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Vascular endothelial cells and angiogenesis

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ABSTRACT

Physiological vascular endothelial cell division and angiogenesis occur during embryonic development, wound healing, in the endometrium during the menstrual cycle, and during placental development. Otherwise, vascular endothelial cells divide less than once per decade. Neoplasms are limited in size (~ 1.0 mm) owing to a deficiency of oxygen and metabolic fuels. To grow larger, new blood vessels form from pre-existing vasculature by angiogenesis (capillary sprouting). During this process, mature endothelial cells replicate and become incorporated into new capillaries resulting in tumor growth. Angiogenesis results in part from the increased production of vascular endothelial growth factors (VEGFs). The human VEGF family consists of VEGF-A/B/C/D and placental growth factor (PlGF). The VEGF family of receptors consists of three protein-tyrosine kinases (VEGFR1/2/3) and two nonprotein kinase receptors (neuropilin-1 and neuropilin-2). Semaphorins 3A-F/4A-G/5 A/B/6A-G/7 A are regulatory ligands that interact with their neuropilin and plexin receptors (PlxA1-A4/B1-B3/C1/D1) and regulate angiogenesis. Angiopoietin-1/2/4 interact with their Tie1/2 receptor protein-tyrosine kinases to modulate vasculogenesis and angiogenesis. Ephrin ligands (EfnA1/A2/A3/A4/A5/B1/B2/B3) and Ephrin receptors (EphA1/A2/A3/A4/A5/A6/A7/A8/A10/B1/B2/B3/B4/B6/) also contribute to angiogenesis. Platelet-derived growth factors, fibroblast growth factors, hepatocyte growth factor (c-Met), stem cell growth factor (Kit) receptor protein-tyrosine kinases, PKB/Akt, Src, and MAP kinases also participate in angiogenesis. Owing to its importance in tumor progression, the inhibition of angiogenic signaling represents an attractive cancer treatment. Ponatinib, regorafenib, and vandetanib are FDA-approved VEGFR, Tie2, and Ephrin receptor blockers used in the treatment of various malignancies. Other disorders characterized by aberrant angiogenesis include diabetic retinopathies and neovascular age-related macular degeneration.

1. Vasculogenesis and angiogenesis

The circulatory system includes the heart, blood vessels, and blood which is circulated throughout the body [1]. The network of blood vessels includes arteries, small arterioles, capillaries that join with small veins or venules, and veins [2]. Blood consists of plasma, erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets). Blood is circulated throughout the body carrying oxygen and nutrients to the tissues and collecting and disposing of spent metabolites [3]. Circulating nutrients consist of carbohydrates, lipids, proteins, minerals, and other components including hormones and gases such as oxygen (bound to hemoglobin) and carbon dioxide (as CO_2 and HCO_3^-). The circulation provides nourishment, sustains the immune system, and maintains homeostasis by stabilizing temperature and pH. The lymphatic system complements the circulatory system. It carries excess plasma (filtered from capillaries as interstitial fluid between cells) away from the body tissues and returns excess fluid back to blood circulation as lymph. The lymphatic system maintains fluid balance, absorbs fats from the gastrointestinal tract, and plays a crucial role in immunity. The circulatory and lymphatic systems transport leukocytes and antibodies

Abbreviations: Ang, angiopoietin; AS, activation segment; CML, chronic myelogenous leukemia; DAG, diacylglycero; EGFR, epidermal growth factor receptor; Efn, ephrin ligand; eNOS, endothelial nitric oxide synthase; Eph, ephrin receptor; FDA, United States Food and Drug Administration; FGFR, fibroblast growth factor receptor; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; HCC, hepatocellular carcinoma; HIF, hypoxia inducible factor; HUVEV, human umbilical vein endothelial cells; Ig, immunoglobulin-like; IGF-1, insulin-like growth factor-1; Il, interleukin; JAK, Janus kinase; Kit, stem cell growth factor receptor; MAPK, mitogen associated protein kinase; c-Met, hepatocyte growth factor receptor; Nrp, neuropilin; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; Ph^+, Philadelphia chromosome positive; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidyl inositol 4,5-bisphosphate; PIP_3, phosphatidyl inositol 3,4,5-trisphosphate; PlGF, placenta growth factor; PKB, protein kinase B; Plx, plexin receptor; PTB, phosphotyrosine binding; RCC, renal cell carcinoma; Sema, semaphorin; TGF, transforming growth factor; VEGFR, vascular endothelial growth factor receptor.

