]. The lymphatic capillaries feed into

lymph nodes where antigen presentation and clonal expansion of immune cells takes place. Lymph continues into larger collecting ducts, the left and right thoracic ducts and finally into the systemic blood circulation. The development of embryonic lymphatic vessels has been less studied than vasculogenesis and angiogenesis. In 1902, Sabin proposed that during embryogenesis, the lymphatic vasculature develops by sprouting lymphangiogenesis from the lymph sacs [21]. Recent data on the expression of early markers of lymphatic endothelium on the endothelium lining the budding lymphatic sacs in mice have supported Sabin's theory [22-25]. The lymphatic vascular system in peripheral tissues has been suggested to develop from the fusion of mesenchymal lymphatic spaces that develop separately from blood vessels [26]. In a quail-chick chimera model, mesodermal lymphatic progenitor cells may participate in lymphatic vessel development [27-29]. Lymphatic EC are thought to develop from venous EC. This theory has been supported by studies on the ability of the *prospero* related homeobox protein-1 (Prox1) to re-program blood vascular EC into lymphatic EC and also by studies on the differences and similarities in the gene expression profile of lymphatic and blood vascular EC [30-33]. Studies on the VEGF-C knockout mice have offered insight into the interplay between VEGF-C and VEGFR-3 in the development of the lymphatic vasculature [34]. Lymphangiogenesis in the adult has not been extensively studied and the cell-cell communication between angiogenic and lymphangiogenic EC *in vivo* is unclear.

Vascular endothelial growth factor family

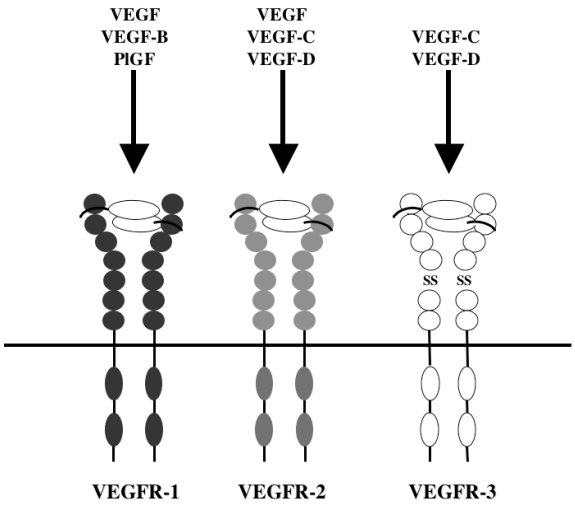

Figure 1. A simplified scheme of ligand-receptor binding in the VEGF gene family in humans.

VEGF

In mammals, the Vascular Endothelial Growth Factor (VEGF) gene family comprises five dimeric glycoproteins able to bind and activate VEGF receptor tyrosine kinases (VEGFRs): VEGF, VEGF-B, VEGF-C, VEGF-D and the placenta growth factor (PlGF). VEGF binds VEGFR-1 and VEGFR-2. VEGF is secreted by a broad range of cells including PC/SMC, fibroblasts and hematopoietic cells. VEGF induces the proliferation, sprouting, migration and tubule formation of EC [35]. VEGF is also a potent survival factor for angiogenic EC during physiological and tumor angiogenesis and induces the expression of anti-apoptotic proteins in the EC [36-38]. VEGF was originally described as a permeability factor [39]. VEGF induces potent changes in the permeability of EC through formation of transcellular gaps, vesico-vascular organelles, vacuoles and fenestrations [40]. VEGF causes vasodilatation through the induction of the endothelial nitric oxide (NO) synthase and the subsequent increase of NO production [41-43]. Although VEGF acts mostly on EC it has been shown to also bind VEGF receptors on HSC, monocytes, osteoblasts and neurons [44-47]. In these cells VEGF induces for example HSC mobilization from the bone marrow, monocyte chemoattraction, osteoblast-mediated bone formation and neuronal protection and angiogenesis [48-50]. VEGF stimulates inflammatory cell recruitment and also the expression of proteases implicated in the degradation of the basal lamina in the early stages of angiogenesis [51-53]. VEGF is strongly induced by hypoxia due to hypoxia-inducible factor (HIF) regulated elements of the VEGF gene [54,55]. Many cytokines including platelet-derived growth factor, epidermal growth factor, transforming growth factors and basic fibroblast growth factor induce VEGF expression and may also regulate VEGF mRNA stability [56,57]. Six forms of VEGF are produced through alternative splicing: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$ [50]. $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are the major forms secreted by most cell types [58]. $VEGF_{121}$ diffuses freely, while in general approximately half of the secreted $VEGF_{165}$ binds to cell surface heparan sulfate proteoglycans (HSPGs). $VEGF_{189}$ remains almost completely sequestered by HSPGs in the extracellular matrix and the HSPGs provide a reservoir of VEGF isoforms that may be released by proteolytic enzymes [59-61]. $VEGF_{145}$ and $VEGF_{206}$ are expressed primarily in the placenta and $VEGF_{183}$ is expressed in the retina [62-64]. Recently, also an inhibitory splice form of VEGF, termed $VEGF_{165b}$, has been found and it is downregulated in renal cell carcinoma [65].

VEGF is ubiquitously expressed in embryonic tissues, but expression is stronger at sites of active vasculogenesis and angiogenesis [66]. VEGF is expressed in practically all solid tumors studied and also in hematological malignancies [50,67]. VEGF inhibition in experimental tumors reduces tumor

growth and correlations have also been found in human cancer patients between VEGF expression and various parameters such as disease progression and survival [68-70]. Transgenic mice overexpressing VEGF in the skin develop an inflammatory skin condition resembling psoriasis [71]. Overexpression of VEGF in murine skin also accelerates experimental tumor growth [72]. Homozygous VEGF knockout (KO) mice die at E8-E9 from defects in the very early blood island formation, in EC development, in EC differentiation and in tubule formation (see Table 2) [73]. The levels of VEGF protein during development are crucial since mice lacking even a single VEGF allele also die at E11-E12, displaying defects in early vascular development [74]. The different biological functions of VEGF isoforms were recently demonstrated by studying isoform-specific KO mice. Mice expressing only $VEGF_{120}$ (human $VEGF_{121}$) die in part soon after birth and those that survive develop lethal ischemic cardiomyopathy [75]. Mice expressing only $VEGF_{188}$ (human $VEGF_{189}$) display impaired arteriolar development and approximately half die at birth [76]. Mice expressing only $VEGF_{164}$ (human $VEGF_{165}$) are viable and healthy [76]. These studies underline the importance of $VEGF_{164}$ (human $VEGF_{165}$) as the principal effector of VEGF action.

Gene	**Chromosomal localization**	**Splice variants**	**Major protein size**	**Major mRNA transcript size**
VEGF	6p23.1 [77]	121,145, 165*, 183*, 189*, 206*, 165b	21kDa	3.7kB, 4.5kB
VEGF-B	-	167*, 186	21kDa, 30kDa	1.4kB
VEGF-C	-	-	20-21kDa	2.4kB
VEGF-D	Xp22.31 [78,79]	-	20-21kDa	2.2kB
PlGF	14q24 [80,81]	131, 152*, 219	38kDa, 30kDa	1.7kB 1.2kB
VEGFR-1	13q12-q13 [82]		180kDa	7.5kB, 8.0kB
VEGFR-2	4q11-q12 [83]		230kDa	7.0kB
VEGFR-3	5q33-qter [84]	VEGFR-3, (VEGFR-3 short)	195kDa	4.5kB, 5.8kB

Table 1. VEGF family members and receptors in humans, chromosomal localization, splice variants, major protein and mRNA transcript sizes. * indicates heparin-binding form. kB = kilobase (1000 nucleotide bases), kDa = kilodalton (100 daltons).

PlGF

Placenta growth factor (PlGF) was originally discovered in a human placental cDNA library [85]. PlGF binds VEGFR-1 [86,87]. PlGF is angiogenic and also arteriogenic [88]. It induces recruitment and survival of bone marrow derived VEGFR-1 positive stem cells [89]. Alternative splicing leads to three different forms of human PlGF: PlGF-1 ($PlGF_{131}$), PlGF-2 ($PlGF_{152}$) and PlGF-3 ($PLGF_{221}$) [80]. PlGF

forms homodimers and PlGF/$VEGF_{165}$ heterodimers [90,91]. In mice, PlGF is expressed most abundantly in the heart and lungs [92,93]. In several animal models PlGF induces the formation of mature, leakage-resistant PC-covered vessels [94,95]. PlGF has also been shown to induce dermal angiogenesis, increase permeability and increase induction of dermal inflammation when overexpressed in the skin of transgenic mice [96,97]. PlGF KO mice are viable but exhibit impaired pathologic angiogenesis and arteriogenesis (see Table 2) [98-100].

