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Vascular endothelial growth factor (VEGF) signaling in tumor progression

Robert Roskoski Jr. *

Blue Ridge Institute for Medical Research, 3754 Brevard Road, Suite 116A, Box 19, Horse Shoe, NC 28742, USA

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* Tel.: +1 828 891 5637; fax: +1 828 890 8130.
E-mail address: rrj@brimr.org.

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Abstract

Vascular endothelial cells are ordinarily quiescent in adult humans and divide less than once per decade. When tumors reach a size of about 0.2–2.0 mm in diameter, they become hypoxic and limited in size in the absence of angiogenesis. There are about 30 endogenous pro-angiogenic factors and about 30 endogenous anti-angiogenic factors. In order to increase in size, tumors undergo an angiogenic switch where the action of pro-angiogenic factors predominates, resulting in angiogenesis and tumor progression. One mechanism for driving angiogenesis results from the increased production of vascular endothelial growth factor (VEGF) following up-regulation of the hypoxia-inducible transcription factor. The human VEGF family consists of VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). The VEGF family of receptors consists of three protein-tyrosine kinases and two non-protein kinase receptors (neuropilin-1 and -2). Owing to the importance of angiogenesis in tumor progression, inhibition of VEGF signaling represents an attractive cancer treatment.

Keywords: Angiogenesis; Hypoxia; Neuropilin; Proteolysis; Receptor protein-tyrosine kinase; Vasculogenesis

1. Vasculogenesis and angiogenesis

1.1. Definitions

The intricately branched circulatory network of vascular endothelial and supporting cells is essential for transporting oxygen, nutrients, and signaling molecules to and the removal of carbon dioxide and metabolic end products from cells, tissues, and organs [1]. Neovascularization, or new blood vessel formation, is divided into two components: vasculogenesis and angiogenesis. Embryonic or classical vasculogenesis is the process of new blood vessel formation from hemangioblasts that differentiate into blood cells and mature endothelial cells [2]. In the embryo and yolk sac, early blood vessels develop by aggregation of angioblasts into a primitive network of simple endothelial tubes [3]. As primitive vessels are remodeled into a functioning circulatory system, they undergo localized proliferation and regression, as well as branching and migration. In contrast, angiogenesis is the process of new blood vessel formation from pre-existing vascular networks by capillary sprouting. During this process, mature endothelial cells divide and are incorporated into new capillaries. Vascular endothelial growth factor (VEGF) signaling is required for the full execution of vasculogenesis and angiogenesis.

Many observations associated with tissue ischemia and tumor formation are consistent with the concept that vasculogenesis also occurs during postnatal vessel development [4]. Asahara et al. were the first to describe the existence of endothelial progenitor cells in adult human blood that can differentiate into endothelial cells [5]. These progenitor cells normally reside in the bone marrow but may become mobilized into the circulation by cytokine or angiogenic growth factor signals [6]. During adult vasculogenesis, mobilized progenitor cells promote vessel formation by integrating into vessels and by supplying growth factors. Bone-marrow-derived endothelial progenitor cells may be recruited to sites of infarction, ischemia, or tissue trauma where they differentiate into mature endothelial cells and combine with other cells to form new vessels. These findings suggest that vasculogenesis and angiogenesis might constitute complementary mechanisms for postnatal neovascularization. Not all studies, however, support the concept of adult vasculogenesis [7], and

additional work will be required to sort out the inconsistencies.

1.2. Physiological and non-physiological angiogenesis

Adult human vascular endothelial cells constitute an estimated 1 kg of tissue and line the vessels of every organ [1]. These endothelial cells correspond to an estimated surface area of 1000 m^2, about the size of a tennis court [8]. In adult humans, most endothelial cells are quiescent; only 1 in every 10,000 endothelial cells is in the cell division cycle at any one time [9]. However, there is an increased rate of endothelial cell mitosis and angiogenesis during wound healing and tissue repair, during ovarian corpus luteum formation, and during placental development establishing pregnancy [10]. Inhibition of angiogenesis represents a potential therapy for disorders with non-physiological angiogenesis including neovascular age-related macular degeneration of the eye, diabetic retinopathy, endometriosis, psoriasis, rheumatoid arthritis, and tumor growth and metastasis [10]. Deciphering the mechanisms of developmental, physiological, and aberrant angiogenesis has assumed considerable biomedical importance during the past 35 years.

1.3. Activators and inhibitors of angiogenesis

Angiogenesis, which is regulated by both endogenous activators and inhibitors, is under stringent control [9]. There are about 30 known endogenous pro-angiogenic factors, several of which are listed in Table 1. Three families of receptor protein-tyrosine kinases play pivotal roles in vasculogenesis and angiogenesis. The VEGF/VEGFR (vascular endothelial growth factor/VEGF receptor) family is the most studied regulator of vascular development, and it is the central focus of this review. The angiopoietin/Tie system controls vessel maturation and quiescence [11] while the eph/Ephrin system controls positional guidance cues and arterio-venous asymmetry [12]. Acidic and basic fibroblast growth factors also play important and well-studied roles in angiogenesis [13].

There are about 30 endogenous anti-angiogenic factors; several of these are listed in Table 2. The most studied negative regulators include angiostatin [15], endostatin [16], and thrombospondin [17]. Under most physiological conditions in mature animals, the action of negative regulators predominates and angiogenesis is quiescent. Under certain pathological conditions, for example, during tumor progression, the vasculature undergoes the so-called angiogenic switch, the action of positive regulators predominates, and angiogenesis is active [9]. In the context of this review, tumor progression represents the process of tumor growth occurring in conjunction with new blood vessel formation.

Table 1
Selected endogenous pro-angiogenic factors

Factor	MW (kDa)[a]	Swiss prot accession no.
Acidic fibroblast growth factor (aFGF, FGF1)[b]	17.5	P05230
Angiogenin[b]	16.6	P03950
Angiopoietin-1	57.5	Q15389
Angiopoietin-2	56.9	O15123
Basic fibroblast growth factor (bFGF, FGF2)[b]	17.3	P09038
Ephrin-A1	23.8	P20827
Ephrin-B1	38.0	P98172
Ephrin-B2	36.9	P52799
Epidermal growth factor (EGF)[b]	134	P01133
Granulocyte colony-stimulating factor (GCSF)	16.3	P09919
Macrophage-granulocyte colony-stimulating factor (GM-CSF)	16.3	P04141
Hepatic growth factor (HGF, scatter factor)[b]	83.1	P14210
Interleukin-8 (Il-8, CXCL8)[b]	11.1	P10145
Leptin	18.6	P41159
Placental growth factor (PlGF)[b]	24.8	P49763
Platelet-derived endothelial growth factor (PD-EGF)[b]	50.0	P19971
Platelet-derived growth factor-A (PDGF-A)[b]	24.0	P04085
Platelet-derived growth factor-B (PDGF-B)[b]	27.3	P01127
Transforming growth factor-α (TGF-α)[b]	17.0	P01135
Transforming growth factor-β (TGF-β)[b]	44.3	P01137
Tumor necrosis factor (TNF-α)[b]	25.6	P01375
Vascular endothelial growth factor (VEGF-A)[b]	27.0	P15692
VEGF-B[b]	21.6	P49765
VEGF-C[b]	46.9	P49767
VEGF-D[b]	40.4	O43915

[a] Molecular weight (MW) corresponding to the unprocessed human precursor.
[b] Commonly found in human tumors.

1.4. Sprouting and non-sprouting angiogenesis

Angiogenesis in tumors and elsewhere is an intricate process that involves interactions between regulatory and effector molecules. Pepper divided classical angiogenesis into a phase of sprouting and a phase of resolution [18]. The phase of sprouting consists of six components: (i) increased vascular permeability and extravascular fibrin deposition, (ii) vessel wall disassembly, (iii) basement membrane degradation, (iv) cell migration and extracellular matrix invasion, (v) endothelial cell proliferation, and (vi) capillary lumen formation. The phase of resolution consists of five components: (i) inhibition of endothelial cell proliferation, (ii) cessation of cell migration, (iii) basement membrane reconstitution, (iv) junctional complex maturation, and (v) vessel wall assembly including recruitment and differentiation of smooth muscle cells and pericytes, both of which are mural cells (mural, wall).

Table 2
Selected endogenous anti-angiogenic factors[a]

Inhibitor	Description	MW (kDa)[b]	Swiss prot accession no.
(A) Derived from the extracellular matrix			
Anastellin	Fragment of fibronectin	263	P02751
Arresten	Fragment of type IV collagen α1 chain	161	P02462
Canstatin	Fragment of type IV collagen α2 chain	168	P08572
Chondromodulin-1	Secreted cartilage glycoprotein	37.1	O75829
EFC-XV	Endostatin-like fragment from type XV collagen	142	P39059
Endorepellin	Fragment of perlecan, a basement membrane-specific heparan-sulfate-proteoglycan core protein	469	P98160
Endostatin	Fragment of collagen type XVIII (residues 1334–1516)	154	P39060
Fibulin fragments	Fibulins 1–5 are secreted extracellular matrix and basement membrane proteins	≈77	P23142, P98095, Q12805, O95967, Q9UBX5
Thrombospondin-1 and -2	Extracellular matrix glycoproteins that are proteolyzed to produce anti-angiogenic proteins; Tsp-1 was the first recognized naturally occurring angiogenesis inhibitor	129	P07996, P35442
Tumstatin	Fragment of type IV collagen α3 chain	162	Q01955
(B) Non-matrix derived factors			
Angiostatin	Fragment of plasminogen (residues 98–465)	90.6	P00747
Antithrombin III (cleaved)	Fragment of antithrombin III	52.6	P01008
Hemopexin-like domain (PEX)	Fragment of MMP-2	73.9	P08253
Interferon-α, -β, -γ	Cytokines	≈22	P01574, P01574, P01579
Interleukin-1, -4, -12, -18	Cytokines	≈17	P01584, P05112, P29459, Q14116
2-Methoxyestradiol	Endogenous estrogen metabolite		
Pigment epithelium-derived factor (PEDF)	Growth factor	46.3	P36955
Plasminogen kringle-5	Fragment of angiostatin/plasminogen	90.6	P00747
Platelet factor-4	Released by platelets	10.8	P02776
Prolactin fragments	8- and 16-kDa fragments of prolactin	25.9	P01236
Prothrombin kringle-2	Fragment of prothrombin	70.0	P00734
Semaphorin-3F	VEGF family antagonist	88.4	Q13275
Soluble VEGFR1	Fragment of VEGFR1	151	P17948
TIMP-2	Tissue inhibitor of metalloprotease-2	24.4	P16035
Troponin I	Inhibitory subunit of muscle troponin	21.2	P48788
TrpRS	Fragment of tryptophanyl-tRNA synthetase	53.2	P23381
Vasostatin	Fragment of calreticulin	48.1	P27797

[a] Adapted from ref. [14].
[b] Molecular weight (MW) corresponding to the unprocessed human precursor.

Besides classical angiogenesis, various forms of non-sprouting angiogenesis have been described in tumors [19]. These include intussusceptive vascular growth, co-option, formation of mosaic vessels, and vasculogenic mimicry. During intussusceptive vascular growth, a column of interstitial cells is inserted into the lumen of a pre-existing vessel, thereby dividing the lumen and yielding two vessels [20]. The column is invaded by fibroblasts and pericytes and accumulates extracellular matrix proteins. This process does not require the immediate proliferation of endothelial cells but rather the rearrangement and remodeling of existing ones. The advantage of this mechanism of growth over sprouting is that blood vessels are generated in a metabolically economic process because extensive cell proliferation, basement membrane degradation, and invasion of the surrounding tissue are not required. By yet another mechanism, developing tumors can surround vessels in the tissue or organ of origin and incorporate, or co-opt, these vessels [21]. Co-option may be important when tumors arise in or metastasize to vascular organs such as the lung or brain.

Tumor cells, along with endothelial cells, may together form the luminal surface of capillaries thus generating a mosaic vessel [22]. Chang et al. found that about 15% of vessels in human colon carcinoma implants (xenografts) in athymic hairless, or nude, mice and in biopsies of human colon carcinomas were mosaic channels lined with both endothelial and tumor cells [22].

In vasculogenic mimicry, first described in ocular melanoma, vascular channels develop that are extracellular-matrix-rich tubular networks [23]. These tubular networks or channels lack endothelial cells but contain circulating red blood cells. Vasculogenic mimicry has been described in breast, lung, ovarian, and prostate carcinoma and in rhabdomyosarcoma [24]. However, Auguste et al. point out that some investigators disagree with the concept of vasculogenic mimicry [19].

Table 3
VEGF receptor ligands and VEGF family isoforms that bind to heparan sulfate proteoglycans[a]

VEGFR1	VEGFR2	VEGFR3	Neuropilin-1	Neuropilin-2	Syndecan[b]
VEGF	VEGF (110–165)[c]	Pro- and mature VEGF-C	VEGF-165	VEGF (145–165)[c]	VEGF (145–206)[c]
VEGF-B	VEGF-C	Pro- and mature VEGF-D	PlGF-152	PlGF-152	PlGF-152, PlGF-224
PlGF	VEGF-D		Pro-VEGF-C[d]	Pro- and mature VEGF-C[d]	VEGF-B167
	VEGF-E		Pro-VEGF-D[d]	Pro-VEGF-D[d]	
			Semaphorin-3C	Semaphorin-3C	
			Semaphorin-3F	Semaphorin-3F	
			Semaphorin-3A		
			VEGF-B		
			VEGF-E		

[a] From ref. [30] unless otherwise noted.
[b] One form of heparan sulfate proteoglycan.
[c] Isoforms within the range of the specified number of amino acids.
[d] From ref. [31].

Sprouting angiogenesis, which is the most studied mechanism of new blood vessel formation, occurs under physiological and non-physiological conditions as does intussusceptive vascular growth [19]. It is uncertain whether co-option, mosaic vessels, or vasculogenic mimicry play a role in physiological vasculogenesis or angiogenesis. The nature of the factors that determine which combinations of these angiogenic processes occur during tumor progression is unknown.

1.5. Tumor vessel morphology

Tumor vessels exhibit abnormal morphology while normal vessels are organized in a hierarchical fashion with arterioles, capillaries, and venules that are readily distinguishable [2,25]. Pioneering work by Algire and Chalkley using tumors grown in a transparent chamber in rats in vivo demonstrated that capillaries in rapidly growing tumors are about five times the diameter of those in normal tissue [26]. These capillaries rarely differentiated into arterioles or venules. Moreover, three-dimensional microscopy of tumor vascular casts revealed an absence of normal arteriole, capillary, and venule structure with arteriolar–venular shunts, frequent blind endings, and incomplete and abnormal endothelial cell lining [27]. Tumor vessels often develop into disorganized bundles containing numerous vascular sprouts while exhibiting irregular vessel lumen diameters [28]. Owing to the abnormal organization and structure of tumor vessels, blood flow in tumors is chaotic [27].

2. The vascular endothelial growth factor (VEGF) family

The VEGF family plays an integral role in angiogenesis, lymphangiogenesis, and vasculogenesis. The human VEGF family consists of five members: VEGF (or VEGF-A), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) [10,29]. Each of these proteins contains a signal sequence that is cleaved during biosynthesis. Moreover, alternative splicing of their corresponding pre-mRNAs generates multiple isoforms of VEGF, VEGF-B, and PlGF. There are three receptor protein-tyrosine kinases for the VEGF family of ligands (VEGFR1, VEGFR2, and VEGFR3) and two non-enzymatic receptors (neuropilin-1 and -2). Moreover, several of the VEGF family ligands bind to heparan sulfate proteoglycans that are found on the plasma membrane and in the extracellular matrix. See Table 3 for a list of the VEGF receptor ligands and the VEGF isoforms that bind to the extracellular matrix heparan sulfate proteoglycans.

VEGF binding sites were identified on vascular endothelial cells [32,33] corresponding to VEGFR1 (Flt-1) [34] and VEGFR2 (Flk-1/KDR) [35–37]. This distribution on endothelial cells accounts for the selectivity and specificity of action of VEGF. VEGFR3 (Flt-4) [38], which is in the same receptor family, binds VEGF-C and VEGF-D. Each of these receptors is a type V (five) protein-tyrosine kinase that consists of an extracellular component containing seven immunoglobulin-like domains, a single transmembrane segment, a juxtamembrane segment, an intracellular protein-tyrosine kinase domain that contains a kinase insert of 70–100 amino acid residues, and a carboxyterminal tail (see ref. [39] for a description of types I–IX receptor protein-tyrosine kinases). The three VEGF receptors are related to the platelet-derived growth factor receptors (α and β), the fibroblast growth factor receptors (1–4), the stem cell factor receptor (Kit), the Flt ligand receptor (Flt-3), and the colony-stimulating factor-1 receptor (CSF-1R), all of which contain extracellular immunoglobulin domains and a kinase insert [39,40].

3. Properties and expression of the VEGF family

3.1. VEGF-A

The discovery of VEGF (VEGF-A) represents the convergence of work by several groups beginning in the 1980s. In 1983, Senger et al. isolated and partially purified a protein from ascites fluid and from conditioned medium induced by

a guinea pig hepatocellular carcinoma, which was assayed by its ability to induce vascular permeability [41]. In 1989, Ferrara and Henzel purified a protein from media conditioned by bovine pituitary folliculostellate cells, which was assayed by its vascular endothelial cell mitogenic activity [42]. Its amino-terminal sequence was Ala-Pro-Met-Ala-Glu. Gospodarowicz et al. also isolated this factor, which was assayed by its vascular endothelial cell mitogenic activity, and found the same N-terminal sequence [43].