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to thwart infections as part of the immune response.

Neovascularization (new blood vessel formation) consists of two components: vasculogenesis and angiogenesis [2]. Embryonic vasculogenesis is the process of blood vessel formation *de novo* from hemangioblasts that undergo differentiation into mature endothelial cells, blood cells, and platelets. Endothelial cells cover the luminal space of blood vessels forming a continuous layer possessing a basal luminal polarity with the apical surface facing the lumen. These cells selectively regulate the transit of molecules and water and regulate vessel contractility, coagulation, inflammation, and the immune response. In the embryo, blood vessels develop by the aggregation of angioblasts into a primitive network of endothelial tubes. These primitive vessels are remodeled into a functioning circulatory system as they undergo localized proliferation, regression, branching, and migration. In contrast, angiogenesis is the process of new blood vessel formation from pre-existing vascular networks mainly by capillary sprouting. Existing endothelial cells divide and are incorporated into new capillaries. Signaling initiated by VEGF and many other factors is needed for the full execution of vasculogenesis and angiogenesis. See Ref. [2] for lists of more than two dozen physiological (i) pro-angiogenic factors and (ii) antiangiogenic factors. That such a large number of physiologic regulators are at play underscores the complexity of the angiogenic process.

Vascular endothelial cells line the vessels of every tissue, and they make up a mass of about 1 Kg in human adults [2] corresponding to $\approx 10^{12}$ cells. An average endothelial cell exhibits a surface area of about 1000 square microns (20 μm x 50 μm); however, the size and surface area of an endothelial cell varies with its location and age. Such endothelial cells represent an estimated surface area of 1000 square meters (1 x 10^{12} cells x 20 μm (width) x 50 μm (length) x 1 m/10^6 μm x 1 m/10^6 μm), which corresponds to about four tennis courts (260 square meters each); these data correspond to an Enrico Fermi back-of-the-envelope calculation. In adult humans, most endothelial cells are quiescent with only one of every 10,000 cells dividing at any one time [2]. However, there is an increased rate of endothelial cell division during wound healing, ovarian corpus luteum formation, endometrial shedding during menstruation, and throughout placental development. Disorders with nonphysiological angiogenesis include diabetic retinopathy, neovascular age-related macular eye degeneration, endometriosis, rheumatoid arthritis, psoriasis, tumor growth and metastasis [4].

Angiogenesis is an elaborate process that includes interactions between regulatory and effector molecular components. Pepper divided classical angiogenesis into phases of activation, progression, and resolution [5]. Activation can be divided into three components including (i) local vasodilation, increased vascular permeability, and extravascular fibrin deposition, (ii) vessel wall breakdown, and (iii) degradation of the basement membrane of the parent vessel. Progression consists of five phases including (i) extracellular matrix invasion and endothelial cell migration toward the angiogenic stimulus with the formation of capillary sprouts, (ii) endothelial cell proliferation, (iii) capillary lumen formation, (iv) anastomosis of capillary sprouts to form a functional capillary loop with blood flow, and (v) reconstitution of the basement membrane. Resolution consists of four phases including (i) blockade of endothelial cell proliferation, (ii) termination of cell migration, (iii) maturation of junctional complexes, and (iv) completion of the vessel wall including recruitment of pericytes and smooth muscle cells, both of which are mural cells (mural, wall).