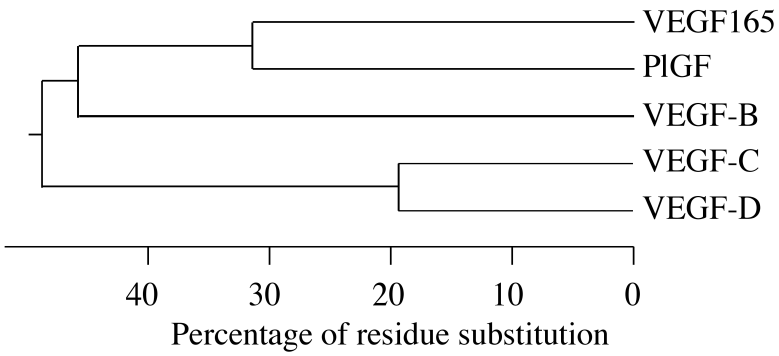

Figure 2. Phylogeny of the VEGF gene family.

VEGF-B

VEGF-B (also called VEGF-related factor/VRF) was discovered independently by two laboratories [101,102]. VEGF-B binds VEGFR-1, but its biological function is not known [103]. Two different splice forms of human VEGF-B exist: the predominant form $VEGF\text{-}B_{167}$ that is partly sequestered by HSPGs and $VEGF\text{-}B_{186}$ [104]. During development, VEGF-B is expressed most prominently in the developing myocardium [105]. In the adult VEGF-B is expressed in most tissues but expression is more abundant in the heart and in the skeletal muscle [106]. VEGF-B has been shown to regulate plasminogen activity in EC [103]. Two independently generated VEGF-B KO mice were healthy and had a slightly prolonged PQ-conduction time or slightly reduced myocardial size (Table 2) [107,108]. The VEGF-B KO mice recover slightly less well from experimental transient myocardial ischemia [107].

VEGF-C

VEGF-C is produced as a single propeptide, the N-terminal and C-terminal ends are proteolytically processed to generate a protein with high affinity for VEGFR-2 and VEGFR-3 [109]. VEGF-C induces mitogenesis and migration of EC [109]. VEGF-C is also a survival factor for lymphatic EC [30]. During development, VEGF-C is expressed along with its receptor VEGFR-3 predominantly in regions where lymphatic vessels develop and also in the developing mesenterium [110]. VEGF-C induces selective lymphangiogenesis *in vivo* in a chick chorioallantoic membrane assay [20]. VEGF-C overexpression in the skin of transgenic mice induces the development of hyperplastic dermal lymphatic vessels *in vivo* with no such effects on blood vasculature [111]. Adenovirus mediated gene transfer of VEGF-C (AdVEGF-C) reduces experimental neointima formation in balloon-neduded rabbit aorta [112]. AdVEGF-C induces growth of functional lymphatic vessels in several different animal models [113-116]. Tumor cells overexpressing VEGF-C induce peritumoral lymphangiogenesis and tumor cell invasion into lymphatic vessels in animal models [117-121]. VEGF-C, the proteolytically processed form in particular, has also been shown to be angiogenic at high concentrations [122,123]. Studies of cancer patients have shown a positive correlation between VEGF-C expression and lymphatic invasion and metastasis and patient survival whereas in a small number of studies no such correlation was found [67]. VEGF-C expression is an independent prognostic factor in cervical cancer and gastric cancer [124,125]. VEGF-C KO mice die prenatally from fluid accumulation in tissues due to lack of development of lymphatic vessels (see Table 2) [34]. The endothelial cells commit to the lymphatic lineage but do not however sprout to form lymphatic vessels. The lymphatic vascular sprouting is rescued by the lymphangiogenic VEGF-C and VEGF-D but not by the angiogenic VEGF, indicating specificity for VEGFR-3 [34]. These results indicate that VEGF-C is a crucial paracrine growth factor for the development of the lymphatic vasculature. VEGF-C is not however essential for the development of blood vessels unlike its receptors VEGFR-2 and VEGFR-3. Heterozygous VEGF-C KO mice also have lymphatic developmental defects indicating that VEGF-C is essential for the development of the lymphatic vasculature in a concentration dependant manner [34].

VEGF-D

VEGF-D is the closest relative of VEGF-C [78,126]. Like VEGF-C, the human VEGF-D also binds and activates VEGFR-2 and VEGFR-3 and is mitogenic for EC and angiogenic and lymphangiogenic *in*

vivo [127-129]. Murine VEGF-D binds only VEGFR-2 indicating a possibly different role for VEGF-D in mice [130]. VEGF-D is processed like VEGF-C in its N-terminal and C-terminal ends [78,131]. VEGF-D is present in most human tissues but it is most abundant in the lung [132]. In experimental tumors VEGF-D increases lymphatic vessel growth and lymphatic metastasis [133]. VEGF-D is expressed by melanoma cells and has been proposed to have a role in tumor angiogenesis and lymphangiogenesis [134]. VEGF-D has been shown to be of prognostic value for lymphatic invasion and also survival in certain human cancers [67]. A VEGF-D KO has not been published.

Gene	**KO lethality**	**Defects in vascular development observed in KO**	**Proposed vascular function**
VEGF	(-/-) E8-E9 (+/-) E11-E12	Defective blood vessel and blood island development † [74]	Induction of vasculogenesis, angiogenesis and hematopoiesis
VEGF-B	survive	No phenotype [108] or slightly smaller myocardium [107]	Unknown
VEGF-C	E15.5-E17.5, no live pups born	Defective lymphatic vessel sprouting, edema [34]	Induction of lymphatic sprouting, lymphatic EC survival
PlGF	survive	Defective pathologic angiogenesis [98]	Recruitment of EPC?
VEGFR-1	E8.5	Defective vasculogenesis and angiogenesis, excess EC progenitors [135,136]	VEGF regulation, pathologic angiogenesis, recruitment and survival EPC?
VEGFR-2	E8.5-E9.5	Defective vasculogenesis, blood island and hematopoietic cell development [137]	Hemangioblast differentiation, proliferation and migration
VEGFR-3	E9.5-E10.5	Defective angiogenesis, pericardial effusion [138]	BV maturation in development, growth and survival of LV
PDGF-B	P0	Hemorrhaging, microaneurysms, defective PC recruitment [139]	SMC growth and PC recruitment and survival
PDGFR	P0	Hemorrhaging, microaneurysms, defective PC recruitment [140]	PC maturation, BV stabilization
Ang-1	E12.5	Defective vascular modeling [141]	Recruitment of PC, BV stabilization
Ang-2	<P14	Defective postnatal angiogenesis, lymphatic vessel abnormalities [142]	BV stabilization, BV and LV remodeling
Tie-1	E13.5-P0	Defective EC integrity, edema, hemorrhaging [143]	BV stabilization and EC survival, EC-PC interactions
Tie-2	E9.5-E10.5	Defective vasculogenesis, angiogenesis, PC recruitment, cardiac defects [144]	BV stabilization and EC survival, EC-PC interactions
Nrp-1	E12.5-E13.5	Defects in neural vascularization, and yolk sac vasculature, cardiac defects [145]	Axon guidance, VEGF co-receptor, VEGF regulation in development
Ephrin B2	E11	Defective vascular remodeling and cardiac defects [146]	Establishment of arterial EC identity
EphB4	E10	Defective vascular remodeling and cardiac defects [147]	Establishment of venous EC identity

Table 2. Mouse models of targeted inactivation of genes involved in vasculogenesis and angiogenesis. EC = endothelial cell, BV = blood vessel, LV = lymphatic vessel, EPC = endothelial progenitor cell, PC = pericyte, SMC = smooth muscle cell. † see text for details on isoform-specific KO-studies.

VEGFR-2

VEGFR-2 is composed of seven extracellular immunoglobulin (Ig) homology domains, a single transmembrane region and an intracellular tyrosine kinase (TK) domain that is interrupted by a kinase-insert domain [148,149]. VEGFR-2 binds VEGF, VEGF-C and VEGF-D. Although the binding affinity of VEGF towards VEGFR-2 is lower than that for VEGFR-1, it is thought that most of the biological effects of VEGF are transduced through VEGFR-2 [150,151]. Using receptor-specific VEGF mutants VEGFR-2 has also been shown to be the sole VEGF receptor signaling EC migration, angiogenesis and vascular permeability [152]. In addition to EC, VEGFR-2 is also expressed on neuronal cells, osteoblasts, pancreatic duct cells, retinal progenitor cells, megakaryocytes and hematopoietic stem cells [50,153]. VEGFR-2 expression is downregulated in the adult blood vascular EC but VEGFR-2 is upregulated in the endothelium of angiogenic blood vessels [154]. VEGFR-2 associates with integrin $\alpha V\beta 3$ upon binding VEGF and this may be associated with integrin-dependent migration of EC [155,156]. During development VEGFR-2 is expressed by the primitive endoderm and embryonic angioblasts [73,74,157]. VEGFR-2 is also expressed in the blood islands and in angiogenic vessels [158,159]. VEGFR-2 KO mice are embryonic lethal at E8.5-E9.5 from developmental defects including lack of development of the blood islands, embryonic vasculature and hematopoietic cells (see Table 2) [137,159].