Connolly et al. purified vascular permeability factor (VPF) from medium conditioned by a guinea pig hepatocellular carcinoma, which was assayed by its permeability enhancing activity, and showed that this factor unexpectedly stimulated vascular endothelial cell proliferation [44]. Its amino-terminal amino acid sequence corresponded to that reported by Ferrara and Henzel [42] and Gospodarowicz et al. [43]. Connelly et al. prepared an antibody directed toward the amino-terminal 21 amino acids of VPF and showed that this antibody blocked both: (i) vascular permeability and (ii) vascular endothelial cell mitogenic activities thereby providing strong evidence that a single entity possesses both activities, a surprising result at the time. Moreover, they showed that ^{131}I-VEGF/VPF binds to vascular endothelial cells with high affinity, and the factor can be chemically cross-linked to a high-molecular weight cell-surface receptor. The factor was specific for enhancing vascular endothelial cell mitogenesis and failed to stimulate the proliferation of bovine smooth muscle cells, human and mouse fibroblasts, bovine chondrocytes, human lymphocytes, or mouse myelomonocytes.

Senger et al. [45] showed that the protein isolated from hepatocellular-carcinoma-conditioned medium has the amino-terminal sequence that corresponds to that described by Ferrara and Henzel [42], Gospodarowicz et al. [43], and Connelly et al. [44]. Moreover, Plouët et al. isolated and characterized a vascular endothelial cell mitogen produced by rat pituitary AtT-20 cells, and they found that its amino-terminal sequence was Ala-Pro-Thr-Thr-Glu [46], which is reminiscent of the sequence reported by the other investigators. All of these groups used heparin-Sepharose chromatography as part of their purification scheme indicating that the chief isoforms produced by these various sources bind to heparin, a negatively charged molecule. Furthermore, Levy et al. isolated an endothelial cell growth factor from medium conditioned by the mouse neuroblastoma NB41 cell line [47]. They demonstrated that this factor, with an amino terminal sequence of Ala-Pro-Thr-Thr-Glu, stimulated human umbilical vein endothelial cell (HUVEC) mitogenesis but not that of fibroblasts. These workers used concanavolin A-Sepharose, a ligand for glycoproteins, in their purification scheme.

Ferrara and Henzel, Gospodarowicz et al., Plouët et al., and Levy et al. reported that the molecular weight of VEGF determined by denaturing gel electrophoresis under non-reducing conditions was about 46 kDa and that under reducing conditions was about 23 kDa [42,43,46,47]. Connelly et al. and Senger et al. reported that the molecular weight under non-reducing conditions ranged from about 34 to 42 kDa and that under reducing conditions was about 17–24 kDa [44,45]. The range of molecular weights may be due to partial proteolysis, different degrees of N-glycosylation, or to the production of isoforms related to alternative splicing of pre-mRNAs. However, all of these groups proposed that VEGF/VPF is a disulfide-linked homodimer based upon the molecular weight differences observed under reducing and non-reducing conditions and the occurrence of a single N-terminal amino acid sequence.

Leung et al. reported the complete sequences of human and bovine VEGF deduced from cDNAs isolated from human HL60 leukemia cells and bovine folliculostellate cells, respectively [48]. Keck et al. independently reported the sequence of this protein based upon a cDNA analysis of a library derived from human histiocytic lymphoma cells (U937) [49]. The deduced amino acid sequence for bovine VEGF corresponds to the reported amino-terminal sequence [42,43]. Moreover, Conn et al. determined the cDNA structure of rat VEGF [50], and the deduced amino-terminal amino acid sequence corresponded to that reported by Plouët et al. [46]. These independent analyses converged and demonstrated that the molecules with vascular endothelial mitogenic activity (VEGF) and that which enhances vascular permeability (VPF) are the same.

VEGF is a mitogen and survival factor for vascular endothelial cells [42,51,52] while also promoting vascular endothelial cell and monocyte motility [53–55]. Moreover, VEGF selectively and reversibly permeabilizes the endothelium to plasma and plasma proteins without leading to injury [2,45]. All of these properties are required for angiogenesis.

VEGF, which contains an N-linkage glycosylation site, consists of nine isoforms that result from alternative splicing of pre-mRNA transcribed from a single gene containing eight exons [29,56,57]. VEGF mRNA and protein are expressed in many tissues and organs [58–61]. Berse et al. reported that the highest level of VEGF mRNA in adult guinea pigs occurs in the lung, a very vascular organ [58]. They reported that guinea pig adrenal, heart, and kidney also express high levels of VEGF mRNA while gastric mucosa, liver, and spleen express lower levels of these transcripts. Moreover, VEGF mRNA and protein are expressed in a wide variety of human malignancies including those of breast, colorectal, non-small cell lung, and prostate carcinomas [29]. As described later, VEGF represents an important anti-cancer target.

The largest human precursor protein contains 232 amino acids. Removal of the signal sequence of 26 residues yields a mature protein, VEGF-206, which contains 206 amino acids (Fig. 1). VEGF-165 is the predominant isoform followed by the 189 and 121 residue molecules as determined by cDNA analysis of a variety of cell types, tissues, and tumor specimens. The other isoforms, which represent minor species in vivo, include VEGF-183, -165b (an inhibitory isoform), -162, -148, and -145. See refs. [29,30,57] for a description of the pre-mRNA alternative splicing that generates each of the isoforms of VEGF. Mouse VEGF isoforms are one residue shorter than the human proteins owing to the deletion of a

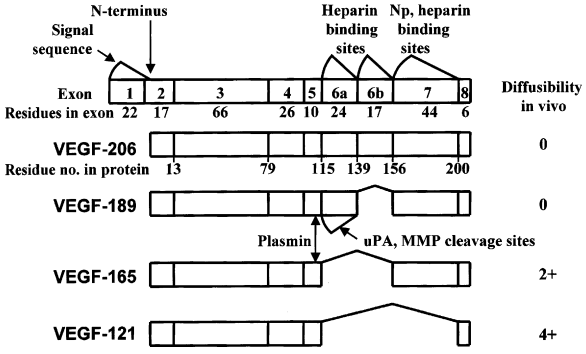

Fig. 1. Major splice variants and functional components of human VEGF. The pre-protein is shown in the upper diagram, and the processed proteins are shown below. The mouse isoforms are one residue shorter owing to a glycine deletion in exon 2. MMP, Matrix metalloprotease; Np, neuropilin; uPA, urokinase type of plasminogen activator.

glycine residue at position 8 in the mature protein or residue 34 in the preprotein.

Although, many first messengers including cytokines and growth factors participate in angiogenesis and vasculogenesis, the VEGF family is of paramount importance. *VEGF* null mice, which die at embryonic Day 8.5, exhibit impaired vasculogenesis and blood-island formation indicating that VEGF participates in the earliest stages of embryonic vasculogenesis [62,63]. Moreover, loss of a single *VEGF* allele in mice leads to vascular deformities and embryonic death between Days 11 and 12. These investigators reported that the formation of blood vessels was abnormal, but not abolished, in heterozygous VEGF-deficient ($VEGF^{+/-}$) embryos. This heterozygous lethal phenotype, which differs from the homozygous lethality in VEGF or VEGF-receptor-deficient embryos, is indicative of an exactingly important dose-dependent regulation of embryonic vessel development by VEGF.

Muller et al. determined the X-ray crystallographic structure of human$VEGF_{8–109}$ and found that it forms an antiparallel homodimer covalently linked by two disulfide bridges between Cys51 and Cys60 [64]. The dominant feature within each monomer is a cystine knot motif that consists of an eight-residue ring formed by the disulfide bridges Cys57–Cys102 and Cys61–Cys104 with a third disulfide bond (Cys26–Cys68) passing through the ring forming a knot.

3.2. VEGF-B

VEGF-B consists of two isoforms that result from alternative splicing of pre-mRNA synthesized from a single gene containing seven exons [65]. After removal of the 21-residue signal peptide, VEGF-B167 (containing 167 amino acids) and VEGF-B186 (containing 186 amino acids) are produced. VEGF-B186 is secreted from cells and is freely diffusible. VEGF-B167, which is the predominant isoform, binds to heparan sulfate proteoglycan and is not freely diffusible in vivo. Both VEGF-B isoforms exist as disulfide-linked homodimers. The tertiary structure of VEGF-B is nearly superimposible with that of VEGF [66]. The ability of VEGF to bind and the inability of VEGF-B to bind to VEGFR2 may be due to subtle structural differences in the β5–β6 loop.

On embryonic Day 14 in mice, a high level of VEGF-B mRNA occurs in the heart and the central nervous system. In adult mice, the highest levels of VEGF-B transcripts occur in brain, heart, kidney, and testes while lower levels are found in liver, lung, and spleen [67]. VEGF-B mRNA is expressed in several human neoplasms including benign thymoma, breast carcinoma, fibrosarcoma, non-Hodgkins lymphoma, and primary and metastatic melanoma [68].

The function of VEGF-B is unclear [69]. *VEGF-B* null mice are viable but exhibit abnormal cardiac conduction [70]. VEGF-B seems to be required for normal heart function in adults but is not required for cardiovascular development or for angiogenesis. However, its expression in human tumors and its ability to activate VEGFR1 and neuropilin-1 indicate that VEGF-B represents a potential anti-cancer target.

3.3. VEGF-C

VEGF-C is synthesized as a prepro-protein that undergoes intricate proteolytic processing to generate the mature form of the growth factor [71,72]. A signal sequence of 31 amino-terminal residues is removed from the 419 amino-acid-residue prepro-protein to yield a pro-protein of 388 residues. Two VEGF-C precursors form an antiparallel homodimer linked by disulfide bonds extending from each of the two C-terminal to N-terminal propeptides. Before secretion, this dimer undergoes proteolysis after dibasic residues (Arg-227, Arg-228) by the Steiner mechanism [73]. This processing may involve subtilisin-like proprotein convertases PC5, PC7, or furin [74]. Each of the C-terminal 228–419 residues remain attached to the opposite N-terminus. The last step of processing, which occurs extracellularly by an ill-defined mechanism, removes residues 32–111 yielding mature VEGF-C. Although, the mature dimeric VEGF-C contains the eight homologous cysteine residues that occur in VEGF and VEGF-B, VEGF-C exists as a homodimer lacking inter-subunit disulfide bonds.

The *VEGF-C* gene contains seven exons [75]. Human adult heart, ovary, placenta, skeletal muscle, and small intestine contain high levels of VEGF-C mRNA [71]. Several other adult tissues produce modest amounts of VEGF-C transcripts including kidney, lung, pancreas, prostate, spleen, and testes. Moreover, mesenchymal cells of mouse embryos express VEGF-C transcripts, particularly in the regions where the lymphatic vessels undergo sprouting from embryonic veins, including the axillary, jugular, and urogenital regions and the developing mesenterium [76]. An unprocessed form of VEGF-C binds to VEGFR3, which is an important participant in lymphogenesis [72]. The fully processed form of VEGF-C binds to both VEGFR2 and VEGFR3 (Table 3). VEGF-C participates in lymphangiogenesis during embryogenesis and in the maintenance of differentiated lymphatic

endothelium in adults [77]. Moreover, VEGF-C is expressed by a significant fraction of human tumors including those of breast, cervix, colon, lung, prostate [74,78], and stomach [79]. Thus, VEGF-C represents a potential anti-cancer target.

About half of *VEGF-C* null mice die between embryonic Days 15.5 and 17.5 and none survive gestation, indicating the essential nature of this factor [80]. Lymphatic vascular development is also defective in *VEGF-C*$^{+/-}$ mice, which exhibit lymphedema. VEGF-C is not needed for cell commitment to the lymphatic endothelial lineage. However, VEGF-C signaling is required for the migration and survival of lymphatic endothelial cells and for the formation of lymph sacs. Although VEGFR2 and VEGFR3, which bind VEGF-C, are essential for blood vessel development, blood vessels develop normally in *VEGF-C* null mice. VEGF-C is thus indispensable for embryonic lymphangiogenesis [80].

3.4. VEGF-D

Like VEGF-C, VEGF-D is synthesized as a prepro-protein that undergoes intricate proteolytic processing to generate the mature form of the growth factor [81]. The precursor for VEGF-D contains amino- and carboxy-terminal extensions that are cleaved to yield the mature product as described for VEGF-C. Mature VEGF-D is a non-covalent homodimer. Although an unprocessed form of VEGF-D binds to VEGFR3, which is important in lymphangiogenesis, the fully processed form binds to both VEGFR2 and VEGFR3 [31,81].

The *VEGF-D* gene contains seven exons and is found on the X chromosome [82,83]. In contrast to *VEGF-C* null mice, *VEGF-D* null mice are viable and exhibit normal lymphangiogenesis during development and normal lymphatics in mature animals [84]. It is clear that VEGF-C and perhaps other factors can substitute for VEGF-D.

Adult colon, heart, lung, skeletal muscle, and small intestine contain high levels of VEGF-D transcripts while ovary, pancreas, prostate, spleen, and testes contain low levels [85]. VEGF-D is up-regulated in breast [86], colorectal [87], gastric [79], and thyroid [88] carcinomas, cervical intraepithelial neoplasia [89], glioblastoma [90], and melanoma [91]. Its expression correlates with lymph node metastasis in colorectal [92], lung [93], and ovarian carcinomas [94]. VEGF-D signaling thus represents a potential anti-cancer and anti-metastasis target.

3.5. Placental growth factor (PlGF)

Placental growth factor is a homodimeric glycoprotein that shares 42% amino acid sequence identity with VEGF [95]. PlGF possesses the VEGF family core of eight cysteine residues that participate in inter- and intra-subunit disulfide bond formation as described for VEGF. The tertiary structure of PlGF is similar to that of VEGF [96]. The *PlGF* gene contains seven exons and expresses four isoforms (PlGF-131, -152, -203, and -224) based upon alternative pre-mRNA splicing [97–99]. PlGF-152 and PlGF-224, which contain basic residues, bind to negatively charged heparan sulfate proteoglycans. PlGF isoform transcripts occur primarily in placenta. However, breast [100], gastric [101], prostate [102], and non-small cell lung cancer cells [103], and normal heart [104], skeletal muscle [105], retina [106], and skin [107,108] express various isoforms of PlGF. *PlGF* null mice are viable and fertile, but they exhibit diminished vascularization of the retina and the corpus luteum [109]. PlGF enhances VEGF signaling, and PlGF expression may obviate anti-VEGF based therapy [109].

3.6. VEGF-E

VEGF-E, a non-human factor, is encoded by the Orf parapoxvirus [110]. VEGF-E stimulates chemotaxis, proliferation, and sprouting of cultured vascular endothelial cells and angiogenesis in vivo. VEGF-E binds with high affinity to VEGFR2 but fails to bind to VEGFR1 (Table 3). This factor supports the angiogenesis associated with parapoxvirus-infected lesions. VEGF-E has vascular permeability activity similar to that of VEGF [110].

4. VEGF receptors

4.1. VEGFR1 (Flt-1)

VEGFR1 (Flt-1, fms-like tyrosyl kinase-1, where fms refers to feline McDonough sarcoma virus) binds to VEGF, PlGF, and VEGF-B (Table 3) [53,111,112]. VEGFR1, which has a molecular weight of about 210 kDa, has variable functions that depend upon the developmental stage and the location of the endothelial cells that produce the receptor [113]. Peters et al. used in situ hybridization to show that, in adult mouse, VEGFR1 is expressed in endothelial cells [114]. Moreover, VEGFR1 is expressed in populations of embryonic cells from which endothelium is derived including early yolk sac mesenchyme.

VEGFR1 has higher affinity for VEGF than VEGFR2 (≈10 pM versus 75–750 pM) [53,113–115]. In contrast to VEGFR2, VEGFR1 has weak tyrosine kinase phosphorylation activity following stimulation by VEGF [53]. Activation of VEGFR1 has no direct proliferative or cytoskeletal effects [53]. However, activation of VEGFR1 is implicated in the increased expression of urokinase type of plasminogen activator and plasminogen activator inhibitor-1 in endothelial cells [112]. As noted later, these molecules play a role in extracellular matrix degradation and cell migration. Moreover, VEGFR1 plays a role in monocyte chemotaxis [55].

The human *VEGFR1* gene, which contains 30 exons, is located at chromosome 13q12. Alternative splicing of VEGFR1 pre-mRNA produces a soluble receptor isoform (sVEGFR1) that can bind to and inhibit the action of VEGF [116]. After the signal peptide is cleaved, sVEGFR1 contains 661 amino acids corresponding to the first six of seven extracellular immunoglobulin domains. Excessive sVEGFR1 that

is generated by human placenta and released into the circulation of the mother leads to the hypertension and proteinuria of preeclampsia [117,118]. Park et al. found that PlGF binds to HUVEC samples, which express both VEGFR1 and VEGFR2, and displaces only a fraction of bound ^{125}I-VEGF-165 [111]. This result is consistent with the supposition that PlGF binds only to VEGFR1. Although high concentrations of PlGF are unable to stimulate bovine adrenal cortical capillary endothelial cell proliferation in culture, PlGF potentiates the mitogenesis of these cells when suboptimal concentrations of VEGF are added. Such potentiation by PlGF may contribute to angiogenesis during tumor progression. Park et al. suggested that VEGFR1 binds to and inhibits VEGF action, acting as a decoy by preventing VEGF binding to VEGFR2 [111].

The level of autophosphorylation of VEGFR1 in response to VEGF is modest and can be detected readily only in cells that overexpress the receptor [53]. Activation of the receptor protein-tyrosine kinases and the initiation of signal transduction involve autophosphorylation of tyrosine residues [40]. Most receptor protein-tyrosine kinases undergo autophosphorylation in the so-called activation loop that leads to increased enzyme activity. However, VEGFR1 fails to undergo significant activation loop autophosphorylation and activation [119]. Six residues in the C-terminal tail of VEGFR1 including tyrosines 1169, 1213, 1242, 1309, 1327, and 1333 have been identified as phosphorylation sites (Fig. 2) [119–121]. Phosphotyrosine 1169 is implicated in the binding and activation of phospholipase C-γ1 (PLC-γ1) leading to the activation of the mitogen-activated protein (MAP) kinase signal transduction pathway [120]. Elucidating the signal transduction mechanisms initiated by VEGFR1 activation has been problematic owing to the low levels of autophosphorylation under physiological conditions.