In addition to classical or sprouting angiogenesis, various forms of nonsprouting angiogenesis occur [2]. These include intussusceptive vessel formation, vascular cooption, mosaic vessel formation, and vasculogenic mimicry. During intussusceptive vessel formation, a column of cells from the connective tissue (interstitium) is inserted into the lumen of a pre-existing vessel, consequently dividing the lumen and yielding two vessels [6]. The column attracts pericytes and fibroblasts and produces extracellular matrix proteins. This process does not require endothelial cell proliferation, but it involves the remodeling and rearrangement of existing cells. The advantage of this mechanism of vessel formation over sprouting is that blood vessels are generated in a metabolically economic process because basement membrane degradation, cell proliferation, and invasion of the surrounding tissue are not required. By yet another process, growing tumors can surround vessels in the tissue of origin and incorporate, or co-opt, these vessels [7]. Co-option may be important when tumors arise in or metastasize to vascular organs such as the lung. Tumor cells, along with endothelial cells, may form the luminal surface of capillaries thus generating a mosaic vessel composed of different cell types [8]. Chang et al. found that about 15 % of vessels in human colon carcinoma biopsies were mosaic channels lined with both tumor and endothelial cells [8]. In vasculogenic mimicry, first described in ocular melanoma, vascular channels develop tumor-cell lined tubular networks [9]. These tumor-cell lined channels, which lack endothelial cells, contain circulating red blood cells. Vasculogenic mimicry has been described in breast, lung, ovarian, colorectal, head and neck, and prostate carcinomas as well as in melanomas and sarcomas [10].

The VEGF family plays an essential role in vasculogenesis, angiogenesis, and lymphangiogenesis [11]. This family consists of five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) [4,12]. Each of these proteins is glycosylated in the endoplasmic reticulum; they contain a signal peptide that is cleaved during biosynthesis leading to the secretion of these factors from the cell [13]. Günter Blobel received the Nobel Prize in Medicine or Physiology in 1999 for discovering that proteins have intrinsic signals (the signal peptide) that guide them to their correct destinations. Multiple isoforms of VEGF-A, VEGF-B, and PlGF are generated by the alternative splicing of their corresponding mRNAs [4,12]. The receptors for the VEGF ligand family include three protein-tyrosine kinases (VEGFR1, VEGFR2, and VEGFR3) and two nonenzymatic receptors (neuropilin-1/2 – Nrp1/2). Moreover, several members of the VEGF family bind to negatively charged heparan sulfate proteoglycans (HSPGs) that occur in the extracellular matrix and on the plasma membrane. Table 1 lists the VEGF receptor ligands and Fig. 1 depicts the pattern of alternative splicing of the VEGF family of ligands.

VEGF-A binds to VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR) located on vascular endothelial cells [14]. This property accounts for the selectivity and specificity of action of VEGF-A. VEGF-C and VEGF-D bind to VEGFR3 (Flt-4). These three receptors are type V (five) protein-tyrosine kinases that consist of an extracellular section containing seven immunoglobulin-like (Ig) domains, a single helical transmembrane segment, an internal juxtamembrane segment, an intracellular protein-tyrosine kinase domain that contains an insert of 70–100 amino acid residues, and a carboxyterminal tail (see [15] 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 colony-stimulating factor-1 receptor (CSF-1R), the stem cell factor receptor (Kit), the Flt ligand receptor (Flt-3), the fibroblast growth factor receptors (1–4) (Table 2), all of which contain extracellular immunoglobulin domains and a kinase insert [15,16].

Manning et al. reported that the human protein kinase enzyme superfamily contains 518 members including 478 typical and 40 atypical enzymes [17]. Protein kinases catalyze the following reaction;

$$MgATP^{1-} + \text{protein–O:H} \rightarrow \text{protein–O:PO3}^{2-} + MgADP + H^{+}$$

These enzymes are classified as protein-serine/threonine kinases (385), protein-tyrosine kinases (90 members), and protein-tyrosine kinase-like enzymes (43). The protein-tyrosine kinases consist of both intracellular nonreceptor (32) and transmembrane receptor (58) proteins. Moreover, the protein-serine/threonine kinase family includes a small group of enzymes including MEK1/2 that catalyze the phosphorylation of both tyrosine and then threonine residues that occur in the activation segment of their target protein kinases. Consequently, MEK1/2 and related enzymes are identified as dual specificity protein kinases. An additional property indicating the importance of protein kinases is