VEGFR-1

The overall structure of VEGFR-1 is similar to that of VEGFR-2 [160]. VEGFR-1 binds VEGF, VEGF-B and PlGF. Upon ligand binding to VEGFR-1, phosphorylation and mitogenic signaling to EC is much weaker than that of VEGFR-2 [161,162]. VEGFR-1 may act as a decoy receptor regulating the bioavailability of VEGF and it may also modulate VEGFR-2-mediated signaling [163]. VEGFR-1 forms heterodimers with VEGFR-2 *in vivo* [164]. VEGFR-1 is expressed on EC and also on osteoblasts, monocyte/macrophages, pericytes, placental trophoblasts, renal mesangial cells and in some hematopoietic stem cells [50,165]. VEGFR-1 activation induces monocyte migration and may also have an important role in inflammatory angiogenesis [166,167]. VEGFR-1 signaling is also involved in the recruitment and survival of bone marrow derived progenitor cells [89,168]. VEGFR-1 is also expressed along with VEGFR-2 in human testis and myometrium [153]. VEGFR-1 expression is upregulated during angiogenesis and unlike VEGFR-2, hypoxia upregulates VEGFR-1 expression [154,169]. Pathologic angiogenesis e.g. during experimental tumor growth is impaired with anti-

VEGFR-1 antibodies [95]. A soluble form of VEGFR-1 (sVEGFR-1, i.e. the extracellular portion of VEGFR-1) has been described which inhibits VEGF action [170]. sVEGFR-1 has been linked to the development of gestational toxemia and pre-eclampsia [171,172]. Women with pre-eclampsia have elevated circulating sVEGFR-1 levels and decreased circulating PlGF and VEGF [173]. The relative absence of VEGF and PlGF may lead to EC dysfunction in the maternal and placental vasculature. During development, VEGFR-1 is first expressed in angioblasts and in endothelia although less strongly than VEGFR-2, VEGFR-1 expression subsides during later embryonic development [135,158,174]. VEGFR-1 KO mice die at E8.5 due to disorganization of blood vessels and overgrowth of EC in the blood vessels with few hemangioblasts being formed (see Table 2) [135,136]. Although VEGFR-1 KO mice do not survive, mice lacking only the intracellular kinase domain are fertile and normal with impaired pathologic angiogenesis [175,176].

VEGFR-3

VEGFR-3 has only six Ig-homology domains as the fifth Ig-homology domain is proteolytically cleaved soon after biosynthesis and the resulting polypeptide chains remain linked via a disulfide bond [177,178]. VEGFR-3 binds VEGF-C and VEGF-D [131,179]. The VEGF-C/VEGFR-3 pathway is crucial for the development of the lymphatic vasculature [34]. VEGFR-3 is present on all endothelia during development but in the adult it becomes restricted to lymphatic EC and certain fenestrated blood vascular EC [22,180]. VEGFR-3 is upregulated on blood vascular EC in pathologic conditions such as vascular tumors and in the periphery of solid tumors [120,154]. A recombinant soluble extracellular portion of VEGFR-3 can disrupt the development of lymphatic vessels in animal models by inhibiting VEGF-C and VEGF-D actions that leads to lymphatic EC apoptosis [121,181]. Transgenic mice overexpressing the extracellular portion of VEGFR-3 in the skin completely lack dermal lymphatic vessels and also have poorly developed deeper lymphatic vessels especially during development [181]. Missense mutations in VEGFR-3 have been linked to hereditary lymphedema in humans and also to a similar condition in a mouse model of lymphedema [182,183]. VEGFR-3 gene targeted mice die at E9.5 from defective remodeling of the primary vascular plexus and disturbed hematopoiesis [138].

Other factors involved in vascular biology

Platelet derived growth factors

The platelet-derived growth factors (PDGF) belong to the VEGF/PDGF family. PDGF monomers include PDGF-A, PDGF-B, PDGF-C and PDGF-D [184,185]. Through binding their receptor tyrosine kinases PDGFR and PDGFRß, PDGFs act as paracrine growth factors during development and as mitogens for fibroblasts, SMC, EC and many other cell types in the adult [185]. PDGFs promote wound healing and overexpression of PDGFs has been linked to many diseases such as atherosclerosis, fibrotic diseases and neoplasia [186,187]. Mice deficient for PDGF-B or PDGFRß die during development or perinatally from absence of mesangial cells in the kidney, defective vascular development, aortal dilatation, and defective PC recruitment leading to hemorrhaging (see Table 2) [139,140].

Fibroblast growth factors

The fibroblast growth factors (FGF) comprise at least 22 secreted growth factors with four tyrosine kinase receptors and pleiotropic effects during development and adult physiology [188,189]. FGFs induce proliferation of a number of cell types: they are involved in tissue repair, vasculogenesis, angiogenesis, hematopoiesis and tumor growth [190]. FGF-1, FGF-2, FGF-4 and FGF-5 have been shown to induce angiogenesis *in vivo* [188,191,192].

Angiopoietins and Tie-receptors

Angiopoietins (Ang) are involved in the formation and maintenance of the vascular system [193-195]. Ang-1 and Ang-4 activate the Tie-2-receptor and Ang-2 is a Tie-2 antagonist whereas no ligand has been found for Tie-1 [196-199]. The Ang/Tie-2 system mediates vessel stabilization through EC-PC interactions whereas the function of Tie-1 is unknown. During development, Ang-1 is involved in vascular remodeling, maturation and stabilization and also in EC-PC interactions [141,144,195,200]. Ang-2 is involved in postnatal vascular and lymphatic vessel remodeling [142]. In adults, Ang-2 is upregulated during angiogenic sprouting in the presence of VEGF and during vessel regression in the absence of VEGF [197,201,202]. Ang-2 inhibition is anti-angiogenic in a rat corneal angiogenesis

assay [203]. An activating mutation of Tie-2 has been found in patients with venous malformations involving dilated vessels and incomplete vessel PC coverage [204]. Overexpressing Ang-1 in mouse skin results in larger, more branched vessels resistant to leakage [205]. Adenoviral transfer of Ang-1 induced angiogenic vessels resistant to vascular leakage [205]. Ang-1 KO mice are embryonic lethal at E12.5 from defective vascular remodeling (see Table 2) [141]. Tie-2 gene targeted embryos die on E9.5-E12.5 from insufficient formation of the vascular plexus, insufficient sprouting and recruitment of perivascular cells and from myocardial developmental defects [144]. A similar phenotype of disorders in vascular maintenance is also seen in adult mice with a Tie-2 conditional knockout that is activated [206]. Tie-1 deficient mice exhibit defects in EC integrity resulting in edema and hemorrhage and death at E13.5-P0 [143]. Soluble forms of the extracellular domains of Tie-1 and Tie-2 have been described [207,208].

Ephrins and Ephs

Ephrins are membrane-bound multimeric ligands which bind to the Eph tyrosine kinase receptors on opposing cells resulting in bi-directional signaling between cells, in the generation and maintenance of patterns of cellular organization, especially in axonal guidance during neuronal development [209-211]. The Eph-ephrin system is also involved in communication of EC with surrounding mesenchymal cells [212,213]. Murine studies have shown that ephrin B2 is expressed primarily on arterial endothelium while its EphB4 receptor is expressed on venous endothelium [146,147]. Mice lacking ephrin B2 or its cytoplasmic domain and mice lacking EphB4 are embryonic lethal at E10-E11 from defective remodeling of the primary vascular plexus and defective venous-arterial remodeling (see Table 2) [146,147,214].

Neuropilins

The neuropilins, Nrp-1 and Nrp-2, have roles in immunology and neuronal development but they are also involved in angiogenesis [215,216]. Neuropilins bind class 3 semaphorins which are secreted molecules that mediate the repulsive signals during neuronal axon guidance [217]. Nrp-1 also binds VEGF, VEGF-B and PlGF while Nrp-2 binds VEGF, VEGF-C and PlGF [215]. Nrp-1 acts as a co-receptor to enhance the function of VEGF through VEGFR-2, to form complexes with VEGFR-1 and to enhance tumor angiogenesis [218-220]. Overexpression of Nrp-1 in mice leads to excessive formation of capillaries and blood vessels and hemorrhages in addition to cardiac malformations [145].

In chick embryos, endothelial Nrp-1 expression is mostly restricted to arteries, whereas Nrp-2 primarily marks veins, indicating a role in arterial/venous identity of EC [221]. Nrp-2 is specific for arterial and lymphatic ECs and mutated Nrp-2 induces abnormalities in the formation of small lymphatic vessels and capillaries in mice [222]. Nrp-1 gene targeted mice die at E13 from vascular defects such as insufficient development of yolk sac vascular networks, deficient neural vascularization and transposition of large vessels (see Table 2) [223,224]. It is thought that during vascular development, Nrp-1 is required for cardiovascular development because it regulates $VEGF_{165}$ levels [224]. While Nrp-2 gene targeted mice do not have a vascular phenotype, a combined Nrp-1 and Nrp-2 KO mouse is embryonic lethal at E8.5 resembling the VEGF and VEGFR-2 KO phenotypes [225].