Although, VEGF and PlGF activate VEGFR1, the phosphorylation sites differ. For example, Autiero et al. found that human VEGF-165 stimulates Tyr1213 phosphorylation whereas human PlGF-152 stimulates only Tyr1309 phosphorylation as determined by mass spectrometry in cells expressing only mouse VEGFR1 receptors [122]. Although VEGF-165 and PlGF-152 both bind to VEGFR1, these results indicate that they activate this receptor differently. Even though VEGF-165 stimulates VEGFR1 phosphorylation, it fails to alter the gene expression profile of mouse primary capillary endothelial cells. In contrast, mouse PlGF treatment produces changes in the expression of more than 50 genes.

Although VEGF-165 and PlGF bind to VEGFR1, they exert distinct biological effects suggesting that each activates VEGFR1 in a dissimilar fashion. Autiero et al. suggested that the mechanism responsible for these differences may be due to the ability of these ligands to induce different conformational changes in VEGFR1 [122]. However, the X-ray crystal structures of VEGF or PlGF bound to the second immunoglobulin domain of human VEGFR1 fail to reveal any differences in conformation [123,124]. The elucidation of the mechanism for the disparate autophosphorylation patterns of the same receptor in response to stimulation by two different ligands promises to add new insight into protein–protein signaling interactions.

4.2. VEGFR2 (Flk-1/KDR)

VEGFR2 (Flk-1/KDR, Fetal liver kinase-1/Kinase Domain-containing Receptor) binds to lower molecular weight forms of VEGF (110–165 amino acid residues), VEGF-E, and the fully processed forms of VEGF-C and VEGF-D (Table 3). VEGFR2, which has a molecular weight of about 210 kDa [53], is the predominant mediator of VEGF-stimulated endothelial cell migration, proliferation, survival, and enhanced vascular permeability [125–127]. Although VEGFR2 has lower affinity for VEGF than VEGFR1, VEGFR2 exhibits robust protein-tyrosine kinase activity in response to its ligands.

VEGF induces the dimerization of VEGFR2 that leads to receptor autophosphorylation and activation. Autophosphorylation occurs in *trans:* one kinase of the dimer catalyzes the phosphorylation of tyrosine residues in the second, and the second catalyzes the phosphorylation of tyrosine residues in the first. Autophosphorylation of tyrosine residues within the activation loop of the kinase domain stimulates catalytic activity while autophosphorylation of tyrosine residues at other locations generates docking sites for modular Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains that recognize phosphotyrosine in sequence-specific contexts.

Takahashi et al. demonstrated that Tyr1175 and Tyr1214 are the two major phosphorylation sites in VEGFR2 [126]. Other sites of tyrosine autophosphorylation include residues 951, 1054, and 1059 (Fig. 2) [127–129]. Autophosphorylation of residues 1054 and 1059 within the activation loop of VEGFR2 leads to increased kinase activity [130]. VEGFR2 phosphorylation leads to PLC-γ activation that in turn leads to protein kinase C activation. To determine which residue interacts with PLC-γ, Takahashi et al. infected murine spleen stromal (MSS31) cells, which are derived from endothelial cells, with adenovirus vectors expressing wild type and various VEGFR2 mutants [126]. They reported that the Tyr1175Phe mutant receptor fails to phosphorylate PLC-γ in response to VEGF treatment whereas wild type and Tyr1214Phe mutants are effective. They also found that VEGF-induced phosphorylation of MAP kinase is reduced in the Tyr1175Phe mutant but not in the wild type or Tyr1214Phe mutant. Furthermore, they reported that tyrosine 1175 is essential for VEGF-induced proliferation of VEGFR2-expressing bovine aortic endothelial cells. These results emphasize the importance of Tyr1175 in VEGFR2 signaling (Fig. 2).

The adaptor protein Shb is involved in signaling pathways involving several growth factor receptors including VEGFR2 [131]. Shb consists of an SH2 domain, a central PTB domain, four central probable tyrosine phosphorylation sites, and a proline-rich N-terminus. Holmqvist et al. demonstrated that

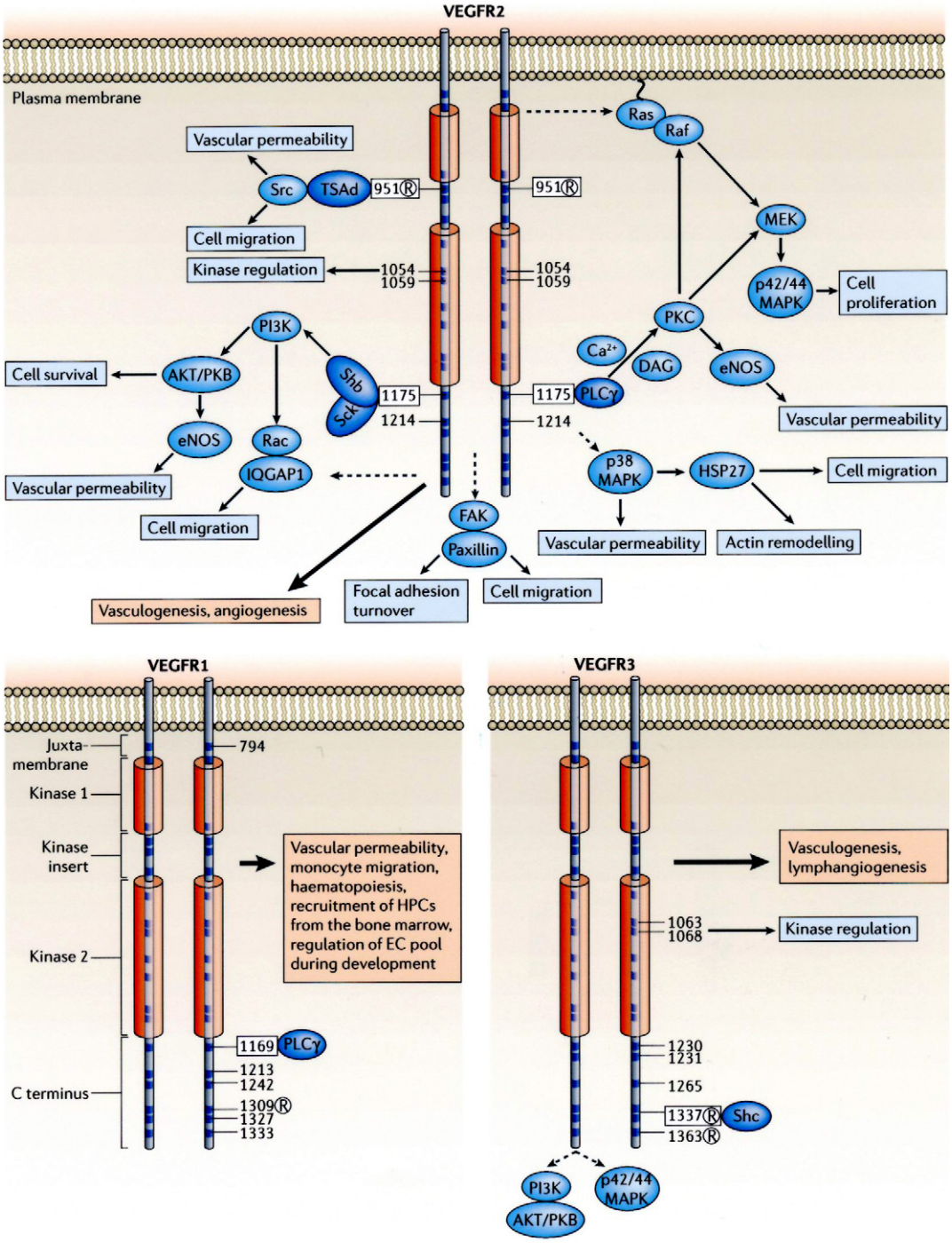

Fig. 2. VEGFR phosphorylation sites and signal transduction. Intracellular domains of VEGF receptors are shown with tyrosine-phosphorylation sites indicated by numbers. A circled R indicates that the use of the phosphorylation site is dependent upon the angiogenic state of the cell (VEGFR2), by a particular ligand (VEGFR1) or by heterodimerization (VEGFR3). Dark blue points in the receptors indicate positions of tyrosine residues. The dashed lines represent ill-defined transduction pathways. DAG, diacylglycerol; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; FAK focal adhesion kinase; HPC hematopoietic progenitor cell; HSP27, heat shock protein-27; IQGAP, GTPase with four IQ protein motifs; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase; p42/44 MAPK, Erk1/2; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLCγ, phospholipase C-γ; Sck, Shc-related protein; Shb, SH2 and beta-cells; TSAd, T-cell-specific adaptor. Reprinted from ref. [121] by permission from Macmillan Publishers Ltd.

Shb is phosphorylated and binds directly to tyrosine 1175 following VEGF stimulation of porcine aortic endothelial cells stably expressing human VEGFR2 [131]. By use of the small interfering RNA (siRNA) methodology directed against Shb, they found that it is required for VEGF-mediated stress fiber formation, cell migration, and activation of phosphatidylinositol (PI) 3-kinase.

Autiero et al. studied the interaction of VEGFR1 and VEGFR2 in immortalized capillary endothelial cells prepared from mice not expressing PlGF; these mouse cells were used because such endothelial cells respond to exogenous PlGF while those prepared from wild-type mice are unresponsive to PlGF [122]. They reported that mouse PlGF (which stimulates VEGFR1 only) fails to increase the phosphorylation of VEGFR2 whereas VEGF-E (which stimulates VEGFR2 only) produces a four-fold increase in VEGFR2 phosphorylation when compared with unstimulated samples. However, a combination of PlGF and VEGF-E produces a 13-fold increase in VEGFR2 phosphorylation. These workers suggested that VEGFR2 is transactivated by VEGFR1 through an intermolecular reaction between VEGFR1 and VEGFR2 homodimer pairs. Transactivation by homodimer pairs represents a novel interpretation in receptor protein-tyrosine kinase research where it is generally assumed that transactivation occurs between heterodimers.

When Sf9 insect cells expressing VEGFR1 and VEGFR2 were treated with human PlGF-152, VEGFR2 phosphorylation was increased by 150% [122]. Its phosphorylation was not increased when cells expressing only VEGFR2 are treated with human PlGF-152. When a kinase-dead mutant of VEGFR2 is co-transfected with VEGFR1, human PlGF-152 stimulates VEGFR2 phosphorylation. However, this phosphorylation fails to occur if the cells are expressing wild-type VEGFR2 and a kinase-dead mutant of VEGFR1. These experiments are consistent with the notion that VEGFR2 is transactivated by VEGFR1. Moreover, these results provide evidence that transphosphorylation of VEGFR1 and VEGFR2 occurs and indicates that cross-talk between receptor signaling pathways is possible.

Autiero et al. then studied the extent of VEGFR1 and VEGFR2 association in immortalized mouse capillary endothelial cells [122]. In the absence of any ligand, VEGFR1 was consistently found in anti-VEGFR2 immunoprecipitates demonstrating that these receptors spontaneously form complexes. They found that mouse homodimeric VEGF-164 and human heterodimeric VEGF/PlGF each increase VEGFR1/VEGFR2 complex formation by about 140%. VEGF-164 and VEGF/PlGF bind to both VEGFR1 and VEGFR2. In contrast, mouse homodimeric PlGF, which only binds to VEGFR1, fails to enhance VEGFR1-VEGFR2 association. Although, VEGFR1 and VEGFR2 are able to form complexes in the absence of activating ligand, increased association results only from stimulation by ligands that bind to both receptors.

Like VEGFR1, Ebos et al. described a soluble and circulating form of VEGFR2 [132]. As noted previously, soluble VEGFR1 is implicated in the pathogenesis of preeclampsia [117,118]. However, the physiological or possible pathological functions of soluble VEGFR2 are obscure, and additional investigation of the actions of sVEGFR2 is certainly warranted. The expression of VEGF and VEGFR1, but not VEGFR2, is augmented by hypoxia [133]. The role of the hypoxia-inducible transcription factor in this regulatory process is described later.

4.3. VEGFR3 (Flt-4)

VEGFR3, which has a molecular weight of about 170 kDa, is the third member of this receptor family [134,135]. VEGFR3 plays a key role in remodeling the primary capillary plexus in the embryo and contributes to angiogenesis and lymphangiogenesis in the adult. This receptor occurs in embryonic vascular endothelial cells where its production decreases during development and is subsequently restricted to lymphatic vessels after their formation [136]. Inactivating mutations in the catalytic loop of the kinase domain of VEGFR3 lead to human hereditary lymphedema (Milroy's disease) that is characterized by a chronic and disfiguring swelling of the extremities owing to defective cutaneous lymphatic vessels; VEGFR3 is the only VEGF receptor for which naturally occurring mutations have been found [121]. VEGFR3 undergoes a proteolytic cleavage in the sixth immunoglobulin domain; the two components of the original chain remain linked by a disulfide bond [134]. Hypoxia increases VEGFR3 expression in differentiating embryonic stem cells in culture [137].

Dixelius et al. studied the phosphorylation catalyzed by VEGFR3 in VEGF-C-stimulated porcine aortic endothelial cells overexpressing VEGFR3, transiently transfected human HEK293T cells expressing VEGFR3, or human primary lymphatic endothelial cells physiologically expressing VEGFR2 and VEGFR3 [138]. After stimulation, the cells were lysed, immunoprecipitated with anti-VEGFR3, and phosphorylation was performed with [γ-^{32}P]ATP in the immunocomplex. They identified five tyrosine residues (1230, 1231, 1265, 1337, and 1363) in the C-terminal tail of human VEGFR3 as autophosphorylation sites (Fig. 2). Phosphotyrosine 1337 serves as the binding site for Shc and Grb2, which occur at the beginning of the MAP kinase signal transduction module. Using human primary lymphatic endothelial cells, Dixelius et al. found that, following VEGF-C treatment (but not VEGF treatment), VEGFR2 was co-immunoprecipitated with VEGFR3 using anti-VEGFR3. Moreover, VEGFR3 residues 1337 and 1363 were not autophosphorylated in the VEGFR2-VEGFR3 immunocomplex. These results suggested that the interaction of the two receptors influenced the pattern of transphosphorylation and signal transduction by preventing the phosphorylation of the Shc and Grb2 binding sites.

Alam et al. studied the phosphorylation catalyzed by VEGFR3 in VEGF, VEGF-C, and VEGF-D-stimulated transiently transfected human HEK293T cells expressing

VEGFR2, VEGFR3, or both receptors. Following various treatments, the cells were lysed, immunoprecipitated with anti-VEGFR2 or anti-VEGFR3. The immunoprecipitates were probed with anti-VEGFR2, anti-VEGFR3, or anti-phosphotyrosine antibodies. These studies provide information on the phosphorylation state that occurs within the cell. In contrast to the results of Dixelius et al. [138], Alam et al. reported that VEGF-C failed to increase the phosphorylation of VEGFR3 expressed in HEK293T cells [139]. These investigators found that VEGF and VEGF-D led to: (i) the formation of a VEGFR2-VEGFR3 complex detected by immunoprecipitation and to (ii) the increased phosphorylation of the receptors. When VEGFR3 and a kinase-dead mutant of VEGFR2 were co-expressed in the HEK293T cells, VEGF and VEGF-C led to increased receptor phosphorylation. These authors concluded that VEGFR3 must interact with VEGFR2 in order to catalyze substrate phosphorylation.

Dixelius et al. reported that VEGF-C induced VEGFR3 phosphorylation in immunoprecipitates derived from cells expressing only this receptor [138], while Alam et al. found that VEGF, VEGF-C, or VEGF-D failed to induce VEGFR3 phosphorylation in such cells [139]. This difference may be related to the cellular versus the immunocomplex phosphorylation methodology performed in vitro. Although both groups found that VEGF-C induced the heterodimerization of VEGFR2 and VEGFR3, only Alam et al. found that VEGF-induced heterodimerization [138,139]. The reason for this discrepancy is unclear. However, the results of both groups point to the formation of VEGFR2-VEGFR3 heterodimers and resultant receptor signaling cross-talk.

4.4. Neuropilin-1 and -2

4.4.1. Properties and expression

Based upon chemical cross-linking studies, Soker et al. identified a VEGF receptor in HUVEC samples that differed from VEGFR1 and VEGFR2 [140]. These investigators purified this receptor from human MDA-MB-231 breast cancer cells and showed that it is identical to neuropilin-1, a surprising result at the time owing to its initial characterization as a neuronal recognition molecule and a neuronal cell adhesion molecule [141,142]. Subsequent work by Chen et al. led to the discovery of the related neuropilin-2 [143]. Numerous observations indicate that these receptors play a crucial role in tumor progression [144,145]. The neuropilins, which occur in several types of tumors, may mediate tumor growth by enhancing angiogenesis or by directly influencing tumor cells per se.

Neuropilins are transmembrane non-protein-tyrosine kinase co-receptors for both the semaphorin family and the VEGF family. Semaphorins are glycoproteins that serve as chemorepulsive axon guidance molecules capable of collapsing axonal growth cones and repelling axons of ganglia during neurogenesis. Of the seven classes [146], neuropilins recognize selected members of class III semaphorins [147]. Semaphorin-3A binds to neuropilin-1 while semaphorins 3C and 3F bind to both neuropilin-1 and -2 (Table 3) [148]. VEGF-165, PlGF-152 (the heparan sulfate-binding isoform), and both isoforms of VEGF-B bind to neuropilin-1 [148,149]. VEGF-145, VEGF-165, PlGF-152 and VEGF-C bind to neuropilin-2 [150,151]. VEGF-121 is not a ligand for either neuropilin-1 or -2.