Table 1
Properties of VEGF receptors.[a]

Receptor	Gene	# Residues	MW (kDa)	Co-receptors	Ligands	Interactions	Signaling	Isoforms	Uniprot ID
VEGFR1	*FLT1*	1338	150.8	Nrp1/2	VEGF-A/B, PlGF	VEGFR2, Src, Yes1, FAK	PI3K/Akt, MAPK, PLCγ	8	P17948
VEGFR2	*KDR*	1356	151.5	Nrp1/2	VEGF-A/C/D	VEGFR1/3, Fyn, Nck1, Src	MAPK, PI3K, PLCγ, FAK	3	P35968
VEGFR3	*FLT4*	1363	152.8	Nrp1/2	Pro- and mature VEGF-C/D	VEGFR2	MAPK, PI3K, PLCγ, JNK	3	P35916
Nrp1	*NRP1*	923	103.1	Nrp2	Sema 3 A/C/F, PlGF2–170, VEGF-A165/B167/186, pro-VEGF-C, pro-VEGF-D. VEGF-E	VEGFR1/2	MAPK, PI3K/Akt, PLCγ via VEGFRs	3	O14786
Nrp2	*NRP2*	931	104.8	Nrp1	Sema 3 C/F, VEGF-A165/145, PlGF1–149, PlGF2–170, pro- and mature VEGF-C, pro-VEGF-D	VEGFR1/2/3	MAPK, PI3K/Akt, PLCγ via VEGFRs	6	O60462

[a] Data from www.uniprot.org

the observation that about one in every 40 human genes (518 protein kinase genes out of an estimated 20,000 human protein-encoding genes) encodes a protein kinase. Other data in support of the importance of protein kinases is the observation of Manning et al. that 244 protein kinase genes correspond to cancer amplicons and other disease loci [17]. The growth, proliferation, and differentiation of cells and the maturation and development of animals can be ascribed to important signaling events triggered by receptor protein-tyrosine kinases.

2. Properties and expression of the VEGF family

2.1. VEGF-A

Leung et al. reported the complete sequence of human VEGF-A based upon cDNAs isolated from human HL60 leukemia cells [18]. Keck et al. independently described the sequence of VEGF-A based upon a cDNA analysis of a library derived from human histiocytic lymphoma cells (U937) [19]. VEGF-A is a mitogen and survival factor for vascular endothelial cells while selectively and reversibly permeabilizing the endothelium to plasma and plasma proteins without leading to injury [2,4,11,14]. VEGF-A also promotes vascular endothelial cell and monocyte motility. All of these actions are essential for angiogenesis.

VEGF-A, which contains an N-linkage glycosylation site, consists of several isoforms that result from the alternative splicing of mRNA transcribed from a single gene containing eight exons (Fig. 1) [12]. VEGF-A mRNA and protein are widely expressed in the vasculature of tissues and organs [4]. Furthermore, VEGF-A is expressed in a wide variety of human neoplasms including those of the breast, brain, colorectum, endometrium, esophagus, liver, lung (NSCLC), bone (osteosarcoma), ovary, and prostate [20]. As described later, the VEGF growth factor and receptor family represent important anticancer and antimetastasis targets.

The largest human VEGF-A precursor contains 232 amino acids [12]. Cleavage of the signal peptide of 26 residues yields mature VEGF-A206 with 206 amino acids (Fig. 1). VEGF-A165 is the predominant isoform followed by the 189 and 121 residue molecules as verified by cDNA analyses of several cell types, tissues, and tumor samples. The other isoforms, which represent minor species *in vivo*, include VEGF-A183, VEGF-A165b (an inhibitory isoform), VEGF-A162, VEGF-A148, and VEGF-A145. See Ref. [12] for a description of the alternative splicing of mRNAs that generate each of the isoforms of VEGF.