Bmx tyrosine kinase

The phosphorylation of proteins in response to extracellular signals is an important regulator of the survival, homeostasis, proliferation, differentiation and apoptosis of most cell types [226]. Protein phosphorylation is also important in cellular communication during development and in immunological responses. The human genome has 518 known protein kinases, 1.7% of all human genes. Mammalian protein kinases comprise of serine-threonine kinases and at least 90 protein tyrosine kinases (PTKs) [226]. PTKs are classified according to cellular localization into non-receptor tyrosine kinases (NRTK or cytosolic TK) and receptor tyrosine kinases (RTK) located on the cell surface. The known 32 NRTKs and 58 RTKs are subdivided according to structure and sequence homology into 10 and 20 subfamilies, respectively [226]. Protein kinases modify substrate activity through the transfer of the phosphate of ATP to specific tyrosine residues. Activation of PTKs requires an enhancement of the intrinsic catalytic activity and the creation of binding sites for the recruitment of downstream signal transduction molecules [227]. On the other hand, phosphorylation of tyrosine residues outside of the activation loop in NRTKs and RTKs can negatively regulate kinase activity [227]. Protein phosphorylation is very specific as approximately only 0.05% of protein tyrosine kinases are phosphorylated at any given moment. Protein phosphatases are capable of regulating protein kinase activity through removal of phosphate groups from target residues [228,229]. The 130 known protein phosphatases are also subdivided into tyrosine and serine/threonine specific kinases. Neoplastic transformation involves inactivating mutations in tumor suppressor genes or activating gain-of-function mutations in proto-oncogenes [230,231]. Approximately 50% of PTKs have been found to be deregulated in various human cancers through for example gain-of-function

mutations, deletions, gene amplification or chromosomal translocations. In this respect, PTKs represent targets for cancer therapy [232].

Tec family of tyrosine kinases

The Tec family of tyrosine kinases is the second largest family of NRTKs after the rous sarcoma oncogene (Src) family. The mammalian Tec family comprises of Tec, Btk, Itk/Tsk/Emt, Txk/Rlk and Bmx/Etk [233]. The Tec family proteins share a characteristic domain structure with a pleckstrin homology (PH) domain in the N-terminal part, followed by a Tec homology (TH), Src homology 3 (SH3) and SH2 domains and a C-terminal tyrosine kinase domain. Tec family members are mostly present in hematopoietic lineages but they are also expressed on other cell types [234]. The Tec kinases are involved in signal transduction in response to growth factor receptor signaling, cytokine receptor signaling, G-protein coupled receptor signaling, antigen-receptor signaling, death receptor signaling and integrin-mediated signaling [235]. Tec kinases are regulated by many other kinases and Tec kinases regulate at least the phosphatidylinositol-3-kinase (PI3K), the protein kinase C and the phospholipase C gamma pathways [236,237]. Mutations in *Btk* gene are associated with a severe immunological B-cell deficiency termed X-linked agammaglobulinemia in humans [238,239]. Similar yet milder immunological phenotypes can be observed in Btk KO mice and in mice with Btk mutations [240,241]. Itk KO mice have disorders in T-cell maturation and thymocyte development [242]. Mice lacking Tec or Txk do not have a clear phenotype [241,242]. Loss of multiple Tec family kinases leads to more pronounced phenotypes compared to the single-gene deficient mice in Itk/Txk and Tec/Btk double KO mice [241,242].

The Bmx tyrosine kinase

The Bone Marrow tyrosine kinase gene in chromosome X (Bmx, also called epithelial and endothelial tyrosine kinase or Etk) was cloned from human bone marrow [243]. Bmx is expressed in arterial endothelium, in the atrial endocardium, in hematopoietic cells and in certain EC [244-247]. Bmx is activated by VEGFR-1 and Tie-2 *in vitro* [247]. Bmx promotes epithelial and endothelial cell survival and proliferation by upregulating VEGF *in vitro*, indicating a VEGF-Bmx autoregulatory loop [248]. *In vitro* Bmx is expressed in EC and in prostate and breast carcinoma cells and it is involved in integrin- and tumor necrosis factor (TNF)-induced cell migration *in vitro* [249-252]. Also, TNF induces transactivation between Bmx and VEGFR-2 [253]. Bmx directly activates the PI3K-Akt

angiogenic signaling independent of the VEGF-induced VEGFR2-PI3K-Akt signaling pathway indicating that Bmx may be involved in TNF-induced inflammatory angiogenesis [253]. Bmx interacts with focal adhesion kinase during integrin-mediated cell migration of normal and cancer cells *in vitro* and Bmx also regulates reorganization of actin filaments *in vitro* [251,252,254,255]. Bmx is involved in src-mediated cellular transformation through signal transducer and activator of transcription (STAT) transcription factors, Bmx associates with STAT and dominant-negative Bmx inhibits cellular transformation [256-258]. Bmx is a target for caspase 3 during apoptosis and following caspase cleavage, Bmx is converted into a proapoptotic form, sensitizing prostate cancer cells to apoptosis [259]. On the other hand, Bmx has been shown to protect prostate cancer cells from apoptosis *in vitro* and Bmx has been shown to be necessary for androgen-independent growth and survival of prostate cancer cells [250,260]. In Bmx KO mice, there was no apparent phenotype [247].

Therapeutic aspects of angiogenesis research

The basis of anti-angiogenic therapy

Pathologists have known for a long time that most solid tumors are highly vascularized [261,262]. Tumor angiogenesis was described early on in several animal models but most notably by Ehrmann and Greenblatt who along with others proposed the existence of “humoral substances” that are produced by tumors and which are able to induce tumor angiogenesis [263,264]. Others had shown in animal models that experimental tumors failed to thrive in the absence of neovascularization [265,266]. Later, Folkman and Williams proposed the presence of a tumor-angiogenesis factor and Folkman also proposed anti-angiogenesis as a therapeutic alternative in cancer [267,268]. Angiogenesis in neoplasia is thought to occur as a result of an imbalance in the pro-angiogenic and anti-angiogenic stimuli. Correlation has been found in human tumors between tumor angiogenesis, tumor VEGF expression, tumor vascularization and prognosis [67]. The ability to induce angiogenesis is thought to be one of the main hallmarks of human neoplasia [269]. Tumor angiogenesis produces structurally heterogeneous, leaky, tortuous vessels with incomplete PC/SMC coverage which grow and regress according to tumor microenvironment needs [270]. Tumor vessels are often mosaic vessels, in part lined by tumor cells but it has also been suggested that blood conducting channels without EC lining exist in certain tumors [271,272]. Hypoxia is seen as one of the major driving forces of the upregulation of VEGF and angiogenesis in the tumor microenvironment [273].

Although nearly all traditional chemotherapeutics also have anti-angiogenic effects, drugs are being developed with direct anti-angiogenic effects (see http://www.nci.nih.gov) [274]. Targets of anti-angiogenic therapy include the proteolytic enzymes involved in angiogenesis, the VEGF-VEGFR system and integrin signaling pathways to name but a few. It is also possible to specifically target angiogenic EC with toxins coupled to homing peptides [275]. VEGF and tumor angiogenesis have been successfully blocked in animal experiments with humanized blocking murine antibodies, soluble VEGFR extracellular domains, RTK inhibitors or ribozyme/antisense molecules [276]. Anti-angiogenic therapy targeting the VEGF-VEGFR-system in human trials has proven to be more challenging than previously thought. Side-effects and complications have involved known biological effects of VEGF and patient responses have also varied, presumably because trials have targeted resistant and highly advanced tumors [276].

Bevacizumab (Avastin, Genentech, USA) is an example of anti-angiogenic therapy based on VEGF. It is a recombinant human monoclonal anti-VEGF antibody well tolerated in early trials. Bevacizumab is in randomized Phase III trials in combination with traditional chemotherapeutic agents in advanced colorectal adenocarcinoma, non-small cell lung cancer and in breast cancer [277] (see http://www.nci.nih.gov). Patients with newly diagnosed metastatic colon cancer who had not received previous therapy and who received bevacizumab (Avastin™) along with the traditional 5-fluoruracil, leucovorin and irinotecan (IFL) regimen had substantially longer overall survival time (by about 5 months) than those that received only the IFL regimen. Bevacizumab is the first anti-angiogenesis agent to prove effective in a randomized Phase III trial.

Angiogenesis in wound healing and rheumatoid arthritis

Wound healing involves the concerted action of cytokines and growth factors including VEGF as summarized in Table 3 [278]. Directly after the injury, platelets and injured cells release wound healing mediators. Polymorphonuclear (PMN) leukocytes are recruited to the site of injury followed by macrophages to remove all necrotic and foreign material: PMN leukocytes and especially macrophages are reservoirs of growth factors and cytokines during the healing process. A provisional matrix is formed through deposition of fibrin, fibronectin, vitronectin and connective tissue. In the adult, EC at the wound edge proliferate and migrate via lamellopodial crawling over the provisional matrix. Fibroblasts and EC become the major cell populations within the wound as the inflammation subsides and a highly vascular granulation tissue is formed [278]. The granulation

tissue matures through deposition of collagen and a decrease in vascularity. VEGF is secreted by the platelets directly after injury, very early by keratinocytes at the wound edge, by macrophages and also possibly by other cells in the granulation tissue [279-281]. Re-epithelialization is delayed in genetically diabetic mice with decreased baseline dermal VEGF expression [280]. Wound healing is also impaired in FGF-2 gene targeted mice partially due to decreased granulation tissue angiogenesis [282]. When chronically overexpressed in the skin of transgenic mice the anti-angiogenic matrix molecule thrombospondin-1 delays wound healing although the anti-angiogenic vasostatin did not affect wound healing even at anti-tumor dosage in a mouse tumor xenograft model [283,284].