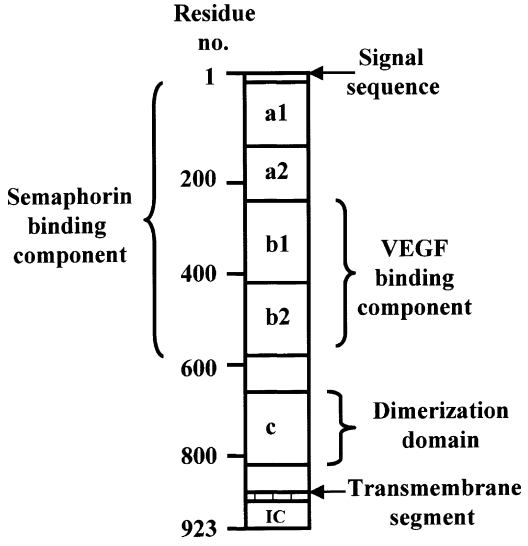

Fig. 3. Architecture of neuropilin-1 and -2. The a1, a2, b1, b2 and c domains are indicated. IC, Intracellular segment.

Neuropilins act as co-receptors with large (≈250 kDa) transmembrane plexins that transduce semaphorin signaling and as co-receptors with VEGFR1, VEGFR2, and VEGFR3 that transduce VEGF family signaling. The neuropilins are thus unusual because they participate as co-receptors in cell signaling initiated by two entirely different families of protein ligands (vascular endothelial growth factors and the semaphorins) in combination with two different classes of co-receptors (VEGF receptors and plexins). The VEGF receptors are protein-tyrosine kinases while the mechanism of action of the plexins is ill defined. Neuropilins also function as receptors for VEGF isoforms independently of VEGFR1, VEGFR2, or VEGFR3.

Neuropilins are glycoproteins with a molecular weight of 120–140 kDa [143]. The neuropilins contain a large extracellular component, a transmembrane segment, and a short (≈40 amino acid residue) intracellular portion [152] Although, the intracellular domain is too small to function as a catalyst, it is possible that it serves as a docking site for downstream signaling molecules alone or in conjunction with co-receptors. Fig. 3 depicts the architecture and functional components of the neuropilins.

Although, neuropilin-1 and -2 received their name from their neuronal localization, they are expressed with partially overlapping patterns in a wide variety of adult human tissues. Neuropilin-1 occurs in sensory and sympathetic neurons while neuropilin-2 occurs in sympathetic but not sensory neurons [153]. Cultured HUVEC samples express both neuropilin-1 and -2 with neuropilin-2 predominating [144].

Neuropilin-1 occurs in a variety of non-neuronal and non-vascular cells types under physiological conditions including bone marrow fibroblasts and adipocytes, dendritic immune cells, osteoblasts, renal mesangial, and renal glomerular epithelial cells [145].

Kärpänen et al. studied the functional interaction of VEGF-C and VEGF-D with the neuropilins [31]. As noted above, the signal peptide is hydrolytically removed from the prepro-protein to yield a pro-protein. Formation of the mature protein requires two additional proteolytic cleavages to yield the C- and N-termini of the growth factors [71,72,81]. Kärpänen et al. prepared soluble neuropilin-Ig (immunoglobulin) fusion proteins and found that partially processed and mature VEGF-C bind to neuropilin-2, whereas the partially processed, but not mature, VEGF-C binds to neuropilin-1 (Table 3). They found that unprocessed but not mature VEGF-D binds to both neuropilin-1-Ig and neuropilin-2-Ig fusion proteins. These investigators found that VEGF-C binds to the b1b2 domains of both neuropilins as well as it binds to the extracellular domains of the a1a2-b1b2 constructs. VEGF-C fails to bind to the a1a2-b2 construct, which indicates that the b1 domain participates in this interaction. These investigators showed that semaphorin-3F competes with VEGF-C binding to the neuropilin-1-Ig and neuropilin-2-Ig fusion proteins.

Kärpänen et al. found that porcine aortic endothelial cells engineered to produce neuropilin-2 and VEGFR3 exhibited VEGF-C-induced internalization of a neuropilin-2-VEGFR3 complex [31]. VEGF-C-induced internalization of VEGFR3 occurs in cells expressing VEGFR3 alone, but induced internalization of neuropilin-2 fails to occur in cells expressing neuropilin-2 alone. Thus, VEGF-C-induced neuropilin-2 endocytosis is dependent on VEGFR3. Moreover, they observed VEGF-D-induced internalization and co-localization of neuropilin-2 and VEGFR3 in endocytic vesicles prepared from human lymphatic or blood vascular endothelial cells. Although, VEGF-165 binds to neuropilin-2, this factor failed to induce the internalization of this co-receptor in lymphatic endothelial cells. The authors suggested that the mechanism by which neuropilin-2 conveys VEGF-C and VEGF-D signaling likely involves its interaction with VEGFR3 [31].

Favier et al. treated porcine aortic endothelial cells that were engineered to express both human VEGFR2 and neuropilin-2 with VEGF-C and performed immunoprecipitation studies. Although there was some association of VEGFR2 and neuropilin-2 in the absence of VEGF-C, they found that VEGF-C increased the formation of the complex. Moreover, they found that neuropilin-2 decreased the concentration of both VEGF and VEGF-C required for VEGFR2 autophosphorylation in aortic endothelial cells.

Favier et al. transfected human microvascular endothelial cells with neuropilin-2 cDNA, a procedure that leads to overexpression of this protein [154]. These cells ordinarily express VEGFR2, VEGFR3, and neuropilin-2. This protein expression pattern most closely resembles that of lymphatic endothelial cells, which was confirmed by the presence of two lymphatic markers (Prox1 and LYVE-1) in these cells. They found that neuropilin-2 overexpression leads to increased cell survival and cell migration evoked by VEGF or VEGF-C, responses that are inhibited by semaphorin-3F. These results are consistent with the finding that semaphorin-3F blocks VEGF and VEGF-C binding to neuropilin-2 [31] and thereby inhibits these growth-factor responses. Moreover, Favier et al. found that VEGF- and VEGF-C-induced VEGFR2 autophosphorylation in untransfected human microvascular endothelial cells and that treatment of these cells with a small interfering RNA targeting neuropilin-2 inhibits ligand-stimulated VEGFR2 autophosphorylation [154].

Evidence exists for the formation neuropilin-1-VEGFR1, neuropilin-2-VEGFR1, neuropilin-2-VEGFR2, and neuropilin-2-VEGFR3 complexes [31,145,154]. Whether neuropilin-1-VEGFR2 or neuropilin-1-VEGFR3 complexes form physiologically or during tumor progression remains to be established.

Table 4
Neuropilin expression by human neoplasms and tumor cell lines[a]

Tumor type/origin	Np-1	Np-2	Citation
Astrocytoma	+	ND	[155]
Bladder	ND	+	[156]
Breast	+	ND	[140]
Colorectum	+	ND	[157]
Esophagus	+	ND	[158]
Gall bladder	+	ND	[158]
Glioma	+	ND	[159]
Melanoma	+	ND	[160]
Neuroblastoma	+	+	[161]
Non-small cell lung cancer	+	+	[162]
Pancreas	+	+	[163,164]
Prostate	+	+	[165,166]
Stomach	+	ND	[167]
Small cell lung cancer	+	+	[162]

[a] Adapted from ref. [145]; Np, neuropilin; ND, not determined.

4.4.2. Tumor progression

Neuropilin-1 and -2 occur in a variety of neoplasms (Table 4). Not surprisingly, the level of expression of the two receptors is often unequal. For example, neuropilin-2 expression in melanoma and glioblastoma exceeds that of neuropilin-1 [144].

In an effort to determine the potential function of neuropilin-1 in tumors, Miao et al. overexpressed it in Dunning rat prostate carcinoma AT2.1 cells using a tetracycline-inducible promoter [165]. Increased expression of neuropilin-1 augments VEGF-165 binding to AT2.1 cells in culture. Following injection of the AT2.1-neuropilin-1 cells into rats, tumor size increased several fold following neuropilin-1 induction by doxycycline when compared with the non-induced controls. The larger tumors with induced neuropilin-1 expression exhibited increased microvessel content and endothelial cell proliferation. These results show that neuropilin-1 expression in tumor cells promotes angiogenesis and tumor progression, a result that indicates that neuropilin-1 represents a potential anti-cancer target.

Parikh et al. examined the role of neuropilin-1 in human colon tumor growth and progression [168]. They reported that neuropilin-1 mRNA and protein were expressed in 20 of 20 human colon adenocarcinoma specimens but not in adjacent non-malignant colon mucosa. Furthermore, they found that neuropilin-1 mRNA and protein were expressed in seven of seven human colon adenocarcinoma cell lines. They prepared a human colon carcinoma cell line (KM12SM/LM2) that was engineered to stably overexpress neuropilin-1 and found that such subcutaneous xenografts in athymic nude mice exhibit increased tumor growth and angiogenesis when compared with cells not overexpressing neuropilin-1. These workers also found that the cells overexpressing neuropilin-1 exhibit a two-fold increase in cell migration in response to VEGF-165. Using cultured human colon carcinoma HT29 cells, they found that epidermal growth factor and insulin-like growth factor-1, but not interleukin-1β or transforming growth factor-α, increased neuropilin-1 mRNA and protein expression. The epidermal growth factor (EGF) response was blocked by anti-EGF receptor antibody. These investigators thus uncovered a potential mechanism for growth factor participation in tumor progression by augmenting neuropilin-1 expression.

In studies designed to determine the identity of signal transduction pathways involved, Parikh et al. found that the phosphatidylinositol (PI) 3-kinase inhibitor, wortmannin, and the extracellular-signal-regulated kinase (Erk 1/2) inhibitor U0126 diminish both basal and epidermal growth factor-stimulated neuropilin-1 mRNA expression in HT29 cells [168]. Inhibition of basal expression implicates an autocrine mechanism for activating these pathways. Because the colon cell lines express neuropilin-1 but not VEGFR2, these investigators hypothesized that VEGF-165 may bind simultaneously to neuropilin-1 on tumor cells and VEGFR2 on adjacent endothelial cells thereby activating endothelial cells and providing a juxtacrine mechanism for neuropilin-1 induction of angiogenesis and tumor progression.

Wey et al. engineered human pancreatic carcinoma cells (FG) to stably overexpress neuropilin-1 [169]. Neuropilin-1 overexpression decreased detachment-induced apoptosis (anoikis) and decreased sensitivity to: (i) gemcitabine and (ii) 5-fluorouracil, cytotoxic drugs that are used to treat pancreatic and other malignancies. They found that neuropilin-1 overexpression increased unstimulated Erk 1/2 phosphorylation six-fold and Jun N-terminal kinase (Jnk) phosphorylation four-fold. The authors surmise that activation of Erk or Jnk signaling may account for the observed chemoresistance to the two cytotoxic agents.

Using a different pancreatic cancer cell line (PANC-1), Wey et al. found that, in contrast to the FG cells, neuropilin-1 expression increased the sensitivity of cells to gemcitabine and 5-flourouracil [169]. Based upon experiments with the FG cells, the authors suggested that expression of neuropilin-1 in pancreatic cancer cells may be one of the factors that leads to their widespread chemoresistance [169,170], and inhibiting neuropilin-1 signaling may lead to increased sensitivity to cytotoxic agents [145,169]. However, the disparate results obtained with FG cells (neuropilin-1 expression leads to decreased sensitivity to cytotoxic agents) and PANC-1 cells (neuropilin-1 expression leads to increased sensitivity) emphasize the importance of the context in which neuropilin-1 signaling occurs, and additional work will be required to sort out the inconsistencies.

Barr et al. examined the role of neuropilin-1 in VEGF-mediated survival of human MDA-MB-231 breast cancer cells [171]. They reported that this cell line expresses neuropilin-1 and -2 but neither VEGFR1 nor VEGFR2. Moreover, this cell line constitutively expresses VEGF. Treatment of these cultured cells with a neuropilin-1 peptide antagonist, which corresponds to the sequence of exon 7 of VEGF-165, produces apoptosis. These workers thus showed that neuropilin-1 plays a crucial role in pro-survival signaling by VEGF in these breast cancer cells and that neuropilin-1 blockade induces tumor cell apoptosis. Using confocal microscopy, these investigators also demonstrated that anti-neuropilin-1 binds to both co-cultured tumor and human umbilical vein endothelial cells whereas anti-VEGFR2 binds only to the endothelial cells. Castro-Rivera et al. reported that human semaphorin-3B inhibits tumor cell growth and induces apoptosis in a human breast cancer cell line (MDA-MB-231) [172]. This effect was reversed by VEGF-165 but not VEGF-121. This finding is consistent with the experiments demonstrating that semaphorin-3B and VEGF-165, but not VEGF-121, bind to neuropilin-1 (Table 3). VEGF, which is widely expressed, thus has the potential to act as a pro-survival factor on a variety of cells expressing neuropilin-1 in the absence of VEGFR1 and VEGFR2.

Besides their participation in apoptotic signaling, the neuropilins play a role in breast cancer cell migration. Nasarre et al. demonstrated that VEGF-165 promotes, while semaphorin-3F inhibits, cell spreading and membrane ruffling in two human breast cancer cell lines: MCF7 and C100 [173]. The MCF7 cell line expresses neuropilin-1 but not neuropilin-2, and the semaphorin-3F inhibition of cell spreading was blocked by anti-neuropilin-1. In contrast, the C100 cell line expresses neuropilin-2 and lower levels of neuropilin-1, and the semaphorin-3F signaling was blocked by anti-neuropilin-2. The VEGF-165 induced membrane ruffling was blocked by semaphorin-3F, which is consistent with the concept that semaphorin-3F competes with VEGF-165 for binding to the neuropilins.

In a follow up study, Nasarre et al. developed an assay to determine whether motile human C100 breast cancer cells would migrate to or from semaphorin-3F-containing zones [174], a procedure initially used to study nerve growth cone guidance. They reported that C100 cells migrate away from semaphorin-3F, and this migration is blocked by anti-neuropilin-1 antibodies. In less motile MCF7 human breast cancer cells, semaphorin-3F induces the loss of cellular contacts with partial delocalization of E-cadherin and β-catenin. Moreover, MCF7 cell proliferation decreases in response to Semaphorin-3F. These investigators suggested that

semaphorins maintain cellular boundaries, and loss of this function would enhance cell migration during tumor progression. Loss of semaphorin-3F expression as a result of chromosomal deletion (see below) is hypothesized to enhance cell migration and contribute to tumor spread and metastasis.

Semaphorin-3B and semaphorin-3F were originally identified from a recurrent 3p21.3 homozygous deletion in small cell lung cancer cell lines suggesting that these might represent tumor suppressors [175–177]. Kusy et al. established human lung cancer cell lines (NCI-H157 and NCI-H460) that stably express semaphorin-3F [178]. Each of these cell lines was implanted via the trachea into the left lobe of athymic nude rats to produce lung tumors. Pulmonary cancer cell lines are generally more tumorigenic when implanted in their usual, or orthotopic, location than when they are when injected into an ectopic subcutaneous location [178]. The tumorigenicity of H157 cells producing semaphorin-3F was diminished when compared with the non-producing cells. In contrast, the tumorigenicity of the H460 was not diminished in the semaphorin-3F producing cells. All of these cell lines express comparable levels of plexin-A1, plexin-A3, plexin-B1, and plexin-B2, VEGFR1, and VEGFR2. The sensitive H157 cells express neuropilin-2 whereas the resistant H460 cells lack this receptor while both the sensitive and insensitive cells express neuropilin-1. The authors thus ascribed the sensitivity of the H157 cells to semaphorin-3F to the expression of neuropilin-2. Overall, these studies suggest that semaphorin-3F has anti-tumor activity in neuropilin-2-expressing cells in this lung cancer paradigm.

Kessler et al. studied the role of semaphorin-3F on angiogenesis in HUVEC samples [179]. They found that it inhibits VEGF-165 and basic fibroblast growth factor stimulation of HUVEC proliferation. Semaphorin-3F inhibits the binding of VEGF-165 to neuropilin-2, but it does not inhibit the binding of fibroblast growth factor to its receptors. The mechanism of inhibition of the latter by semaphorin-3F is thus unclear. Semaphorin-3F also inhibits VEGF-165 and basic fibroblast growth factor stimulation of angiogenesis in vivo. Overexpression of semaphorin-3F in tumorigenic human embryonic kidney (HEK293) cells inhibits their tumor-forming ability in athymic nude mice but fails to alter their proliferation in cell culture. These findings suggest that semaphorin-3F restrains tumor growth by inhibiting tumor angiogenesis and not tumor cell proliferation.

Gagnon et al. reported that neuropilin-1, like VEGFR1 and VEGFR2, exists as a soluble isoform that contains the extracellular ligand binding a1a2 and b1b2 domains but lacks the c, transmembrane, and cytoplasmic components [180]. Soluble neuropilin-1, like the membrane isoform, binds to VEGF-165 but not VEGF-121. The soluble and membrane isoforms have different patterns of expression. The membrane isoform is associated with blood vessels, whereas the soluble form has a more widespread distribution including hepatocytes and renal proximal and distal tubules. Gagnon et al. prepared Dunning rat prostate carcinoma cells that expressed soluble neuropilin-1 and found that expressing and non-expressing cells have the same growth rate in culture. Following subcutaneous injection into rats, however, they found that the tumors expressing soluble neuropilin-1 possessed large hemorrhagic centers and exhibited extensive apoptosis but little proliferation. In contrast, the non-expressing tumor cells were solid, vascular, and exhibited sparse apoptosis but extensive proliferation [180]. Soluble neuropilin-1 presumably binds to VEGF and decreases its signaling, which impairs tumor vascularization. The possible physiologic and tumorigenic roles of soluble neuropilin-1, however, are largely unknown.

Growth factors and oxygen regulate neuropilin expression. For example, VEGF [181], epidermal growth factor [163,167,168], insulin-like growth factor-1 [168], and hypoxia [182] lead to the increased expression of neuropilin-1. Moreover, cerebral ischemia leads to increased expression of both neuropilin-1 and -2 [183]. Whether hypoxia per se leads to increased levels of neuropilin-2 remains to be determined.