Although many first messengers including chemokines, cytokines, and growth factors participate in vasculogenesis and angiogenesis, the VEGF-A family is of paramount importance [4]. *VEGFA* null mice, which die at embryonic day 8.5, exhibit impaired vasculogenesis and blood-island formation indicating that VEGF-A is required for the earliest stages of embryonic vasculogenesis [21,22]. Furthermore, loss of a single *VEGFA* allele in mice leads to vascular abnormalities followed by embryonic death between days 11 and 12. Carmeliet et al. and Ferrara et al. reported that the formation of blood vessels was abnormal, but not abolished, in heterozygous VEGF-A–deficient ($VEGFA^{+/-}$) embryos [21,22]. This heterozygous lethal phenotype, which differs from the homozygous lethality in VEGF-A or VEGFR-deficient embryos, is indicative of a stringent dose-dependent regulation of embryonic vessel development by VEGF-A.

Muller et al. determined the X-ray crystallographic structure of human VEGF-A and found that it forms an antiparallel homodimer covalently linked by two disulfide bridges between Cys51 and Cys60 [23]. The dominant feature within each monomer is a cystine knot motif that consists of an eight-residue ring formed by disulfide bridges Cys57–Cys102 and Cys61–Cys104 with a third disulfide bond (Cys26–Cys68) passing through the ring forming a knot (Fig. 2A). A central four-stranded β-sheet (labeled β1, β3, β4, and β5) extends from this motif. The α2 and β2 segments connect the β1- and β3-strands. The interaction of VEGF-A with VEGFR1 is described later.

2.2. VEGF-B

Alternative splicing of VEGF-B mRNA yields two isoforms encoded by a single gene containing seven exons [24]. After elimination of the 21-residue signal peptide, VEGF-B167 (containing 167 amino acids) and VEGF-B186 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 occur as disulfide-linked homodimers. The X-ray crystal structure of VEGF-B is almost superimposable with that of VEGF-A [25]. The inability of VEGF-B to bind and the ability of VEGF-A to bind to VEGFR2 may be due to subtle structural differences in L1 depicted in Fig. 2B.

A high level of VEGF-B mRNA occurs in the heart and brain of mice on embryonic day 14 [26]. The highest levels of VEGF-B transcripts occur in the heart, brain, kidney, and testes of mature mice while lower levels occur in the spleen, lung, and liver. VEGF-B mRNA is expressed in many human neoplasms including those of the breast, ovary, colorectum, kidney (RCC), prostate, lung, oral squamous cell carcinoma, fibrosarcoma, non-Hodgkins lymphoma, and primary and metastatic melanoma [27]. *VEGFB* null mice are viable but exhibit abnormal cardiac conduction [28]. VEGF-B is required for normal cardiac function, cardiovascular development, and for angiogenesis. Moreover, its presence in human neoplasms and its ability to activate VEGFR1 and neuropilin-1 indicate that VEGF-B represents a potential anticancer target.

2.3. VEGF-C

VEGF-C biosynthesis involves its production as a prepro-protein that undergoes intricate proteolytic processing to generate the mature form of the growth factor [29,30]. A 31 amino-terminal signal sequence is eliminated from the 419 amino-acid-residue prepro-protein to yield a pro-protein containing 388 residues. Then two VEGF-C precursors form