Growth factor/ Cytokine	**Main cell source**	**Function**
FGF-1, FGF-2	Macrophages, EC	Angiogenesis, fibroblast proliferation
Colony stimulating factor (CSF-1)	Many cells	Macrophage activation and matrix synthesis
Epidermal growth factor (EGF)	Platelets	Pleiotropic, cell motility, proliferation
Heparin binding epidermal growth factor-like growth factor (HB-EGF)	Macrophages	Pleiotropic, cell motility, proliferation
Insulin-like growth factor-1 (IGF-1)	Fibroblasts, keratinocytes	Re-epithelialization, matrix synthesis, angiogenesis
Interleukin-1 (IL-1), IL-1	Neutrophils	Pleiotropic expression of growth factors
Keratinocyte growth factor (KGF)	Fibroblasts	Keratinocyte motility and proliferation
PDGFs	Platelets, macrophages, keratinocytes	Fibroblast recruitment and proliferation, macrophage recruitment and activation, matrix synthesis
Transforming growth factor- (TGF-)	Macrophages, keratinocytes	Pleiotropic, cell motility, proliferation
TGF-1, TGF-2	Platelets, macrophages	Keratinocyte motility, macrophage/fibroblast recruitment, matrix synthesis, remodeling, angiogenesis
TGF-3	Macrophages	Anti-scarring effects
Tumor necrosis factor (TNF-)	Neutrophils	Pleiotropic expression of growth factors
VEGF	Macrophages, keratinocytes	Angiogenesis, permeability changes

Table 3. A list of important growth factors and cytokines involved in wound healing, their main cellular sources during wound healing and their proposed functions.

Infectious diseases and autoimmune disorders involve the recruitment of inflammatory cells which express angiogenic molecules such as VEGF [285]. Rheumatoid arthritis (RA) is an autoimmune chronic inflammatory disorder with increased angiogenesis in the synovial membrane and in the proliferating pannus tissue and increased expression of angiogenic peptides in the synovial membrane and fluid [286]. VEGF and VEGFRs are expressed in the synovial membrane [287,288]. High VEGF concentrations are found in RA synovial fluid and inflammatory cells and synovial fluid

VEGF has been shown to correlate with disease activity in juvenile RA [289-291]. Most anti-rheumatic drugs also have indirect anti-angiogenic effects. Direct anti-angiogenic therapy has also been suggested to possibly be beneficial in RA treatment [292]. In RA, targeting angiogenic factors ameliorates experimental disease and human trials are expected to follow soon [293,294].

Other human disease involving angiogenesis

Excessive ocular neovascularization contributes to visual loss in retinopathy of prematurity and diabetic retinopathy affecting the retinal vasculature and in age-related macular degeneration affecting primarily the choroidal vasculature. Retinal hypoxia and inappropriate secretion of angiogenic factors like VEGF leads to inappropriate retinal neovascularization and hemorrhages [295-297]. Experimental retinal neovascularization can be inhibited with anti-angiogenic therapy [298-300]. Clinical trials are underway to treat disease involving ocular neovascularization with bevacizumab and ranibizumab (Lucentis®, Genentech, USA). Psoriasis is a chronic inflammatory skin disorder characterized by keratinocyte hyperproliferation, dermal inflammation, dermal angiogenesis and overexpression of angiogenic factors such as VEGF [301,302]. Animal models have supported the role of VEGF in psoriasis: transgenic mice with sustained overexpression of murine VEGF in the skin have many of the classical features of psoriasis [71,303]. Pathologic angiogenesis is also involved with endometriosis, several uterine bleeding disorders and other gynecological disorders [304]. Atherosclerotic plaque progression has been associated with increased VEGF and angiogenesis [305]. Experimental atherosclerosis can be induced with VEGF and the experimental atherosclerotic changes can be reduced with anti-angiogenic therapy [306,307]. Vascular malformations also involve angiogenesis and the first successful treatment with anti-angiogenic agents involved regression of pulmonary hemangiomas with administration of interferon-α2a in 1989 [308]. Furthermore, Tie-2 mutations have been found in hereditary vascular malformations [204].

Endogenous stimulators of angiogenesis
Angiogenin
Ang-1
FGF-1, FGF-2
Hepatocyte growth factor (HGF)
Insulin-like growth factor -1, -2 (IGF-1, -2)
Platelet-derived endothelial cell growth factor (PD-ECGF)
PDGF-BB
PlGF
TGF-β
TNF-α
VEGF

Endogenous inhibitors of angiogenesis
Angiostatin (fragment of plasminogen)
Antithrombin III
Arrestin (fragment of type IV collagen)
Canstatin (fragment of type IV collagen)
Endostatin (fragment of type XVIII collagen)
Heparinases
Interferon-inducible protein-10 (IP-10)
Interferon-α (IFN-α), IFN-β, IFN-γ
Interleukin-12 (IL-12)
Kringle 5 (fragment of apolipoprotein a)
Plasminogen activator inhibitor (PAI)
Platelet factor-4 (PF-4)
Restin (fragment of type XV collagen)
Retinoids
TGF-β
Thrombospondin-1 (TSP-1), TSP-2
Tissue inhibitors of matrix metalloproteinases (TIMPs)
Tumstatin (fragment of type IV collagen)
Vasostatin (fragment of calreticulin)
2-methoxyestradiol

Table 4. Table of known endogenous stimulators and inhibitors of angiogenesis.

Pro-angiogenic therapy

Angiogenic growth factors may also be useful in increasing collateral vessels in coronary heart disease, other ischemic heart conditions and in critical limb ischemia and claudication or in ameliorating diabetic neuropathy of the lower extremities [309]. Trials utilizing pro-angiogenic therapy have shown some successful endpoints [310-312]. Unrelated gene therapy has however involved

serious side-effects in human trials [313,314]. In patients with critical limb ischemia and claudication, injection of VEGF plasmid induced the growth of functional collateral vessels and reduced morbidity due to the vascular insufficiency [315-317]. Inoperable myocardial ischemia has been treated with naked VEGF plasmid injections resulting in clinical benefit [318-320]. Adenoviral gene transfer of VEGF has also been used as a pro-angiogenic therapy in human patients with ischemic heart disease [321]. Currently therapy is being developed utilizing different viral vectors for gene delivery of angiogenic molecules [309]. Because of the side-effects of VEGF, it has been suggested that use of a combined approach with several growth factors such as VEGF and angiopoietins or the more arteriogenic PlGF may lead to better results [94,322,323].

Lymphangiogenic therapy

Studies in mice have provided evidence on the possibility of manipulating tumor lymphangiogenesis and thus possibly also lymphatic metastasis in cancer [118-121]. It is unclear which subgroups of cancer patients would benefit from such therapy. Conversely, also lymphedematous states may be treated with pro-lymphangiogenic therapy. Inadequate lymphatic vessel functioning may be acquired or hereditary [324,325]. In acquired lymphedema, the lymphatic vessels may be destroyed as the result of infection such as recurrent cellulitis, filiriasis or following surgical or radiation therapy induced trauma resulting in blockage of lymph flow and lymphedema [324-326]. Hereditary lymphedema disorders involve either a partial or complete absence of lymphatic vessels and some hereditary lymphedema syndromes have been linked to missense mutations in VEGFR-3 [182,327,328]. In all its forms, lymphedema is a chronic disease of tissue swelling, increased risk of infection in the affected area, dermal fibrosis, thickening of the skin and accumulation of adipose tissue [324,325]. In animal experiments, the formation of functional lymphatic vessels has been documented in several studies and pro-lymphangiogenic therapy may be beneficial also in human lymphedema disorders [67].

AIMS OF THE STUDY

The goal of this work was to contribute to the knowledge of the biology of VEGFs and to elucidate the *in vivo* role of the intracellular tyrosine kinase Bmx. The specific aims of the present study were:

I. To study the chromosomal localization, genomic amplification in cancer and relation to known human amplicons of VEGF-B and VEGF-C.

II. To study the regulation of VEGF-B and VEGF-C expression in relation to VEGF.

III. To study lymphangiogenesis and VEGFR-3 expression *in vivo*, particularly in wounds, using an animal model.

IV. To study the expression of lymphangiogenic growth factors VEGF-C and VEGF-D and their receptors in arthritis.

V. To study the function of the Bmx tyrosine kinase *in vivo* using mouse transgenic and gene-targeted mice.

MATERIALS AND METHODS

More detailed description of the materials and methods is in the original publications.

Polymerase chain reaction (PCR) and quantitative reverse transcription-PCR. (I, IV, V)

PCR was performed with appropriate primers according to standard PCR protocols. A DNA panel of 24 interspecies somatic cell hybrids, which retained one or two human chromosomes, was used for the chromosomal localization of VEGF-B and VEGF-C by PCR using appropriate primers. PCR products were visualized in 1% agarose gel with UV light and ethidium bromide. For quantitative PCR, a LightCycler PCR machine was used, amplicons were verified by sequencing and beta-actin was used as an internal control for determining amplicon copy number.