In summary, there is considerable evidence that neuropilin-1 and -2 participate in tumor progression. As co-receptors for VEGFR1, VEGFR2, and VEGFR3, the neuropilins function as part of protein-tyrosine kinase cascades. As co-receptors for the semaphorins, the neuropilins interact with plexins where the nature of signal transduction is ill defined. Perhaps the neuropilin-plexin complex interacts with downstream components directly or after the complex is phosphorylated by yet unidentified protein kinases. Moreover, the activated neuropilins per se initiate a signaling cascade independently of the VEGF receptors or the plexins. The nature of neuropilin signal transduction warrants further study. Whether neuropilin-2 (like neuropilin-1) participates in pro-survival signaling and whether the neuropilins participate in pro-survival signaling under physiological conditions as opposed to tumorigenic conditions are open questions.

4.5. Essential nature of the VEGF receptors

Fong et al. showed that *VEGFR1* null mice are embryonic lethal [184]. Endothelial cells form normally in both embryonic and extra-embryonic sites in these mice, but the cells fail to assemble into organized blood vessels. However, Hiratsuka et al. found that mice expressing the VEGFR1 extracellular ligand-binding and transmembrane segments but lacking the tyrosine kinase (TK) and its insert domain (*VEGFR1-TK*$^{-/-}$) are viable [185]. This observation indicates that the ligand-binding domain is the essential part of the receptor during development. This finding is consistent with the concept that the chief function of VEGFR1 in embryos is to sequester VEGF and modulate the concentration of the free ligand.

Shalaby et al. reported that *VEGFR2* null mice die between embryonic Days 8.5 and 9.5 as a result of defects in the development of hematopoietic and endothelial precursors [186]. Yolk-sac blood islands were absent at 7.5 days, organized blood vessels were not observed in the embryo or yolk sac at any stage, and hematopoietic progenitors were severely reduced. These findings indicate that VEGFR2 is essential

for yolk-sac blood-island formation and vasculogenesis in the mouse embryo and are consistent with the concept that VEGFR2 is one of the earliest markers of embryonic endothelial cells.

Dumont et al. showed that *VEGFR3* null mice died by embryonic Day 9.5 and exhibited defective blood vessel development [187]. Vasculogenesis and angiogenesis occurred, but large vessels became abnormally organized with defective lumens, leading to fluid accumulation in the pericardial cavity, and cardiovascular failure. Thus, VEGFR3 has an essential role in the development of the embryonic cardiovascular system before the emergence of the lymphatic vessels where VEGFR3 also plays a pivotal role.

Neuropilin-1 is an active participant in angiogenesis [188]. Kitsukawa et al. demonstrated that its overexpression in mice results in excessive capillary and blood vessel formation and lethal hemorrhaging in embryos [189]. This group showed that *Np1* null mice die in utero at Days 10.5–12.5 owing to anomalies in the cardiovascular system as well as in the nervous system [190]. The anomalies include: (i) impairment of neural vascularization, (ii) transposition of large vessels, and (iii) inadequate development of vascular networks in the yolk sac.

Np2 null mice have an apparently normal vascular phenotype [191,192]. However, Yuan et al. reported that neuropilin-2 expression is restricted to veins and lymphatic vessels and *Np2* null mice exhibit an absence or severe reduction of small lymphatic vessels during development [193]. Thus, strong cases can be made that neuropilin-1 plays an important role in vascular development and angiogenesis and that neuropilin-2 plays a crucial role in lymphatic development.

Takashima et al. studied the effects of mutations of both neuropilin-1 and -2 in mice [194]. $Np1^{-/-}NP2^{-/-}$ mice died in utero at embryonic Day 8.5 with totally avascular yolk sacs. $Np1^{+/-}Np2^{-/-}$ or $Np1^{-/-}Np2^{+/-}$ were also embryonic lethal and survived to embryonic Day 10. The yolk sac lacked capillary beds and branching arteries and veins. The embryos possessed unconnected blood vessel sprouts and blood vessels heterogeneous in size. Moreover, the mutant embryos were about half the length of wild-type mouse embryos and exhibited multiple hemorrhages. These mice, which have a more severe vascular phenotype that either *Np1* or *Np2* null mice, resemble those of *VEGF* and *VEGFR2* null mice. The authors concluded that both neuropilin-1 and -2 are required for embryonic vessel development.

5. Proteolysis of VEGF isoforms and release from heparan sulfate proteoglycans

5.1. VEGF isoforms

All VEGF isoforms are hydrophilic and freely diffusible in simple buffered solutions. However, VEGF-165, VEGF-189, and VEGF-206 contain heparan sulfate proteoglycan-binding domains that limit their diffusibility in vivo because of their affinity for anionic proteoglycans located on the plasma membrane of cells and in the extracellular matrix [30]. Exons 6a, 6b, and 7 each encode heparan sulfate-binding domains (Fig. 1) that limit VEGF diffusibility. VEGF-121 lacks these binding domains and is freely diffusible in vivo. VEGF-165, which is the predominantly expressed isoform, lacks exons 6a and 6b. However, it contains a heparan sulfate-binding segment that is encoded by exon 7. Fifteen of the 44 amino acids encoded by exon 7 are basic. As a result, between 50% and 70% of VEGF-165 remains cell and extracellular-matrix associated thereby making this isoform partially diffusible in vivo [195]. The larger isoforms, which contain residues encoded by exons 6a, 6b, and 7, contain heparan sulfate-binding domains and are not freely diffusible. These isoforms are completely sequestered at the cell surface and in the extracellular matrix.

The action of growth factors is regulated at several levels including transcription, translation, and secretion, translocation to the site of action, receptor activation, and inactivation by endocytosis and proteolytic degradation. Kit ligand, transforming growth factor-α, epidermal growth factor, colony-stimulating factor-1, and tumor necrosis factor-α are initially anchored in the plasma membrane of the biosynthetic cell and are released via proteolysis [196]. Other growth factors including basic fibroblast growth factor, platelet-derived growth factor [196], and several VEGF splice variants [30] bind to extracellular matrix components. Regulated growth factor release from these sites is mediated by extracellular proteases including plasmin and matrix metalloproteases [197].

5.2. Plasminogen activators, plasmin, and matrix metalloproteases

Plasminogen activators, plasmin, and matrix metalloproteases (MMPs) play an integral role in angiogenesis [198]. The enzymatic active site of plasmin contains a serine residue thereby accounting for its designation as a serine protease. Plasmin catalyzes the hydrolysis of several extracellular proteins including fibronectin, laminin, and the protein core of proteoglycans [197]. Active plasmin is derived from inactive plasminogen following proteolytic activation by tissue plasminogen activator (tPA) or by the urokinase type of plasminogen activator (uPA) [196]. Tissue plasminogen activator plays a pivotal role in generating plasmin for fibrinolysis whereas uPA plays a key role in generating plasmin for the degradation of the extracellular matrix during tissue remodeling [199]. The activity of uPA is abolished by plasminogen activator inhibitor-1 and -2, which are protein in nature. Plasminogen is widely distributed in the extracellular compartment, which provided the rationale for examining its possible role in angiogenesis.

The human family of matrix metalloproteases consists of 23 zinc-dependent enzymes that can be divided into two distinct groups: secreted MMPs and membrane-type

MMPs (MT-MMPs). The secreted MMPs include MMP 1–3, 7–13, 19, 20, 23A, 23B, 24–28 and the membrane-associated MMPs include MMP 14–17 and 21 [200,201]. These enzymes can digest a large number of extracellular matrix proteins in the connective tissue stroma including the basement membrane and various types of collagen. They are secreted as inactive proenzymes and are activated extracellularly by various enzymes including plasmin. Moreover, tissue inhibitors of metalloproteases (TIMPs), four of which have been described [202], regulate MMP activity. Owing to the irreversible nature of proteolysis, it is imperative that the catalytic activity of all of these proteases is exactingly regulated.

5.3. VEGF isoform proteolysis by plasmin

Plasminogen and plasmin are widely distributed extracellularly [198]. Houck et al. demonstrated that VEGF-165 and VEGF-189, bound isoforms that were stably expressed and secreted by human embryonic kidney CEN4 cells, are cleaved by exogenous plasmin to produce a product that lacks heparan sulfate-binding domains and diffuses freely from cells [195]. Keyt et al. showed that plasmin cleaves VEGF-165 between Arg110 and Ala111 to yield an amino-terminal mitogenically active fragment (VEGF-110) and an inactive carboxyterminal fragment (111–165) [203]. VEGF-110 fails to bind whereas VEGF-165 and the 111–165 fragment bind to heparin. These experiments were the first to show that heparin-binding domains occur in residues 111–165.

Keyt et al. measured the affinity of VEGF isoforms to chimeric VEGFR1-Ig and VEGFR2-Ig proteins by determining the concentration (IC_{50}) of the isoform required to inhibit 50% of ^{125}I-VEGF-165 binding to the soluble receptor [203]. The carboxyterminal portion from residues 111–165 or 122–165 increases the affinity of VEGF-165 for VEGFR1-Ig or VEGFR2-Ig by a modest factor of two. Keyt et al. determined the effective concentration (EC_{50}) of the various isoforms required to produce 50% of the maximal proliferation of endothelial cells derived from bovine adrenal cortex [203]. The EC_{50} of VEGF-165 is about 5 pM and that of VEGF-110 and VEGF-121 is about 2600 pM, a 500-fold difference. This observation indicates that residues 122–165 (from exons 7 and 8) play a major role in mediating VEGF-165-mitogenic action. Such a dramatic increase in biological response cannot be explained by the modest changes in VEGF isoform affinity for VEGFR1-Ig or VEGFR2-Ig.

Whitaker et al. reported that the EC_{50} of VEGF-121 for stimulating the phosphorylation of VEGFR2 in HUVEC samples is 300-fold greater than that for VEGF-165 (2450 pM versus 7.76 pM), indicating that VEGF-165 is more potent than VEGF-121 [204]. They expressed human VEGFR2 in COS-1 cells and found that the affinity of VEGF-121 and VEGF-165 for the receptor was nearly identical ($IC_{50} \approx 300$–600 pM). They hypothesized that the lack of differences in binding affinity but the differences in potency were due to the expression of neuropilin-1 in the HUVEC samples. They showed that formation of a neuropilin-1-VEGFR2 complex does not alter its affinity for VEGF-165; rather there is an increase in ligand efficacy. They demonstrated that VEGF-165, but not VEGF-121, binds to neuropilin-1. They proposed that VEGF-165 binding to the neuropilin-1-VEGFR2 complex increases the local concentration of VEGFR2 relative to that achieved with VEGF-121 binding and thereby increases the potency of the larger ligand [204]. This concept may also apply to the work of Keyt et al. described previously [203].

Lauer et al. performed studies to determine whether plasmin plays a role in VEGF cleavage in humans. They showed that the expression of total VEGF protein and VEGF-121, VEGF-165, and VEGF-189 mRNAs is increased in chronic cutaneous ulcers when compared with normal skin [205]. They demonstrated that exogenous VEGF-165 is degraded when incubated with wound samples, and this degradation is inhibited by phenylmethylsulfonyl fluoride, a serine protease inhibitor. Inhibitors of matrix metalloproteases (EDTA, phenanthroline) and cysteine proteases (leupeptin) were without effect. Moreover, α2-antiplasmin inhibited the degradation of exogenous VEGF-165 catalyzed by the wound samples. These studies show that VEGF-165 expression is increased during wound healing, but the potential beneficial effects of the more potent VEGF-165 may be abolished by endogenous plasmin activity [205].

Roth et al. studied the role of plasmin-mediated proteolysis of VEGF-165 during wound healing in wild type and db/db mice [206]. The db/db mice possess an autosomal recessive mutation in the leptin receptor gene (*ObR*) resulting in a type II diabetes phenotype. The mutant mice exhibit severely impaired cutaneous wound repair when compared with wild-type mice. These workers showed that plasmin activity is increased at the wound site during repair in mutant mice when compared with wild-type C57BL/6 mice. They also showed that a transgenically expressed VEGF-165 Ala111Pro construct, which contains a mutation at the site of plasmin-mediated hydrolysis of VEGF, is more stable than wild-type VEGF-165 in the wound tissue. In animals expressing VEGF-165 Ala111Pro, wound closure is accelerated, vessels are stabilized, pericyte recruitment is increased, and capillary endothelial cell apoptosis is delayed when compared with animals expressing wild-type VEGF-165. This study provides evidence that plasmin-mediated proteolysis of VEGF-165 occurs in vivo.

5.4. VEGF isoform proteolysis by urokinase type of plasminogen activator

Plouët et al. studied the release and extracellular proteolytic maturation of VEGF-189 in wild-type Chinese hamster ovary cells and in mutant cells that were glycosaminoglycan deficient [207]. They found that VEGF-189 is displaced from wild-type cell membranes by heparin or by synthetic peptides with the sequence corresponding to that of a part of VEGF exon 6a (a heparin-binding component of VEGF). VEGF-

189 is secreted from the mutant cells into the medium and is not retained by the glycosaminoglycan-deficient membranes. While native glycosylated VEGF-189 has a molecular weight of 52 kDa, their studies showed that VEGF-189 is released from mutant cells as a 40-kDa form that is generated by endogenous protease activity. Inhibition of intrinsic urokinase type of plasminogen activator (uPA) with an antibody inhibits VEGF-189 cleavage in the mutant Chinese hamster ovary cell culture. On the other hand, inhibition of any intrinsic plasmin activity does not prevent VEGF-189 proteolysis. In contrast to the studies with human [205] and mouse [206] wound healing, plasmin-mediated proteolysis of the larger VEGF isoforms in Chinese hamster ovary cells is not as prevalent as that catalyzed by uPA [207].

Plouët et al. found that uPA converts VEGF-189 from the 52-kDa form to the 40-kDa form in vitro [207]. In contrast, VEGF-165 is not degraded by this protease. These results indicate that uPA cleavage occurs in residues encoded by exon 6a, which is not expressed in VEGF-165 (Fig. 1). A recombinant form of VEGF-189 (50 kDa) was expressed and purified from baculovirus-infected Sf9 cells. This recombinant protein, which binds to VEGFR1 but not VEGFR2, is not mitogenic in endothelial cells derived from bovine adrenal cortex. After treatment with uPA, however, the resulting 38-kDa product binds to VEGFR2 and is mitogenic. These results explain, in part, why *VEGF*$^{188/188}$ mice have an abnormal phenotype (see below).

Plouët et al. found that the proteolysis of VEGF-165 and VEGF-189 by plasmin results in the generation of identical 34-kDa products [207]. An enzyme-linked immunosorbent assay (ELISA) targeting exon-6-encoded residues was negative, consistent with the generation of VEGF-110 observed by Keyt et al. [203]. Plouët et al. determined the affinity of VEGF-165, VEGF-189, and the fragments resulting from plasmin-mediated and uPA-mediated proteolysis of the proteins for VEGFR2 [207]. They reported that VEGF-189 is unable to bind to VEGFR2-expressing Chinese hamster ovary cells and is not mitogenic for endothelial cells derived from bovine adrenal cortex. VEGF-165 and the uPA fragment of VEGF-189 (38-kDa) bind to VEGFR2-expressing cells with high affinity and have robust mitogenic activity for the bovine endothelial cells. These observations indicate that VEGF-189 contains a segment encoded by exon 6a (residues 115–139) that prevents its binding to VEGFR2.

5.5. VEGF isoform proteolysis by matrix metalloproteases

There is increasing evidence that matrix metalloproteases play a role in VEGF maturation. Lee et al. [208] characterized the proteolysis of mouse VEGF-164 and VEGF-188 in vitro by several matrix metalloproteases (MMPs). These investigators found that VEGF-164 and VEGF-188 are cleaved by MMP-3, -7, -9, and -19 [208]. MMP-1 and -16 are somewhat less effective whereas MMP-2, -8, -14, and -26 are without effect. Proteolysis by MMP-3 is inhibited by tissue inhibitor of metalloprotease-1 and -2 (TIMP-1 and -2) but not by aprotinin, a serine protease inhibitor. TIMP-1 and TIMP-2 each form a 1:1 stoichiometric complex with MMP-3 leading to its inhibition. These investigators found that heparin-bound VEGF-164 is a good substrate for MMP-3, which liberates the amino-terminal product from heparin. Using mass spectrometry, Lee et al. determined that VEGF-164 proteolysis occurs between residues 135–136, 120–121, and finally 113–114 [208].

Lee et al. generated VEGF-164$_{\Delta 108–118}$, which contains an 11 amino-acid-residue deletion [208]. This form is resistant to MMP-3 proteolysis despite the presence of the amino acids corresponding to the wild-type protein at residues 120 and 135. They found that VEGF-164, VEGF-164$_{\Delta 108–118}$, and VEGF-113 are equally effective in activating VEGFR2 by demonstrating comparable autophosphorylation of receptor expressed in porcine aortic endothelial cells [208]. All three forms elicit an angiogenic response, but the morphology of the resulting vessels differs. VEGF-164 produces vessels with wild-type morphology, whereas VEGF-113 produces enlarged, dilated vessels. VEGF-164$_{\Delta 108–118}$, the MMP-resistant form, produces thin vessels with multiple branch points [208]. These studies suggest that matrix-bound and freely diffusible VEGF provide dissimilar signaling outcomes even though they act through the same cell-surface receptor. It is possible that these agonists induce different autophosphorylation patterns in VEGFR2. Different signaling outcomes occur in mice expressing only specific VEGF isoforms as described below.