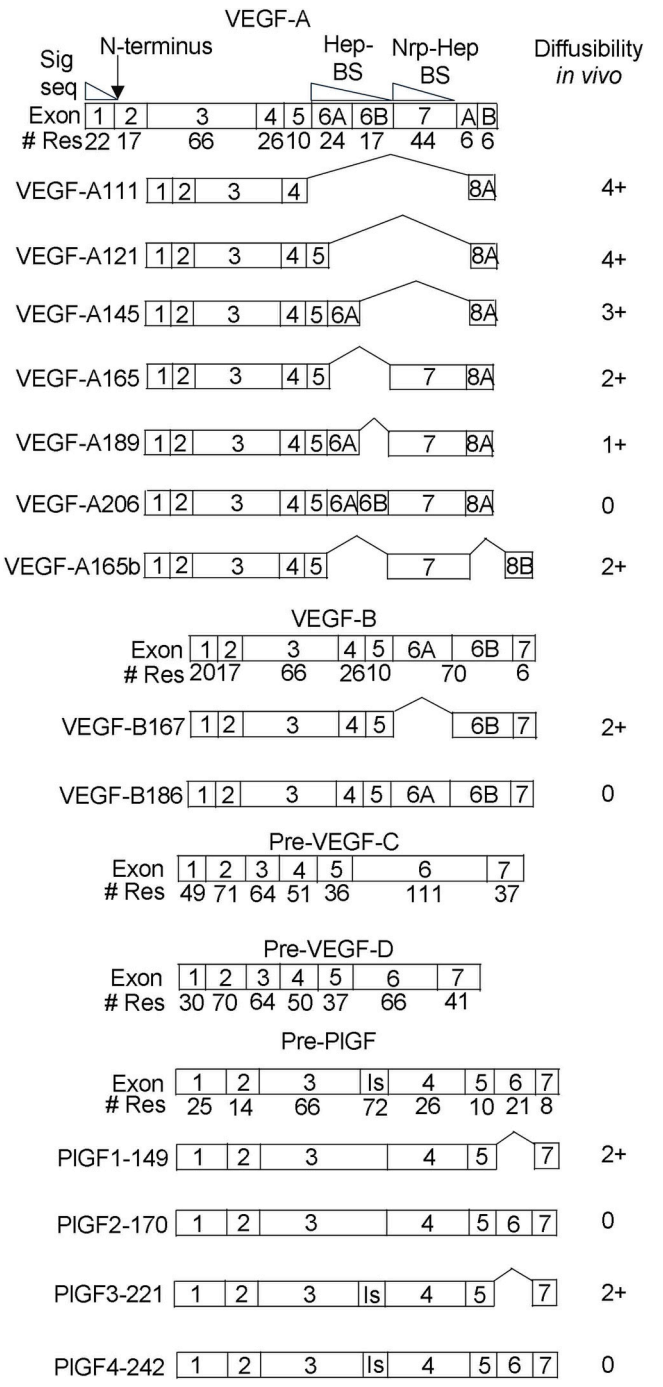

Fig. 1. Alternative splicing of the VEGF family of ligands. Hep BS, heparin-binding segment; Is, insert; Nrp, neuropilin; #Res, number of residues in each exon; Numbers and letters in boxes are exon labels. Relative lengths are not to scale.

an antiparallel homodimer attached by disulfide bridges extending from each of the two amino-terminal to carboxyterminal pro-peptides. Prior to secretion, this homodimer undergoes proteolysis after dibasic residues (Arg-227, Arg-228). Each of the carboxyterminal 228–419 residues remains attached to the opposite amino-terminus. The last step of processing, which occurs extracellularly by an undefined mechanism, cleaves residues 32–111 yielding mature VEGF-C. Although the mature dimeric VEGF-C contains the eight homologous cysteine residues that are found in VEGF-A and VEGF-B, VEGF-C exists as a homodimer lacking the corresponding inter-protomer disulfide bonds.

The human *VEGFC* gene contains seven exons [31]. Human adult heart, skeletal muscle, small intestine, ovary, and placenta contain high levels of VEGF-C mRNA [29]. Several other adult tissues contain modest amounts of VEGF-C transcripts including lung, kidney, pancreas, spleen, prostate, and testes. Furthermore, mouse embryo mesenchymal cells express VEGF-C transcripts, particularly in the regions where the lymphatic vessels undergo sprouting from embryonic veins, including the jugular, axillary, and urogenital regions and the developing mesenterium [32]. An unprocessed form of VEGF-C interacts with VEGFR3, which together are important components in lymphogenesis [30]. The mature form of VEGF-C interacts with both VEGFR2 and VEGFR3 (Table 1). VEGF-C participates in embryonic lymphangiogenesis and stabilizes differentiated lymphatic endothelium in adults [33]. Furthermore, VEGF-C is expressed by numerous human neoplasms including those of the breast, cervix, colon, lung, pancreas, prostate, thyroid, stomach, and in acute myeloid leukemia [34,35].