Southern blotting (I, V)

□-32P-dCTP-labelled cDNA inserts of plasmids representing complete VEGF-B and VEGF-C coding domains were used as probes in Southern blotting and hybridization analysis of the somatic cell hybrid DNAs according to manufacturer's instructions. Mouse tail DNA Southern blotting and analysis was performed in a similar fashion with plasmids representing Bmx coding domain.

Northern blotting (II, V)

Total RNA was isolated using kits from a commercial source. Alternatively the poly(A) mRNA fraction was isolated by binding to oligo(dT) cellulose. 5-20 □g of total RNA or mRNA was denatured and electrophoresed through a 1.6% formaldehyde-agarose gel, the gels were transferred to nylon membranes which were hybridized with appropriate radiolabeled fragments and visualized on film.

Isolation and analysis of RNA from cell culture and tissues (II, IV, V)

Tissues and cell cultures were homogenized and lysed in appropriate lysis buffer. RNA was isolated either with oligo(dt) cellulose or with commercially available kits according to

manufacturer's protocol. For RT-PCR mRNA was obtained from total RNA with magnetic (dt)25-polystyrene beads and a magnetic collector. The mRNA was used to prepare primary cDNA with (dt)12-18 primers and SuperScript enzyme followed by RNase treatment. RNAs were transferred to a nylon membrane and analyzed by Northern blot hybridization with □-32P-dCTP-labelled cDNA inserts of appropriate plasmids.

Fluorescent *in situ* hybridization (I)

For fluorescent *in situ* hybridization, P1 clones (obtained from a commercial source) verified by Southern blotting to contain part of the VEGF-B and VEGF-C genes were labeled with nick translation with biotin-11-dUTP, biotin-14-ATP or digoxigenin 11-dUTP according to standard protocol. Peripheral blood lymphocytes were stimulated with phytohemagglutinin and treated with 5-bromodeoxyuridine at an early replicating phase to induce G-banding. Hybridization was carried out with the labeled probes and specific hybridization signals were detected by incubating the hybridized slides in labeled anti-deoxigenin antibodies followed by counterstaining. The signal was detected with fluorescent markers and image analysis was performed with personal computer with appropriate image analysis programs and basic statistical methods.

Cell culture (II)

Cells were cultured on petri dishes in the appropriate media with added growth factors or serum. Before stimulation cells were starved overnight. Growth factors for stimulation experiments were obtained from commercial sources. For hypoxia treatment, cells were exposed to hypoxia in an Anaerocult A anaerobic culture jar. For mRNA half-life studies, cells were kept in low-serum medium, transcription was blocked with actinomycin D (10□g/ml) treatment and translation was blocked with cycloheximide (10□g/ml).

Immunohistochemistry (III, IV, V)

For immunohistochemistry, either Tissue-Tek-embedded frozen samples or paraffin-embedded samples were cut with a microtome into sections. Frozen sections were fixed in acetone or methanol. For paraffin-embedded sections, tissues were fixed in paraformaldehyde or neutral formalin, dehydrated and embedded in paraffin. Sections were then de-parafinized and rehydrated. Antigen retrieval was performed when necessary with microwave treatment in 0.02 M sodium citrate, trypsin digestion or commercial kit digestion followed by treatment with hydrogen peroxide for inhibition of background peroxidase activity. Sections were blocked by incubating them with appropriate serum or commercial blocking kit and stained with primary and secondary antibodies obtained from our laboratory or from commercial sources as detailed in the publications. Immunohistochemistry kits for assaying apoptosis and cell proliferation were used according to the manufacturer's protocol. The signal was amplified with signal amplification kits, staining was visualized with a chromophore. Negative staining controls were by omitting the primary antibodies, by using irrelevant primary antibodies of the same isotype or by preabsorption of the primary antibody using an excess of the immunizing antigen. Sections were counterstained in hematoxylin when required. Immunohistochemistry results were analyzed by microscope and digital image analysis using a personal computer and appropriate image analysis software.

Wound experiments (III, V)

Young domestic pigs were anesthetized with intravenous ketamine hydrochloride, atropine sulfate and diazepam. Circular punch-biopsy wounds with a 6 mm diameter or incisional wounds were made on the backs of the pigs and the wounds were allowed to heal. The pigs were sacrificed with a lethal dose of intravenous pentobarbital and the wounds were collected. Alternatively, mice were anesthetized with subcutaneous ketamine hydrochloride and xylazine hydrochloride. Circular punch-biopsy wounds with a 5 mm diameter or incisional wounds were made on the backs or ears of the mice and the wounds were allowed to heal. The mice were sacrificed with carbon monoxide and cervical dislocation and the wounds were collected. Pain medication in the experiments was with either diclofenac for pigs or buprenorphine for mice. Infected wounds were discarded. Samples were snap-frozen in liquid nitrogen and embedded in Tissue-Tek or fixed in neutral formalin or 4% paraformaldehyde, dehydrated and embedded in paraffin for immunohistochemical

analysis. Permits for animal experiments were acquired from the Provincial State Office of Southern Finland or from the Helsinki University Central Hospital Ethics Committee.

Generation and analysis of transgenic mice by Western blotting (V)

The cDNA encoding full-length Bmx fused to a C-terminal hemagglutinin (HA) epitope was cloned into a human keratin-14 (K14) promoter expression cassette and injected into fertilized FVB/NIH mouse oocytes. The progeny were genotyped using PCR of tail DNA, Southern blotting of tail DNAs, and Bmx expression was verified by Northern blotting and by immunoprecipitation and Western blotting using anti-Bmx antibodies. For immunoprecipitation, frozen tissue samples were homogenized and lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF) and sodium vanadate. Immunoprecipitations were carried out from equal lysate aliquots by incubation with anti-Bmx abs, followed by incubation with protein-G-sepharose, elution into Laemmli sample buffer and separation in 7.5% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and detected by using anti-Bmx abs.

High-throughput gene expression array (V)

For high-throughput gene expression analysis, total RNA from skins of newborn mice was extracted as mentioned above, treated with DNAse I and labeled according to the manufacturer's protocol. Murine genome microarray gene chips were hybridized with the labeled RNAs according to the manufacturer's protocol and results were analyzed with a personal computer and software provided by the manufacturer.

RESULTS AND DISCUSSION

1. Chromosomal localization of VEGF-B and VEGF-C

VEGF-B and VEGF-C presented as possible candidate genes for mutations leading to vascular malformations or other diseases. Mutations in Tie-2, endoglin and activin are for example associated with venous malformations and hereditary hemorrhagic telangiectasia 1 (HHT1) and HHT2 [204,329-331]. Cutaneous and cerebral capillary-venous malformations are associated with the Krev1 interaction trapped (KRIT1) [332].

The chromosomal location of VEGF-B and VEGF-C was studied using several different overlapping techniques. Their relation to possible amplicons present in human cancer was also studied. PCR was used to amplify VEGF-B and VEGF-C from human rodent somatic cell hybrids containing defined sets of chromosomes. Chromosomal localization was also studied by using fluorescent *in situ* hybridization (FISH) of metaphase nuclei. VEGF-C was localized to 4q34, in the proximity of the aspartylglucosaminidase gene locus. VEGF-B localized to 11q13, near to the cyclin D1 locus. The 11q13 region containing the genes for cyclin D1 and EMS-1 is amplified in many human mammary and squamous cell carcinomas [333] but VEGF-B in our analysis was not found to be a part of this amplicon in the breast cancer cell lines analyzed.

2. Regulation of expression of VEGF, VEGF-B, VEGF-C and Ang-1

The mRNA regulation of VEGF-B and VEGF-C and angiopoietin-1 was studied in relation to the VEGF mRNA regulation. The effects of serum and different serum component growth factors such as PDGF, EGF and TGF-ß on the mRNA expression of VEGF, VEGF-B, VEGF-C and angiopoietin-1 were also studied. In a Northern blotting panel of tumor and normal cell lines, VEGF-B mRNA was present in all cell lines analyzed, VEGF mRNA was present in most and VEGF-C mRNA was present only in certain cell lines. Stimulation of serum-starved human fibroblast cells with serum, PDGF, EGF or TGF-ß induced the expression of VEGF and VEGF-C but not of VEGF-B. Angiopoietin-1 expression decreased upon serum stimulation or treatment with the growth factors. While VEGF has a short half-life, VEGF-B mRNA levels were stable

throughout the experiments and VEGF-C was found to have a half-life of 3.5 hours; this was increased in the presence of serum.

Hypoxia is known to induce the activation of the HIF complex and to subsequently increase VEGF protein through increased transcription but also through post-transcriptional mechanisms such as stabilization of the VEGF mRNA [334]. Previously it had also been shown that stimulation with the tumor promoter phorbol myristate 12,13-acetate (PMA) and expression of the Ras oncoprotein increases VEGF mRNA expression [335,336]. Additionally the mutant p53 tumor suppressor had been shown to increase VEGF expression *in vitro* [337]. Hypoxic treatment of rat glioma cells, human and mouse embryo fibroblasts or fibroblasts with a dominant-negative p53 tumor promoter had no effect on levels of VEGF-B or VEGF-C mRNA. VEGF was strongly induced in all these cell lines while Ang-1 mRNA expression was down-regulated by hypoxia. Treatment with the tumor promoter PMA increased the steady-state mRNA levels of VEGF and VEGF-C but not of VEGF-B while the expression of the Ras oncoprotein had no effect on mRNA levels of VEGF-B or VEGF-C.