Lee et al. addressed the important question of whether such proteolytic maturation of VEGF occurs in vivo [208]. Using affinity chromatography on a polyclonal VEGF antibody column, they purified VEGF from ascites fluid obtained from women with ovarian cancer. The isolated fractions were evaluated with epitope-specific antibodies. They found that 80–90% of isolated VEGF lacks the carboxyterminal portion encoded by exon 8 (Fig. 1). An antibody against the amino-terminus recognized a group of monomeric fragments ranging from 13 to 16 kDa. This observation provides further evidence that proteolytic fragments occur in vivo. They reported, moreover, that the ascites fluid contains plasmin and MMP-3 enzyme activity. The matrix metalloproteases represent bona fide anti-neoplastic drug targets [200].

5.6. Differential stimulation of VEGF isoform action by heparin

Ashikari-Hada et al. compared the effects of heparin and various desulfated derivatives of heparin on VEGF-mediated human umbilical vein endothelial cell (HUVEC) mitogenesis [209]. They showed that heparin, but not the desulfated derivatives, increases VEGF-165-induced proliferation of HUVEC samples. In contrast, heparin has no effect on VEGF-121-induced proliferation. They also reported that heparin increases VEGF-165-induced endothelial cell tube formation on type I collagen gels.

Ashikari-Hada et al. found that heparin nearly doubles the tyrosine phosphorylation of VEGFR2 in VEGF-165-treated HUVEC samples, but not in VEGF-121-treated samples [209]. Moreover, they found that treatment of HUVEC samples with heparinase decreases VEGF-165-induced VEGFR2 tyrosine phosphorylation by 75%. Heparinase treatment has no effect on VEGF-121-induced VEGFR2 phosphorylation. Addition of heparin to heparinase-treated cells increases VEGF-165-stimulated phosphorylation to a level equivalent to cells not treated with heparinase. These findings suggest that heparin-like components in the extracellular matrix augment the action of VEGF-165. Addition of heparin to heparinase-treated cells does not increase VEGF-121-mediated receptor phosphorylation.

Heparin thus potentiates the outcomes of VEGF-165 on VEGFR2 autophosphorylation and on endothelial cell proliferation. These results are consistent with the observation that VEGF-165 contains but VEGF-121 lacks heparin-binding segments. Ashikari-Hada et al. suggested that the heparin-like domain of heparan sulfate forms a complex with both VEGF-165 and VEGFR2 thereby enhancing VEGF-165 signaling [209]. This attractive idea warrants further biochemical study.

6. Phenotypes of mice expressing specific VEGF isoforms

Carmeliet et al. generated mice expressing only VEGF-120 ($VEGF^{120/120}$), VEGF-164 ($VEGF^{164/164}$), or VEGF-188 ($VEGF^{188/188}$) using the Cre/loxP system to remove exons 6a, 6b, and 7 (VEGF-120), exons 6a and 6b (VEGF-164), or exon 6b (VEGF-188) (Fig. 1) [210,211]. This important approach relies on the expression of viral Cre recombinase in mice, where it is not usually expressed. This protein is capable of mediating recombination of DNA by recognizing pairs of DNA binding sequences, LoxP sites, and catalyzing the deletion of the DNA located between them.

The heterozygous mice ($VEGF^{+/120}$, $VEGF^{+/164}$, and $VEGF^{+/188}$) appear normal, healthy, and have a normal life span [210,211]. About half of the $VEGF^{120/120}$ homozygous mice die perinatally, and the others die within 2 weeks after birth owing to impaired myocardial angiogenesis resulting in cardiac failure. These mice also suffer from reduced renal angiogenesis and decreased bone formation. The $VEGF^{164/164}$ mice gain weight normally, are fertile, and have normal litter sizes (10 ± 3 pups). In contrast, about half of the $VEGF^{188/188}$ mice die in utero. Survivors gain less weight, are less fertile, and have smaller litter sizes (7 ± 2 pups).

Stalmans et al. reported that retinal vascular development is normal in $VEGF^{164/164}$ mice, thus indicating that this isoform possesses all of the necessary properties required for normal development of these blood vessels [211]. $VEGF^{188/188}$ mice have normal retinal venous development but impaired arterial outgrowth. $VEGF^{120/120}$ mice have faulty retinal venous development and severely defective arterial vascular development. Stalmans et al. found that $VEGF^{+/+}$, $VEGF^{164/164}$, and $VEGF^{188/188}$ mice have comparable kidney glomerular and heart arteriolar content. In contrast, $VEGF^{120/120}$ mice have fewer heart and kidney arterioles.

VEGF-164 binds to the extracellular matrix but is also able to diffuse. This isoform can produce a concentration gradient from cells that express this factor, and such gradients may be important in vasculogenesis and angiogenesis [211,212]. VEGF-164 may provide matrix-associated guidance cues that support physiological cell migration during development. VEGF-120, in contrast, is freely diffusible in vivo and is unable to establish a physiological growth-factor gradient. Moreover, VEGF-120 is less potent in inducing endothelial cell proliferation and has modestly reduced affinity for VEGFR1 and VEGFR2 in the presence of heparin when compared with VEGF-164 [203].

VEGF-188 is non-diffusible and may be unable to provide guidance cues to target endothelial cells. Moreover, neuropilin-1 and -2, co-receptors for VEGF, bind to residues expressed in exon 7. VEGF-120 lacks these residues (Fig. 1), and this deficiency and inability to bind to neuropilins may also explain the severity of the $VEGF^{120/120}$ phenotype. That the $VEGF^{164/164}$ animals are normal under laboratory conditions fails to provide any clues for the existence of the other VEGF isoforms. Although, VEGF-164 provides all of the information necessary for development, perhaps these animals would reveal deficiencies if subjected to trauma (to elicit wound healing) or to other stresses.

7. Regulation of VEGF gene expression by oxygen, growth factors, and oncogenes

7.1. Hypoxia-inducible transcription factor (HIF) family

The cellular concentration of oxygen plays a key role in regulating the expression of 50–500 or more genes [213,214]. When the oxygen concentration is low (hypoxia), the expression of many proteins including glucose transporters, enzymes of the glycolytic pathway, erythropoietin, and VEGF increases. The augmentation of these gene products constitutes an adaptive response to hypoxia. Glucose transporters and enzymes of the Embden-Myerhof-Parnas glycolytic pathway promote glucose catabolism; this is the only metabolic pathway in vertebrates that can generate substantial amounts of ATP in the absence of oxygen. Erythropoietin increases red blood cell production leading to increased oxygen transport. Increased VEGF production promotes angiogenesis, which in turn leads to increased red blood cell and oxygen delivery.

The HIF family lies at the center of the adaptive response to hypoxia in embryos, growing animals, and adults [215,216]. HIF is a heterodimer composed of HIF-1α (or the paralogs HIF-2α or HIF-3α) and HIF-1β. HIF-1α is expressed in most cells, whereas HIF-2α and HIF-3α display more restricted expression. HIF-1β is the aryl hydrocarbon receptor nuclear translocator (ARNT) that dimerizes with the aryl hydrocarbon receptor after activation of the latter by binding with compounds such as dioxin. Whereas HIF-1β expression and activity are unaffected by changes in oxygen concentration, the amount of cellular HIF-1α is augmented during hypoxia [215,216] by an unusual mechanism described below.

Both HIF-1α and HIF-1β subunits are members of the basic helix-loop-helix (bHLH) containing Per-ARNT-Sim (PAS), or bHLH-PAS, domain family of transcription factors [214]. Each subunit contains two conserved hydrophobic PAS domains of about 70 amino acids designated as PAS-A and PAS-B. PAS refers to the first three proteins (Per, ARNT, and Sim) in which this domain was identified. The PAS domains mediate heterodimer formation between HIF-1α and HIF-1β. HIF-1α possesses two transactivation domains (TAD). One is at the C-terminus and the other is toward the N-terminus accounting for the C-TAD and N-TAD nomenclature (Fig. 4). An inhibitory domain (ID), which represses TAD activity during normoxia, separates N-TAD and C-TAD. Overlapping N-TAD is an oxygen-dependent degradation (ODD) domain. Oxygen regulates HIF-1α cell content and activity by mechanisms that involve: (i) hydroxylation of proline 564 (preferred) or proline 402 in the human ODD domain and (ii) hydroxylation of asparagine 803 in the human C-TAD segment.

7.2. *HIF-1α prolyl hydroxylation and proteosomal degradation*

HIF-1α prolyl 4-hydroxylation regulates the level of HIF-1α in cells by promoting its proteosomal degradation. Under normoxic conditions, the product of the von Hippel-Lindau tumor suppressor gene (pVHL) recognizes the oxygen-dependent degradation (ODD) domain within the HIF-1α subunit [217]. pVHL is a component of a protein-ubiquitin ligase complex that targets the α-subunit for degradation by the proteasome. pVHL recognition of HIF-1α is dependent on the hydroxylation of proline 564 or proline 402 within the ODD domain. The half life of HIF-1α under normoxic conditions is less than 5 min. Under hypoxic conditions, 4-prolyl hydroxylation fails to occur resulting in decreased binding of HIF-1α to pVHL, decreased HIF-1α proteosomal degradation, and resultant HIF-1α accumulation. The stability of HIF-1α varies as a function of time of exposure to hypoxia. Using human HeLa cells, Berra et al. found that that the half-life of HIF-1α increased to 19 min after 1 h of hypoxia (1–2% oxygen) whereas the half-life progressively decreased to 5 min after 8 h of hypoxia [218]. A family of three prolyl hydroxylase domain-containing enzymes (PHD-1-PHD-3) catalyzes this posttranslational modification [216]. These enzymes, which are dioxygenases, catalyze the following reaction:

$$\text{HIF-1}\alpha\text{-proline} + \alpha\text{-ketoglutarate} + O_2 \rightarrow \text{HIF-1}\alpha\text{-proline-OH} + \text{succinate} + CO_2$$

Because oxygen is a substrate for these enzymes, a decrease in oxygen concentration (hypoxia) leads directly to less prolyl hydroxylation and diminished ubiquitinylation and proteosomal degradation.

Based upon results with HeLa cells treated with small interfering RNAs directed against each of the three main 4-prolyl hydroxylases, Berra et al. concluded that PHD-2 is the chief HIF-1α regulatory enzyme [219]. Moreover, Takeda et al. found that *Phd2*$^{-/-}$ embryos died between embryonic Days 12.5 and 14.5, whereas *Phd1*$^{-/-}$ or *Phd3*$^{-/-}$ mice were apparently normal, thereby confirming the essential nature of PHD-2 [220]. The *Phd2*$^{-/-}$ mice exhibited cardiac and placental defects.

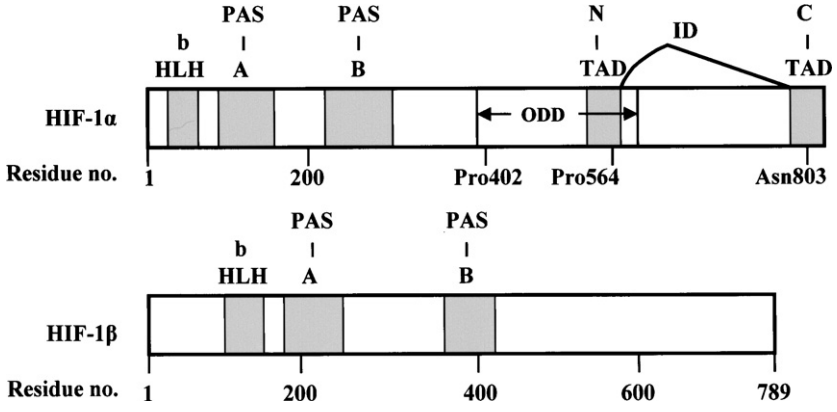

Fig. 4. Functional components of human HIF-1α and HIF-1β. The asparagine (Asn) and two proline (Pro) residues that can be hydroxylated are indicated. PAS-A and PAS-B, where PAS refers to Per-ARNT-Sim, participate in protein dimerization. bHLH, basic helix-loop-helix; ODD, oxygen-dependent degradation; C-TAD, C-terminal transactivation domain; ID, inhibitory domain; N-TAD, N-terminal transactivation domain.

7.3. HIF-1α asparaginyl hydroxylation and transcription

Hypoxia promotes the ability of the HIF-1α C-terminal transactivation domain (C-TAD) of HIF to interact with transcriptional co-activators such as p300 and CBP (CRE binding protein, where CRE is the cyclic AMP response element of genes) [215]. CBP and p300 are histone acetyltransferases that promote transcription. Under normoxic conditions, hydroxylation of asparagine 803 within C-TAD inhibits the association of HIF-1α with p300. Hypoxia diminishes asparaginyl hydroxylation allowing HIF to recruit the larger transcriptional apparatus to hypoxia-responsive target genes. An asparaginyl hydroxylase catalyzes the modification of a key residue (Asn803) within the HIF-1α C-TAD, thereby suppressing HIF activity. The factor inhibiting HIF (FIH), the asparaginyl 3-hydroxylase, is a dioxygenase that uses Fe^{2+} to bind oxygen and add it to both the target asparaginyl residue and α-ketoglutarate according to the following chemical equation:

$$\text{HIF-1}\alpha\text{-asparagine} + \alpha\text{-ketoglutarate} + O_2 \rightarrow \text{HIF-1}\alpha\text{-asparagine-OH} + \text{succinate} + CO_2$$

Asparaginyl 3-hydroxylase uses molecular oxygen as substrate, and a decreased oxygen concentration (hypoxia) leads directly to less hydroxylation owing to the diminished substrate concentration. Vitamin C, moreover, maintains iron in the reduced, or ferrous (Fe^{2+}), state in the HIF-1α prolyl and asparaginyl hydroxylases [221].

The K_m values of the HIF prolyl hydroxylases for oxygen are about 240 μM and that of HIF asparaginyl hydroxylase is about 90 μM [221]. A possible consequence of this difference is that HIF-1α would be stable owing to the absence of prolyl hydroxylation and yet would be transcriptionally inactive because of asparaginyl hydroxylation [215]. Note that the concentration of dissolved oxygen in blood plasma at 37 °C in equilibrium with air ($pO_2 \approx 150$ Torr) is about 200 μM [222]. However, the oxygen concentration in the circulation (pO_2 ranging from 40 to 100 Torr) and in the interstitial compartment is less. That in the cell is even lower owing to the consumption of oxygen by oxidative phosphorylation and other oxygen-requiring reactions.

7.4. Responses to hypoxia

Hypoxia inducibility of genes is conferred by a hypoxia response element (HRE) that occurs in the promoter or enhancer of target genes [223]. Although the human *VEGF* gene encodes multiple splice variants, analysis of its promoter reveals that a single hypoxia response element is located at nucleotide positions −975 to −968 (5′-TACGTGGG-3′) relative to the common transcription start site [56]. Hypoxia decreases HIF-1α prolyl and asparaginyl hydroxylation. As a result of diminished prolyl hydroxylation, HIF-1α is stabilized and forms a dimer with HIF-1β, thereby forming the transcription factor. As a result of diminished asparaginyl hydroxylation, HIF-1α complexed with HIF-1β is able to interact with CBP or p300 and initiate the transcription of target genes. The presence of hypoxia response elements has been demonstrated directly in more than 50 genes [214].

Using a systems biology approach, Manalo et al. compared the gene expression profiles of human pulmonary artery endothelial cells after 24 h in hypoxic (1% oxygen) versus normoxic (20% oxygen) conditions and after 24 h in normoxic cells infected with an adenovirus encoding a constitutively active form of HIF-1α versus adenovirus encoding β-galactosidase [213]. Following cDNA and biotin-tagged cRNA synthesis, the cRNA was hybridized to a human genome array containing 22, 283 human gene probe sets. They identified 245 gene probes with increased expression (>1.5-fold) and 325 gene probes with decreased expression in both the: (i) hypoxia and (ii) HIF-1α-expressing groups.

The genes with increased expression included VEGF, VEGF-C, PlGF, and PDGF-B [213], all of which are implicated in angiogenesis. VEGF has a hypoxia response element [214], but the mechanism of induction of the other three of these growth factors is ill defined. The *VEGFR1* gene, which has an HRE [214], was not reported to be induced in this study [213]. The observed increase in prolyl hydroxylase domain-containing PHD-2 and PHD-3 mRNAs provides a potential feedback mechanism for repressing HIF-1α expression. Other growth factors, receptors, and signal transduction molecules exhibited increased expression. More than a dozen genes encoding collagens and collagen-modifying enzymes were induced. Genes that were repressed by hypoxia and HIF-1α expression include cyclins, DNA and RNA polymerases, and replication factors.

More than two dozen genes encoding transcription factors in both the hypoxia and HIF-1α-expressing groups were up-regulated while about one dozen transcription factors were down-regulated [213]. These data suggest that HIF-1α may be at the top of a hierarchy of oxygen-regulated gene expression within pulmonary artery endothelial cells. Moreover, the authors point out that some of the genes that were induced in this study may represent secondary HIF targets owing to the relatively long 24-h treatments [213].

7.5. Growth factors and hormones

Although the role of hypoxia has been more thoroughly investigated, it is clear that growth factors, hormones, oncogenes, and tumor suppressor genes regulate *VEGF* gene expression [10,29]. For example, epidermal growth factor stimulation (Table 5) of several human glioma cell lines results in a 25–125% increase in the secretion of bioactive VEGF [224]. Heparin-binding epidermal growth factor-like growth factor (HB-EGF), which is a member of the epidermal growth factor family, increases the expression of VEGF mRNA in human bladder carcinoma EJ cells in culture [225]. In EJ cancer cells containing a tetracycline-regulated soluble HB-EGF or membrane-anchored HB-EGF expression

Table 5
Selected growth factors and hormones that increase VEGF expression

Growth factor/hormone	Citation
Activin A	[230]
Basic fibroblast growth factor (bFGF)	[231]
Epidermal growth factor (EGF)	[224]
Follicle-stimulating hormone (FSH)	[237]
Heparin-binding epidermal-like growth factor (HB-EGF)	[225]
Insulin	[235]
Insulin-like growth factor-1 (IGF-1)	[236]
Interferon-β (IFN-β)	[233]
Interleukin-1 (IL-1)	[231,232]
Interleukin-6 (IL-6)	[233,234]
Luteinizing hormone (LH)	[237]
Platelet-derived growth factor (PDGF)	[226]
Progesterone	[240]
Testosterone	[238,239]
Transforming growth factor-β (TGF-β)	[227–229]
Tumor necrosis factor-α (TNF-α)	[231,233]

system, tetracycline induction following subcutaneous injection of the cells enhances tumor progression and increases the content and size of tumor blood vessels in athymic nude mice. Moreover, platelet-derived growth factor produces an increase in VEGF mRNA expression in NIH 3T3 fibroblasts, which is mediated by protein kinase C-α [226].

Transforming growth factor-β (TGF-β) treatment of quiescent cultures of mouse embryo-derived AKR-2B cells and human lung adenocarcinoma A549 cells increases VEGF mRNA and protein content [227]. In contrast, the related PlGF mRNA is not induced by TGF-β in these cells. TGF-β also stimulates *VEGF* gene transcription in human cholangiocellular carcinoma cells in culture [228]. Studies using 5′ deletion and mutational analysis of the human VEGF promoter revealed that TGF-β stimulates VEGF protein production through Sp1-dependent transcriptional activation. Furthermore, TGF-β also stimulates VEGF expression in human hepatocellular carcinoma (HuH-7) cells in culture by a mechanism that involves downstream signaling by Smad3-Smad4 [229]. Activin A, a member of the TGF-β cytokine superfamily, increases VEGF protein expression in hepatocellular carcinoma cells in culture by a process that involves Sp1-Smad2 interaction [230].

Ryuto et al. demonstrated that tumor necrosis factor-α, basic fibroblast growth factor, and interleukin-1 enhance VEGF mRNA expression in U251 human brain glioma cells [231]. Kawaguchi et al. showed that treatment of the TMK-1 human gastric cancer cell line with interleukin-1β increases VEGF mRNA expression [232]. This increase, which does not involve a change in mRNA half life, lasts for at least 24 h. Cohen et al. showed that interleukin-6, a proinflammatory cytokine, increases VEGF mRNA levels in human epidermoid carcinoma A431 cells, L8 cells (skeletal muscle myoblasts), and C6 glioma cells in culture [233]. Moreover, these workers showed that interferon-β and tumor necrosis factor-α also augment VEGF mRNA levels in the A431 cells. The level of VEGF mRNA induction was similar to that produced by hypoxia. Huang et al. reported that interleukin-6 increased VEGF expression in four gastric carcinoma cell lines [234]. These workers also showed that the level of interleukin-6 was positively correlated with VEGF expression and tumor vascular content in 54 human surgical specimens.

Bermont et al. showed that insulin increases the amount of VEGF protein in the conditioned medium derived from endometrial carcinoma cells (HEC-1A) in culture [235]. They also showed that insulin increases the rate of VEGF transcription and prolongs the half-life of VEGF mRNA. Warren et al. reported that insulin-like growth factor-1 (IGF-1) increases the expression of VEGF mRNA and protein by COLO 205 colon carcinoma cells [236]. IGF-1 also induces expression of VEGF mRNA in SW620, LSLiM6, and HCT15 colon carcinoma cells. IGF-1 augments the cellular content of VEGF mRNA by increasing the rate of transcription and by increasing the half-life of VEGF mRNA. These experiments suggest that IGF-1 and its receptor represent potential targets in the treatment of colorectal cancer.

Wang et al. showed that both follicle-stimulating hormone and luteinizing hormone increase VEFG-165 expression in serous ovarian tumor cells [237]. This observation suggests that VEGF may play a role in the pathogenesis of ovarian cancer and that the elevated gonadotropins, as found in menopause and in most ovarian cancer patients after surgery, could accelerate tumor progression and recurrence by inducing VEGF expression in ovarian neoplasms.

Haggstrom et al. demonstrated that VEGF mRNA and protein levels are significantly decreased in rat ventral prostate epithelium by castration while testosterone restores VEGF expression [238]. However, VEGFR1 and VEGFR2 are unaffected by castration and testosterone treatment. Later work demonstrated that these responses are inhibited by chimeric VEGFR1-Ig, a VEGF, PlGF, and VEGF-B binding protein, or trap [239].

Hyder et al. demonstrated that progesterone increases VEGF protein in T47-D human breast cancer cells [240]. In addition to progesterone, a number of synthetic progestins used in oral contraceptives (norethindrone, norgestrel, and norethynodrel), hormone replacement therapy (medroxyprogesterone acetate), and high-dose progestin treatment of breast cancer (megestrol acetate) also increase VEGF activity in the media of cultured T47-D cells. This effect is hormone specific and is not produced by estrogens, androgens, or glucocorticoids. The induction of VEGF by progestins is also cell type specific and does not occur in human breast cancer cell lines MCF-7, ZR-75, or MDA-MB-231. The T47-D cells express a mutant form of the p53 tumor suppressor. More recent work showed that stable transfection of wild-type p53 in the T47-D cells inhibits the progestin-dependent induction of VEGF [241]. See Table 5 for a summary of selected growth factors and hormones that increase VEGF expression.

Mutations that result in the inactivation of tumor suppressor genes occur in a variety of cancers [242]. Zhang et al. demonstrated that the restoration of wild-type p53 expression in human leiomyosarcoma SKLMS-1 cells, which

ordinarily express mutant p53, markedly inhibits angiogenesis induced by tumor cells in vivo [243]. Microvessel content was lower in tumor xenografts from cells expressing wild-type p53 than in xenografts from cells expressing mutant p53. Conditioned medium from sarcoma cells expressing wild-type p53 decreased both the growth and migration of murine lung endothelial cells when compared with conditioned medium from sarcoma cells expressing mutant p53. The introduction of wild-type p53 into the SKLMS-1 sarcoma cells producing mutant p53 significantly reduces the expression of VEGF. Thus, failure to express wild-type p53, mutations of which occur in the majority of human cancers, allows for increased expression of VEGF, a driver of angiogenesis.

7.6. Oncogenes

Mutations that result in the conversion of proto-oncogenes to oncogenes occur in a variety of cancers [244]. It would not be surprising if these gain-of-function mutations lead directly or indirectly to angiogenesis because of increased pro-angiogenic signaling and to decreased anti-angiogenic signaling [245]. Mutations of the *ras* proto-oncogene occur in a large fraction of human cancers [245]. One function of Ras is to activate the Raf-Mek-Erk MAP kinase cascade [246]. Ras activates Raf, an intracellular serine/threonine protein kinase. Raf catalyzes the phosphorylation and activation of Mek. Mek is a dual specificity kinase that catalyzes the phosphorylation of threonine and tyrosine in the activation loop of Erk1/2 thereby leading to their activation. Kinase Suppressor of Ras (KSR) is a scaffold protein that links Raf-Mek-Erk to Ras [246]. Erk1/2, in turn, catalyze the phosphorylation of several transcription factors that result in cell proliferation. Several studies support the notion that Ras signaling leads to the increased expression of VEGF.

In 1989, Thompson et al. retrovirally transfected 0.1% of mouse fetal urogenital sinus cells, which develop into the prostate gland, with the *H-ras* oncogene [247]. The resulting composite cells (transfected and non-transfected) were grafted under the renal capsule of syngeneic adult male hosts, and the reconstituted organs were removed after 28 days. The explants exhibited both angiogenesis and dysplasia. This observation suggested that a *ras* oncogene may contribute to the angiogenic switch during tumor progression. However, similar transfection of these cells with another oncogene, *myc*, failed to induce angiogenesis but did induce hyperplasia when compared with uninfected cells [247].

Grugel et al. stably transfected NIH3T3 cells with activated *v-H-ras* or *v-raf* [248]. They found that these serum-starved transformed cell lines expressed increased levels of VEGF mRNA and protein when compared with untransformed cells. Ras leads to an increase in the expression of Jun and Fos that form the AP-1 transcription factor [249]. Erk1/2 catalyze the phosphorylation of Jun [250]. Jun/Fos dimers bind to AP-1 transcription sites, four of which occur in the VEGF promoter [251]. These results support the notion that Ras and Raf not only drive cell proliferation but they also mediate angiogenesis.

Rak et al. studied an immortalized non-tumorigenic line of rat intestinal epithelial cells (IEC-18) transfected with activated human *H-ras* or chicken *v-src* [252]. Subcutaneous injection of the *H-ras* transformed cells leads to the formation of highly vascularized tumors in athymic nude mice. In culture, these transformed cells produce VEGF mRNA and protein whereas the non-transfected cells do not. Anti-VEGF monoclonal antibody (Ab 617) suppressed the mitogenic activity of conditioned medium of both *H-ras* and *v-src* transformed IEC-18 cells as determined in HUVEC samples, indicating that these cells release VEGF.

Rak et al. also studied two human colon cancer cell lines (DLD-1 and HCT 116) that both express a human *K-ras* allele and two sublines in which this mutant allele was disrupted by targeted homologous recombination [252]. The DLD-1 and HCT-116 cell lines form tumors in athymic nude mice whereas the disrupted *K-ras* sublines do not. They found that VEGF in conditioned medium from the disrupted *K-ras* sublines was 20–25% that of the parental sublines. Moreover, they found that the medium from the parental cell lines was more active in inducing mitogenesis in HUVEC samples than that from the disrupted mutant sublines. They also reported that L-739–749, a Ras farnesyltransferase inhibitor that blocks Ras function, suppressed the HUVEC growth-stimulatory activity of conditioned medium from the *H-ras* transformed IEC cells. Based upon these genetic and pharmacological studies, Rak et al. concluded that *ras* oncogenes induce VEGF production.

Arbiser et al. immortalized mouse endothelial cells by infecting them with an ecotropic retrovirus encoding SV40 large T antigen [253]. Injection of these cells (MS1) into the flank of athymic nude mice led to the development of small non-progressive tumors. These investigators infected these cells with a second retrovirus encoding activated *H-ras* to produce SVR cells. Injection of these cells into athymic nude mice led to rapidly progressive tumors. They demonstrated that the *H-ras* expressing cells (SVR) produce considerably more VEGF mRNA and more MMP-2 and MMP-9 activity in culture than the non-*H*-ras MS1 cells. Moreover, the *H-ras* SVR cells express less TIMP activity than the non-*H-ras* MS1 cells. Treatment of the activated *H-Ras*-expressing cells with wortmannin, an inhibitor of PI 3-kinase, decreased MMP expression. Furthermore, wortmannin injection intralesionally into Ras-expressing cellular implants decreased tumor growth in mice by 66%. The authors suggested that the failure of wortmannin to completely inhibit the activated *H-ras*-induced tumor growth indicates that mechanisms in addition to the PI 3-kinase pathway are involved in promoting tumor progression [253].

Amplification of *ras* leads to increased VEGF expression in a variety of tumors [254]. For example, high VEGF levels occur in about half of human non-small cell lung carcinomas bearing a *K-ras* mutation and in one-quarter of those lacking a *K-ras* mutation [255]. Moreover, about three-quarters of pan-

creatic carcinomas with *K-ras* mutations and 40% of tumors with wild-type *K-ras* exhibited elevated VEGF mRNA levels [256]. Thus, there is not a strict correlation of Ras signaling and VEGF expression. In addition to increased VEGF expression driven by Ras, Raf, and Src noted here, Folkman adds Myb, Myc, HER-1 (EGFR), HER-2, Fos, Bcl-2, and other oncoproteins as direct or indirect stimulators of angiogenesis [257].

8. VEGF and tumor progression

8.1. Tumor growth and angiogenesis

Tumor growth requires new blood vessel formation, and Folkman proposed in 1971 that inhibiting angiogenesis might be an effective antitumor strategy [258]. The proof of the requirement for angiogenesis in tumor progression came from experiments involving the placement of tumor fragments or cultured tumor cells into the cornea of a rabbit eye, an avascular site (reviewed in ref. [9]). The implants attracted new capillaries that grow in from the marginal region of the cornea of the eye to vascularize the expanding tumor mass. If the capillaries are physically prevented from reaching the implant, the tumor nodule does not exceed a diameter of about 0.4 mm. Further growth is not possible because of an inadequate supply of oxygen and nutrients. When angiogenesis is absent or blocked, experimental tumors range in diameter from 0.2 to 2.0 mm (depending upon the tumor type), which corresponds to about 10^5–10^6 tumor cells [8].

The occurrence of new blood vessel growth suggested that tumors release diffusible activators of angiogenesis that signal a normally quiescent vasculature to begin capillary sprouting. New capillary growth requires both endothelial cell division and migration. Bioassays for these two processes led to the identification of acidic and basic fibroblast growth factors [9] and to the identification of vascular permeability factor/vascular endothelial growth factor [41–49]. Acidic and basic fibroblast growth factors, their receptors, and VEGF are widely expressed in normal adult human and mouse organs (see ref. [259] for a review). In contrast, the VEGF family of receptor protein-tyrosine kinases is generally, although not exclusively, restricted to endothelial cells in the cardiovascular and lymphatic circulatory systems [32–38,76].

8.2. VEGF expression in tumors

VEGF is expressed in a wide variety of tumors (see ref. [260] for a comprehensive list). VEGF mRNA is expressed in neoplastic cells but little occurs in endothelial cells. In contrast, endothelial cells express VEGFR1 and VEGFR2 mRNAs and proteins. These findings are consistent with the concept that VEGF functions as a paracrine mediator: VEGF secreted from neoplastic cells influences nearby endothelial cells. Immunohistochemical studies showed that VEGF is localized on both neoplastic and endothelial cells [260,261]. These observations indicate that VEGF accumulates on the endothelial target cells, which express little VEGF mRNA. Moreover, Dvorak et al. found that VEGF protein occurs in the vessels near solid tumors in guinea pigs and humans but is absent in vessels more than 0.5 mm from the tumor [261]. When transplanted guinea pig tumors undergo immune rejection, VEGF protein is lost within 24–48 h.

9. Inhibition of VEGF family signaling

9.1. Anti-VEGF antibodies

Diseases that are candidates for therapeutic inhibition of VEGF signaling include neovascular age-related macular degeneration of the eye, diabetic retinopathy, endometriosis, psoriasis, rheumatoid arthritis, and tumor growth and spread [10,29]. Of these disorders, the role of VEGF in tumor angiogenesis has received the greatest attention. Various strategies for restraining tumor growth and progression include curbing VEGF signaling by using antibodies directed against VEGF or VEGFR2 or by using small molecule inhibitors directed against VEGF receptor kinases [262].

In a pioneering study, Kim et al. found that injection of a mouse monoclonal antibody (Mab A.4.6.1) directed against all of the human VEGF isoforms suppresses the growth of human xenografts in athymic nude mice in vivo [263]. They showed that this antibody inhibits the growth of the SK-LMS-1 leimyosarcoma, the A673 rhabdomyosarcoma, and the G55 glioblastoma multiforme between 70% and 95%. Neither VEGF nor Mab A.4.6.1 has any effect on the growth of these neoplastic cells in culture, indicating that the antibody does not target the tumor cells per se. These observations demonstrated directly that inhibition of endogenous VEGF suppresses tumor progression in vivo.

A comparison of the X-ray crystallographic structures of VEGF bound to the antigen-binding fragment (Fab) of bevacizumab (a humanized Mab A.4.6.1 derivative) and to the VEGFR1 binding site in the second extracellular immunoglobulin domain (D2) provides insight on the mechanism of action of the antibody [64,123,264]. Because the overall structure of free $VEGF_{8–109}$ is identical to that in the complex with the antigen-binding fragment (Fab), the inhibitory action of anti-VEGF is not the result of an induced conformational change of the factor. Of the 19 residues of $VEGF_{8–109}$ that occur at the antigen-Fab interface, 9 also occur at the $VEGF_{8–109}$-$VEGFR1_{D2}$ interface. The inhibitory effect of this monoclonal antibody thus results from sterically blocking the interaction of VEGF with VEGFR1 and most likely with VEGFR2.

The growth of human tumor cells implanted in athymic nude mice depends upon the development of supporting stroma (fibroblasts, inflammatory, and vascular cells) from mouse host cells. Various human tumor xenografts differ in their sensitivity to Mab A.4.6.1 [263,265]. Liang et al. tested the idea that one source of this variation results from

VEGF produced by mouse stromal cells (Mab A.4.6.1 and the humanized bevacizumab recognize human but not mouse VEGF). They generated antibodies that bind to both human and mouse VEGF. They found that these antibodies completely prevent the growth of cell implants corresponding to human colorectal (HM-7 cells), pancreatic (HPAC cells), and skeletal muscle (A673 cells) tumors in athymic nude mice [265]. In contrast, bevacizumab is ≈90% effective at inhibiting the growth of the skeletal muscle and colorectal tumors but is less than 50% as effective at inhibiting the growth of the pancreatic tumor. They measured human and mouse VEGF in these three tumors and found that the two more sensitive tumors contained little mouse VEGF whereas the less sensitive pancreatic neoplasm contained appreciable mouse VEGF. These results implicate stromally produced VEGF as an important participant in tumor progression, a finding that that is germane to human neoplasms [266].

Bevacizumab is less effective as a single agent (monotherapy) when used in the treatment of human cancers when compared with human tumor xenografts in athymic nude mice [8,267]. Although, bevacizumab monotherapy significantly increased the time to progression in patients with metastatic renal cell cancer, it did not increase overall survival [268]. In clinical studies that compared the efficacy of standard metastatic colorectal chemotherapy (irinotecan, 5-fluorouracil, and leucovorin) with and without bevacizumab, median survival with bevacizumab was increased from 15.6 to 20.3 months. Similar increases were seen in progression-free survival, response rate, and duration of response. Although, the benefits observed in these studies were modest, the U.S. Food and Drug Administration approved bevacizumab (February, 2004) as a part of the first-line treatment along with cytotoxic agents for metastatic colorectal cancer. Bevacizumab is undergoing clinical trials in the treatment of breast, head and neck, non-small cell lung, pancreatic, prostate, and renal cell cancer as well as hematological malignancies [269].