This study showed that VEGF, VEGF-B and VEGF-C were differentially regulated, suggesting that they have distinct functions in normal tissues and possibly also in the tumor environment. Although the hypoxia-regulated VEGF is thought to be an important angiogenic driving force in the growth of certain solid tumors, the related VEGF-B or VEGF-C were not regulated by hypoxia. On the other hand, VEGF-C was stimulated by several of the growth factors indicating that it may have a role in processes such as angiogenesis, inflammation and tumors, where such factors are overexpressed.

VEGF-B was constitutively expressed in many tissues and most cell lines studied. The levels of VEGF-B did not respond to growth factor stimulation and the degradation of the VEGF-B mRNA was very slow. VEGF-B may be needed to regulate VEGFR-1 activation in concert with VEGF and PlGF.

3. VEGFR-3 in wound healing and chronic inflammation

VEGFR-3 is essential for cardiovascular development [138]. This receptor is expressed on all endothelia during early embryogenesis, whereas expression in the adult is restricted to lymphatic EC and certain fenestrated EC [22,138,180]. We studied the expression of VEGFR-3 during angiogenesis and lymphangiogenesis *in vivo* during wound healing granulation tissue development and chronic inflammation of chronic wounds. The hypothesis was that VEGFR-3 may be upregulated on blood vascular endothelium during inflammation and angiogenesis and VEGFR-3 may also provide a tool for studying lymphangiogenesis *in vivo*.

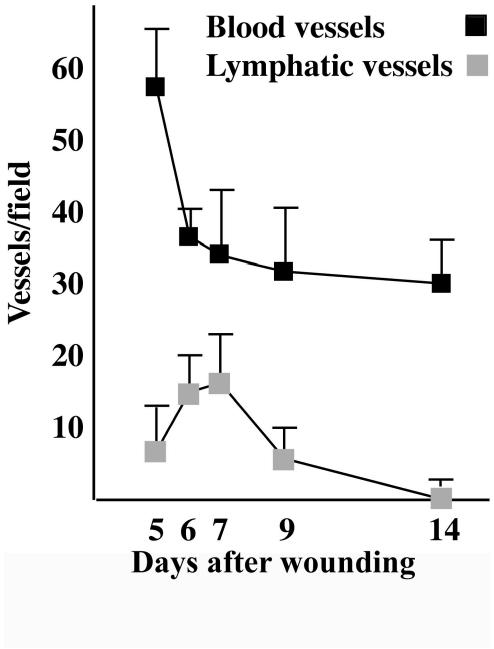

Figure 3. A graph depicting the growth of blood vascular and lymphatic vessels into wound granulation tissue during the first two weeks after wounding. See article for details.

VEGFR-3 positive lymphatic vessels, distinct from angiogenic blood vessels by immunohistochemical analysis, sprouted into the wound granulation tissue shortly after the blood vessels, on days 5-7 (Fig. 4). These vessels regressed completely thereafter (Fig. 3). Blood vessels preceded lymphatic vessels, reached a maximum density on day 5 and regressed afterwards.

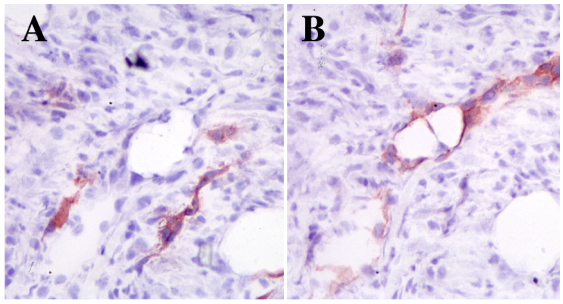

Figure 4. Figure showing concurrent angiogenesis (A, blood vessel staining for PAL-E) and lymphangiogenesis (B, lymphatic vessel staining for VEGFR-3) in wound granulation tissue. The vessels stain red. Background staining with hematoxylin (blue).

Few blood vessels were seen in later stages of granulation tissue maturation and scar formation. In human samples of various chronic wounds such as a decubitus wound, a diabetic ulcer and a leg ulcer few lymphatic vessels were seen. Surprisingly, blood vascular EC stained for VEGFR-3 in chronic wounds.

This study showed that lymphatic vessels develop concurrently with the blood vessels but with different kinetics during granulation tissue formation. In this study porcine skin was studied primarily because it is structurally close to human skin unlike for example murine skin. Lymphangiogenesis may be necessary for normal wound healing. The lag before the in-growth of lymphatic vessels into the granulation tissue may be due to the fact that the lymphangiogenic stimulus during wound healing is through VEGF-C secretion from pre-existing blood vessel structures and inflammatory cells that need to accumulate at the wound site. The kinetics of lymphatic vessel growth during the resolution of wound healing suggested that lymphatic vessels have only a transient role during normal wound healing. Strong transient wound lymphangiogenesis may be necessary for the resolution of inflammation i.e. evacuation of inflammatory cells, fluid, cellular debris and proteins. Lymphangiogenesis is closely associated with angiogenesis and these two processes may have similar regulatory pathways. The relative lack of lymphatic vessels in chronic wounds indicated also that the VEGF-C/VEGFR-3 interplay may be disturbed during the abnormal healing process of chronic wounds and that lack of lymphatic vessels during the healing phase may lead to impaired wound healing. Stimulation of lymphatic vessel growth may be of benefit in the treatment of chronic wounds. The upregulation of VEGFR-3 on blood vascular EC in chronic wounds may be due to inflammatory growth factors and cytokines.

Cytokines and growth factors of inflammation may also change the EC phenotype towards that of fenestrated EC and VEGFR-3 could be needed in maintaining fenestrations on blood vascular EC.

4. VEGF-C and VEGF-D and their receptors in rheumatoid arthritis

VEGF-C is upregulated by cytokines and growth factors involved in inflammation [338]. VEGF-C and VEGF-D and their receptor VEGFR-3 were therefore studied in arthritis and control synovial membranes. The hypothesis was that the lymphangiogenic growth factors are upregulated *in vivo* during the inflammation involved in arthritis. The expression of VEGF-C, VEGF-D, VEGFR-2 and VEGFR-3 was studied by RT-PCR and immunohistochemistry in synovial samples surgically removed from patients with osteoarthritis, ankylosing spondylitis, rheumatoid arthritis or from healthy joints sampled from surgery due to trauma.

VEGF-C staining was strong in the hypertrophic synovial lining of arthritic joints while VEGF-D staining was weak. By RT-PCR analysis, VEGF-C was found to be more abundant than VEGF-D in healthy and arthritic synovium. VEGF-C and VEGF-D were found to be expressed in SMC/PC of blood vessels. VEGFR-2 was present on blood vessel endothelium and VEGFR-3 was expressed in lymphatic EC and also in the fenestrated synovial blood vascular endothelium.

VEGF-C was strongly expressed in tissues obtained from arthritic joints. VEGF-C may have a role in the development and maintenance of the angiogenic inflammation seen in arthritis. The function of VEGF-C may also be different in different environments depending on the expression levels of VEGF-C and the localization of its receptors. VEGF-C may be more angiogenic than lymphangiogenic in the setting of chronic inflammation where VEGFR-3 is expressed also on fenestrated blood vascular EC. This would have to be considered when possible therapeutics targeting the VEGF-C/VEGFR-3 pathway are designed for example to treat arthritis. VEGF-D on the other hand was little expressed in RA, indicating that although VEGF-C and VEGF-D are structurally related proteins binding the same receptors, their biologic function is different *in vivo* at least in arthritis and possibly also in inflammation. This would also have implications on therapy aimed at affecting the growth and/or development of the lymphatic vasculature in different diseases.

Relatively few lymphatic vessels were seen in the healthy synovium and they appeared to decrease during the arthritic inflammation. The lack of lymphatic vessels may actually be contributing to the joint swelling, inflammation and accumulation of exudate in arthritis. The expression of VEGFR-3 on synovial blood vascular EC further underlined the possible role of VEGFR-3 in the maintenance of fenestrations of blood vascular EC.

5. The Bmx tyrosine kinase in cell proliferation, migration and angiogenesis *in vivo*

The Bmx tyrosine kinase is expressed primarily in arterial endothelia and the Bmx gene targeted mice have no obvious phenotype [246,247]. Bmx also increases the migratory potential of cancer cells *in vitro* according to several studies. The goal was to study Bmx in *in vivo* angiogenesis and in cellular migration in a mouse model.

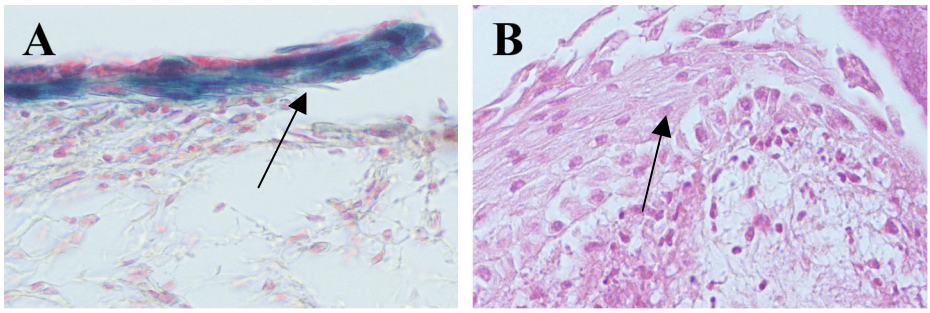

Figure 5. Bmx is expressed in the leading edge of the migrating epidermal cells of the healing wound. The epidermal edge is migrating left-to-right over the wound bed in the day 4 KO (A, arrow) and WT (B, arrow) mouse skin wound. Bmx expression is indicated by the blue color due to the ß-galactosidase reporter activity in the KO mouse whereas no staining is seen in the WT mouse. A nuclear red counterstaining was used.

In skin wounds of the Bmx gene targeted mice, Bmx was not expressed in angiogenic blood vessels. Bmx expression was upregulated in migrating epithelial keratinocytes during the re-epithelialization process as visualized by ß-galactosidase staining of skin wounds (Fig. 5). The significance of Bmx *in vivo* in epithelial cell migration was studied. Transgenic mice expressing Bmx under the control of the keratin 14 promoter in skin keratinocytes were generated. The mice had strong keratinocyte hyperproliferation in the skin indicating that overexpression of Bmx induces epidermal keratinocyte proliferation. *In vivo*, Bmx did not appear to affect keratinocyte apoptosis or differentiation. The mice also exhibited accelerated wound healing due to accelerated

epidermal migration during the wound healing. The mice also had dermal inflammation and strong dermal angiogenesis due to upregulation of many angiogenic and chemotactic/pro-inflammatory chemokines and cytokines in the skin (Fig 6). The gene expression changes were verified by high-throughput gene expression analysis.

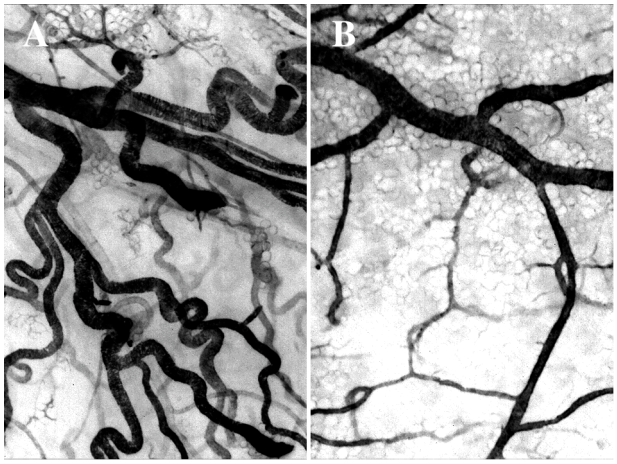

Figure 6. Large tortuous blood vessels in the skin of K14-Bmx TG mice (A) compared to vessels in WT littermate skin (B). Black and white inverted photo to illustrate the blood vessels in a whole mount staining for smooth muscle actin.

The study provided a proof of concept for studying intracellular tyrosine kinases in a simple transgenic model. Bmx has long been thought to be involved in neoplastic cell migration and proliferation on the basis of results from *in vitro* studies. The study provides functional *in vivo* data for the first time concerning the Bmx tyrosine kinase. Since marked Bmx expression was noted in the migrating epithelial edge of the healing wound, overexpressing Bmx in keratinocytes was a logical step in studying the biological function of Bmx. Bmx induces hyperproliferation of the epidermis when overexpressed in keratinocytes. Bmx overexpression also accelerates the keratinocyte migration observed in the re-epithelialization process. These studies thus support the previous *in vitro* observations.

It has previously also been shown that the Bmx TK is activated through angiogenic pathways involving ligand-binding to VEGFR-1 and Tie-2. It has also been reported that Bmx may be involved in TNF-mediated angiogenesis. In our experiments, Bmx overexpression in the TG mice appeared to induce the secretion of pro-inflammatory and angiogenic molecules from keratinocytes. No increased VEGF secretion was seen despite the tortuous dilated vessels

reminiscent of VEGF-induced angiogenesis in the skin of the K14-Bmx mice. Several angiogenic inflammatory cytokines were however induced very strongly. Although the strong angiogenic effects seen in the K14-Bmx TG mice may also be secondary due to inflammation it has been reported that Bmx is involved in TNF-mediated angiogenesis. Bmx is transactivated with VEGFR-2 independent of VEGF/VEGFR-2 signaling resulting in TNF-induced EC migration [253]. Our model indicates that Bmx may be involved in inflammatory angiogenesis and may present a therapeutic target in that setting.

The study also provided a model for future studies of Bmx in experimental epithelial carcinogenesis assays. Comparing tumor responses in the skin of KO and TG mice will provide important functional data on the role of the Bmx tyrosine kinase *in vivo* in tumor biology.

CONCLUDING REMARKS

Research in the field of angiogenesis and vascular biology has provided tools for therapeutic intervention by either inhibiting angiogenesis in diseases such as solid tumor growth or by stimulating angiogenesis in diseases such as ischemic heart disease. Research on VEGFs has provided basic knowledge on the complex nature of angiogenesis.

The first generation of anti-angiogenic agents are in clinical trials and pro-angiogenic therapy with angiogenic agents has provided promising results. Anti-angiogenic therapy is a promising therapeutic alternative or adjunct therapy in the treatment of angiogenic solid tumors. Whether this would apply to all human tumors is unclear. It is not well known how much active angiogenesis is involved in the survival and growth of some of the most typical neoplasias. Mammary adenocarcinoma for example may take over five years to grow from a single neoplastic cell into a macroscopic tumor. Anti-angiogenic therapy may be beneficial in advanced disease inducing the "dormancy" of metastases. On the other hand, the ongoing cancer trials often involve end-stage cancer patients with metastasized cancer that is highly resistant to other forms of therapy. Anti-angiogenic trials may need to be conducted in smaller populations of earlier stage tumors that have been selected on the basis of gene-expression analysis. The traditional clinical trial study design may also not be well suited for such purposes. Medical professionals must keep in mind that anti-angiogenic therapy may paradoxically make tumors less accessible to conventional cytostatic agents, more resistant to radiation therapy and anti-angiogenic therapy may also have serious side-effects. It is also not yet clear which sub-groups of cancer patients would benefit most from anti-angiogenic therapy with minimal side-effects and long-term clinical benefits. The many questions concerning anti-angiogenic and pro-angiogenic therapies will hopefully be answered in the years to come.

The discovery of the VEGFR-3 and other markers with specificity for lymphatic vascular endothelium has provided the scientific community with tools for studying the biochemical mechanisms of lymphangiogenesis and possibly also lymphatic metastasis of tumors. VEGF-C and VEGF-D have been shown to be lymphangiogenic *in vivo* and inhibiting the VEGF-C/-D-VEGFR-3 interaction has resulted in regression of lymphatic vessels from tumors in mice. Many solid tumors have been shown to secrete lymphangiogenic factors and this may help cancer cells to

metastasize to local lymph nodes and to distant locations [67,339]. Although intratumoral lymphatic vessels have been documented in certain cancers, generally it is thought that there are few clinically relevant lymphatic vessels inside tumors because of high tumor interstitial pressure [340]. Anti-lymphangiogenic therapy may be of benefit in cancer. Inhibiting tumor lymphangiogenesis may however favor angiogenic metastasis instead. It is not known whether angiogenic metastases are more aggressive than lymphangiogenic metastases and what differences exist between the two.

Previously it was thought that lymphatic metastasis is a passive event of tumor cells being shed and being washed into lymphatic vessels and lymph nodes. However, it has also been proposed that neoplastic cells metastasize to specific locations depending on the tumor cell characteristics and local factors, the so-called "seed and soil theory" [341]. Lymphangiogenic metastasis may actually be an active event with neoplastic cells binding to lymphatic endothelium via specific interactions and also surviving in the lymphatic circulation through active mechanisms [342,343]. One of the most important implications of studies on lymphangiogenesis in cancer are the specific interactions between malignant cells and EC. It is important to understand why only a tiny fraction of certain neoplastic cells are able to metastasize to regional lymph nodes and beyond.

Recent studies have also highlighted the role of tyrosine kinases and their deregulation in cancer and studies have provided specific targets on cancer cells for therapeutic intervention with for example tyrosine kinase inhibitors. The Bmx tyrosine kinase has been shown to be involved *in vitro* in many even opposing cell functions in cancer cells such as the regulation of cell survival, apoptosis, differentiation, migration and transformation. This study suggests that Bmx may be involved *in vivo* in the proliferation and migration of cells and in inflammatory angiogenesis. Future studies must address in detail the role of Bmx in such scenarios.

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This work is dedicated to my wife, Aino, the joy of my life now and for evermore.

No worries,

Helsinki
December, 2003

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