9.2. VEGF traps (genetically engineered VEGF-binding proteins)

Other strategies to inhibit VEGF family signaling have been documented [270]. For example, constructs modeled after the VEGF receptors have been produced. One of these is a chimeric protein consisting of the second extracellular immunoglobulin domain of VEGFR1, the third extracellular immunoglobulin domain of VEGFR2, and the Fc portion of a human antibody. The resulting protein acts as a decoy receptor, or trap, that binds both VEGF (K_d = 1–10 pM) and PlGF (K_d = 45 pM). This VEGFR1/2-trap is a potent inhibitor of experimental angiogenesis that has the advantage of targeting VEGF and PlGF. Recall that PlGF can increase the cellular response to VEGF [109,111]. The VEGFR3-trap, which is a chimeric VEGFR3-Ig, has high affinity for VEGF-C and -D. This protein inhibits tumor lymphangiogenesis and tumor spread to lymph nodes in experimental animals. Metastatic spread of tumors via lymphatic vessels to lymph nodes occurs in a variety of human carcinomas. Furthermore, antibodies against VEGFR2 and VEGFR3 have been developed that block VEGF family signal transduction [270].

9.3. VEGF receptor protein-tyrosine kinase inhibitors

Small molecule inhibitors of VEGF receptor protein-tyrosine kinases represent another approach for inhibiting angiogenesis [271,272]. The U.S. Food and Drug administration approved BAY 43-9006 (sorafenib, Nexavar) monotherapy for the treatment of metastatic renal cell carcinoma in December 2005 [273]. BAY 43-9006 inhibits several protein kinases including VEGFR2, VEGFR3, Flt-3, Kit, PDGFR-β, and Raf [274]. Advanced renal cell cancer, which is often associated with up-regulated Raf, epidermal growth factor receptor, VEGF and VEGF receptor activity, is a highly vascularized tumor thus making it an attractive anti-angiogenic target [273]. Moreover, the U.S. Food and Drug Administration approved SU-11248 (sunitinib, Sutent) monotherapy for the treatment of: (i) metastatic renal cell carcinoma and (ii) gastrointestinal stromal tumors (GIST) in January 2006 [275]. SU-11248 inhibits several receptor protein-tyrosine kinases including VEGFR1, VEGFR2, VEGFR3, colony-stimulating factor-1 receptor, Flt-3, platelet-derived growth factor receptors (α and β), and Kit, the stem cell factor receptor [276]. STI-571 (imatinib, Gleevec), which is a Kit inhibitor, was previously approved for the treatment of gastrointestinal stromal tumors [277], and SU-11248 is approved for people with tumors resistant to STI-571 or who otherwise cannot take the drug. STI-571 also inhibits platelet-derived growth factor receptor and Abl protein-tyrosine kinases. Other compounds under development and in clinical studies as anti-angiogenic agents include PTK787/ZK222584 (vatalanib, a VEGFR1, VEGFR2, VEGFR3, PDGFR-β, Flt-3, and Kit inhibitor) and ZD6474 (a VEGFR1, VEGFR2, VEGFR3, Abl, and epidermal growth factor receptor inhibitor) [271,272,276].

The hypothesis that tumor angiogenesis could serve as a target for cancer therapy [258] is now strongly supported by numerous human clinical trials. VEGF pathway inhibitors are now being explored in combination with chemotherapy for virtually every type of solid tumor. For a current overview of cancer clinical trials with VEGF signal transduction inhibitors, see http://www.cancer.gov/clinicaltrials/developments/anti-angio-table.

10. Tumor metastasis, the pre-metastatic niche, and VEGFR1

Hiratsuka et al. studied Lewis lung carcinoma growth in primary subcutaneous injection sites and metastatic sites that occur following tail vein injection in wild type and mutant mice lacking the intracellular protein-tyrosine kinase domain of VEGFR1 (*VEGFR1-TK*$^{-/-}$) [278]. They found that tumor

growth in the primary site was the same in wild type and mutant mice. However, when they injected cancer cells intradermally and allowed the tumor to grow to 1.5 cm in diameter and then injected the neoplastic lung cells by tail vein, they found that the metastatic tumor mass in wild-type mice was three times that of the *VEGFR1-TK*$^{-/-}$ mice. Thus, wild-type VEGFR1 expression results in an increased metastatic tumor mass.

Hiratsuka et al. found that expression of matrix metalloproteinase-9 (MMP-9) in lung endothelial cells at the mRNA and protein levels is increased in wild-type tumor-bearing mice when compared with mutant tumor-bearing mice [278]. Furthermore, they found that macrophages occurring in lungs contain twice the amount of MMP-9 in wild type compared with *VEGFR1-TK*$^{-/-}$ mice in tumor-bearing animals. The matrix metalloproteases have the potential to enhance angiogenesis by liberating non-diffusible VEGF from the extracellular matrix [198], and they also mediate extracellular matrix breakdown that promotes angiogenesis and metastasis [200,201]. MMPs thus represent bona fide anti-neoplastic drug targets. AG-3340 (prinomastat) and BMS-275291, which are broad-spectrum MMP inhibitors, are under development and in clinical trials for the treatment of non-small cell lung cancer [200,272].

Hiratsuka et al. reported that human lung samples in people with various tumors including colon, esophagus, liver, pancreas, ovary, and stomach express increased MMP-9 when compared with people without tumors [278]. The mechanism for tumor-induced up-regulation of MMP-9 in mouse lung requires VEGFR1 protein-tyrosine kinase activity, but the identities of the signaling pathways responsible for enhanced MMP-9 expression are unclear.

Metastasis is a sequential process, contingent on tumour cells breaking off from the primary tumour, intravasating into and travelling through the bloodstream, and stopping at a distant site. At the new site, the cells extravasate and establish a blood supply and grow to form a life-threatening mass. Kaplan et al. tracked the movements of various cells as tumors metastasized to the lungs of mice [279]. The mice were first irradiated to eliminate their bone-marrow cells; these cells were replaced by bone-marrow cells that express green fluorescent protein that can be visualized microscopically. Once the new bone-marrow cells were established, the mice were injected intradermally with Lewis lung carcinoma or B16 melanoma cells, each expressing red fluorescent protein. Lewis lung carcinoma cells metastasize to the lungs and occasionally the liver whereas the B16 melanoma cells disseminate more widely.

Following subcutaneous injection, Lewis lung carcinoma cells form a primary tumor in the skin that subsequently metastasizes to the lungs. Kaplan et al. found that the green bone-marrow-derived cells appear in the lungs 12–14 days after injection of the red Lewis lung carcinoma cells [279]. However, the red tumour cells appear in lung 18 days after injection and micrometastases form 23 days after injection with more than 95% of the tumour cells occurring in precisely the same sites as the bone-marrow-derived cells. These experiments suggested that the bone-marrow-derived cells establish a pre-metastatic niche in the lungs.

Using immunohistochemistry, Kaplan et al. reported that the bone-marrow cells expressVEGFR1 [279]. Moreover, treatment of mice with an antibody to VEGFR1 prevents the pre-metastatic sites from forming. If the experiment was performed with bone marrow cells that do not express VEGFR1, neither pre-metastatic niches nor metastases form. Anti-VEGFR1 antibody treatment in mice eliminates the formation of the initiating pre-metastatic sites and completely prevents metastasis. Thus, anti-VEGFR1 prevents the pre-metastatic niche from forming. Treatment with anti-VEGFR2 antibody does not prevent the formation of VEGFR1 postive sites but limits metastatic progression. Based upon the use of conditioned medium, the authors found evidence that PlGF, which activates VEGFR1 but not VEGFR2, plays a role in the formation of pre-metastatic sites.

Kaplan et al. found that other signaling components are required for the formation of the pre-metastatic niche [279]. They reported that the VEGFR1-expressing cells contain VLA-4, or integrin $\alpha_4\beta_1$. This integrin interacts with fibronectin, and fibronectin expression increases in pre-metastatic niches. They also found that MMP-9 is expressed in the pre-metastatic sites and suggested that this expression may result from enhanced $\alpha_4\beta_1$-integrin signaling through VEGFR1-expressing hematopoietic progenitor cells.

The authors found VEGFR1-expressing cellular clusters in a number of primary and metastatic human tumors including those of breast, gastroesophageal junction, and lung. There were increased VEGFR1-expressing cellular clusters in common sites of metastasis before tumor spread. They proposed that these might also be pre-metastatic niche cells [279].

These findings suggested that inhibitors of this multifaceted pathway have the potential to block metastasis. But if such preventative therapy were to be tested in human clincial trials, it should not be directed at patients with metastatic disease, which is where most clinical testing begins. In such patients, the pre-metastatic niches and metastases have already formed. Rather, inhibition of VEGFR1 would be more germane in patients in the adjuvant setting, for instance those at high risk of metastatic disease. It is in these patients that the formation of metastases might be interrupted. However, the contribution of bone-marrow-derived cells to human tumors may be significantly lower than that for mouse tumors [8], and the efficacy of this strategy in humans remains to be established.

11. VEGF and vascular endothelial cell survival

Although, the role of VEGF family signaling during vasculogenesis and angiogenesis during development is well documented, the function of VEGF in adults is less clear [61]. Kamba et al. used a variety of VEGF signal trans-

duction inhibitors to determine which vascular beds in adult mice depend upon VEGF signaling for survival [280]. These inhibitors include AG-013736 (an inhibitor of VEGFR1, VEGFR2, VEGFR3, PDGFR-β, and Kit protein-tyrosine kinases), soluble VEGFR1 (a VEGF, PlGF, and VEGF-B trap), and soluble VEGFR2 (a VEGF, VEGF-C, and VEGF-D trap). These agents failed to produce capillary regression in adrenal medulla, heart, and tongue muscle. However, all of these agents produced capillary regression in adrenal cortex, anterior and posterior pituitary, choroid plexus, pancreatic islets, renal glomeruli and peritubular capillaries, small intestinal villi, and thyroid. VEGF-dependent capillaries contained diaphragm-covered fenestrations and strongly expressed both VEGFR2 and VEGFR3. These investigators found that non-fenestrated capillaries express low levels of VEGFR2 and no VEGFR3 immunoreactivity. These results implicate VEGFR3 in VEGF-dependent capillary stabilization. Although VEGF fails to bind to VEGFR3, it may induce VEGFR2-VEGFR3 complex formation and VEGFR3 signaling [139]. Surprisingly, VEGF-dependent capillaries had normal pericyte coverage, which contradicts the adage that pericytes stabilize endothelial cells and makes them less VEGF-dependent [281].

Maharaj et al. used mice expressing the β-galactosidase (*lacZ*) reporter gene with a nuclear localization signal and an internal ribosome entry site inserted into the 3′ untranslated region of the *VEGF* gene [61]. This gene yields a bicistronic mRNA that produces functional VEGF and β-galactosidase in the same cells. They found that these proteins are co-expressed in a wide variety of adult mouse cells including brainstem, cerebellum, cerebrum, and olfactory bulb. Moreover, secretory glands including the adrenal, the endocrine and exocrine pancreas, prostate, salivary gland, and testes also produce VEGF. Furthermore, adipocytes, aorta, liver, lung, skeletal, and cardiac muscle express the factor. They found that VEGF is robustly expressed in epithelial cells that directly overlay fenestrated vessels in the choroid plexus. Moreover, podocytes in the kidney, which contact the basement membrane of the fenestrated glomerular endothelium, express high levels of VEGF. These investigators examined VEGFR2 in selected tissues and found that this receptor occurs in its phosphorylated and activated form in adipocytes, aorta, kidney, liver, and lung.

Maharaj et al. postulated that VEGF stabilizes vessels in adults, which has important implications in the treatment of diseases with VEGF inhibitors [61]. Two of the more common grades 3 and 4 side effects of bevacizumab (a therapeutic antibody against VEGF) include proteinuria and hypertension [282]. Proteinuria occurred in a few percent of patients, and hypertension occurred in 3–12%, depending upon the study. Hypertension also occurred in people treated with SU-11248 (sunitinib) [283] and BAY 43-9006 (sorafenib) [284], small molecule drugs that inhibit VEGF receptors and other protein kinases. Those with hypertension responded to standard therapies. However, the occurrence of hypertension and proteinuria in people treated with the VEGF-trap led to its discontinuance from clinical trials [285]. Both the studies of Maharaj et al. and Kamba et al. suggest that VEGF influences the kidney vasculature in adults [61,280]. Moreover, proteinuria and hypertension occur in pregnant women with preeclampsia as a result of the placental production of soluble VEGFR1 that binds maternal VEGF [117,118]. It appears that hypertension and proteinuria are related to the blockade of VEGF action on the renal vasculature as a result of sequestering VEGF (bevacizumab, soluble VEGFR1, VEGF-trap) or by inhibiting the VEGF receptor kinases. However, the biochemical and pharmacological mechanisms that produce these effects are ill defined. The hypertension may involve increased vascular tone mediated by decreased endothelial nitric oxide synthase activation and decreased nitric oxide (endothelium-derived relaxing factor) generation as a result of blockade of VEGFR2 (Fig. 2) [8].

12. Epilogue

Following an injury or wound, activated cells (mast cells, platelets) release several cytokines and growth factors including VEGF. VEGF attracts circulating neutrophils and monocytes. Activated cells also release tissue and urokinase type of plasminogen activators, which in turn catalyze the conversion of plasminogen to plasmin. As noted previously, plasmin can mobilize VEGF-165 and VEGF-189 and uPA can mobilize VEGF-189 from the extracellular matrix [195,198,199,205–207]. Several matrix metalloproteases are also released and may liberate heparan sulfate-bound VEGF-165 and VEGF-189 [208]. VEGF increases vascular permeability, and this action aids in the movement of proteins and cells, which are needed for repair, from the vascular compartment into the extracellular matrix.

The interrelationships among the members of the VEGF signaling family are elaborate. There are five members of the human VEGF family of growth factors, and several of these (VEGF, VEGF-B, and PlGF) possess multiple isoforms. The mechanisms that regulate the production of the isoforms by alternative splicing remain unknown. PlGF and VEGF are able to function synergistically [109,111], and it is likely that other growth factor interactions occur. Semaphorins and VEGF family members interact with the neuropilins, and the semaphorins antagonize VEGF action [173,178,179]. There are three receptor-protein tyrosine kinases and two non-protein-tyrosine kinase receptors. VEGFR2 interacts with both VEGFR1 and VEGFR3 [122,138]. The neuropilins have the ability to modulate the action of the VEGF receptor kinases [31,147,154,204]. These interactions represent only the first layer of communication. The occurrence of about 30 endogenous pro-angiogenic and about 30 endogenous anti-angiogenic factors exponentially increases the possibilities for signaling interactions. A systems biology approach will be required to decipher these interrelationships.

Owing to the large number of angiogenic regulators, it should not be surprising that bevacizumab monotherapy,

which is directed only toward VEGF, rarely extends the lives of people with solid tumors [8]. Perhaps it is surprising that bevacizumab monotherapy effectively inhibits human xenograft growth in athymic nude mice [263,267]. Decreased VEGF signaling leads to tumor vessel pruning [286]. In response to decreased blood flow, up-regulation of other pro-angiogenic factors such as basic fibroblast growth factor and down-regulation of anti-angiogenic factors such as thrombospondin-1 may occur. Although little work on the mechanisms of tumor resistance to anti-VEGF monotherapy or combination therapy has been performed, it is likely that this problem will receive increased attention. Multi-targeted drugs such as sorafenib offer the advantage of inhibiting Raf in the mitogen-activated Raf-Mek-Erk protein kinase signaling module as well as inhibiting VEGFR2 and VEGFR3 [276]. Whether sorafenib leads to better clinical outcomes, however, remains to be established.

Because endothelial cell division is very limited in adults, inhibition of VEGF and its receptors for the treatment of disorders involving non-physiological angiogenesis such as tumor progression represents a sound and developing strategy. An alternative to inhibiting VEGF and other pro-angiogenic signaling factors is to administer anti-angiogenic factors such as angiostatin or endostatin [8]. A long-term goal of anti-angiogenic therapy is to treat cancer with minimal toxicity and a low incidence of drug resistance [8]. It may then become possible to convert cancer to a chronic manageable disease. Whether a safe and effective anti-angiogenic therapy can be developed that can be given chronically to asymptomatic people to prevent: (i) primary tumor growth and (ii) metastasis is an open question. Potential drawbacks of anti-angiogenic therapy in adults include diminished fertility in reproductive females and faulty wound healing, tissue remodeling, and tissue repair.

VEGF mRNA and protein occurs in many organs and tissues [10,29]. The wide spread distribution of VEGF and phosphorylated VEGFR2 in adult mice suggests that VEGF has functions besides stimulating endothelial cell proliferation such as stabilization of mature vessels [61]. Considerable progress in elucidating the structure and function of VEGF has been made since its discovery. The observations cited in this review indicate that angiogenesis is a coordinated, intricate, and regulated process.

Reviewer

Professor Emile E. Voest, University Hospital Utrecht, Dept. Internal Medicine, Postbus 85500, Utrecht, NL-3508 GA, The Netherlands.

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Biography

Robert Roskoski, Jr. graduated from the University of Chicago School of Medicine in 1964 and received his Ph.D. from the same institution in 1968 under the direction of Donald F. Steiner. He performed postdoctoral studies with Fritz Lipmann at the Rockefeller University from 1969 to 1972. He was a faculty member in the Department of Biochemistry at the University of Iowa from 1972 to 1979 and was a member of the faculty in the Department of Biochemistry and Molecular Biology at Louisiana State University in New Orleans from 1979 to 2006. He is currently the Scientific Director of the Blue Ridge Institute for Medical Research in Horse Shoe, North Carolina (USA).