The essential nature of this factor is confirmed by the finding that 50 % of *VEGFC* null mice die between embryonic days 15.5 and 17.5 and none survive gestation [36]. *VEGFC*$^{+/-}$ mice exhibit defective lymphatic vascular development and lymphedema. VEGF-C is not required for the commitment to the lymphatic endothelial cell lineage. Nevertheless, VEGF-C action is required for the formation of lymph sacs from mesoderm and the subsequent relocation and survival of lymphatic endothelial cells. Although VEGFR2/3, which bind VEGF-C, are essential for the development of arteries, veins, and capillaries, perhaps surprisingly blood vessels develop normally in *VEGFC* null mice. VEGF-C is thus indispensable for embryonic lymphangiogenesis, but not vasculogenesis [36].

2.4. *VEGF-D*

Similar to VEGF-C, VEGF-D is produced as a prepro-protein that undergoes intricate proteolytic processing to generate the mature growth factor [37]. The precursor for VEGF-D contains N-terminal and C-terminal extensions that are cleaved to yield the mature product as described for VEGF-C. Mature VEGF-D is likewise a noncovalent homodimer. Although unprocessed VEGF-D binds to VEGFR3, which is important in lymphangiogenesis, the fully processed form binds to both VEGFR2/3 [37,38]. The *VEGFD* gene contains seven exons and is found on the X chromosome at Xp22.31 [39,40]. This gene was initially named *FIGF* for c-fos induced growth factor [40]. Unlike *VEGFC* null mice, *VEGFD* null mice are viable and exhibit normal lymphangiogenesis during development and normal lymphatics in mature animals [41]. Thus, VEGF-C and perhaps other factors replace VEGF-D.

Adult heart, skeletal muscle, lung, colon, and small intestine contain high levels of VEGF-D transcripts while spleen, pancreas, ovary, prostate, and testes contain low levels [2]. VEGF-D is found in high levels in gastric, colorectal, breast, and thyroid carcinomas, cervical intraepithelial neoplasia, glioblastoma, and melanoma. Its expression correlates with lymph node metastases in lung, oro-esophageal, gastric, colorectal, head and neck squamous cell carcinomas, melanomas, ovarian carcinomas, and soft tissue sarcomas.

2.5. *Placental growth factor (PlGF)*

Placental growth factor is a small homodimeric N-linked glycoprotein that shares 42 % amino acid sequence identity with VEGF-A [42]. Each PlGF monomer possesses the VEGF family core of eight cysteine residues that participate in one inter-chain (C60-C69) and three intra-chain (C35-C77, C66-C111, C70-C113) disulfide bridges as are found in VEGF-A. The tertiary structure of PlGF is similar to that of VEGF-A [43]. The PlGF gene contains seven exons that encode four isoforms (PlGF-149/170/221/242) based upon alternative mRNA splicing [44]. PlGF-149 and PlGF-221, which contain basic residues, bind to negatively charged heparan sulfate proteoglycans. PlGF isoform transcripts occur primarily in placenta [2]. However, normal heart, skeletal muscle, retina, skin as well as breast, stomach, prostate, and non-small cell lung

Table 2
Selected protein kinases that participate in angiogenesis.[a]

Kinase	Gene name	Type	# Residues	MW (KDa)	Signaling	Function	Iso-forms	Uniprot ID
VEGFR1	*FLT1*	RY	1338	150.8	Negative regulator of VEGFA signaling; ↑MAPK & Akt pathways; phosphorylates Src & Yes.	Vasculogenesis & angiogenesis	8	P17948
VEGFR2	*KDR*	RY	1356	151.5	↑MAPK & Akt, phosphorylates PLCγ; Promotes phosphorylation of Fyn, Nck1, eNOS, PI3K, PTK2/FAK1 & Src.	Vasculogenesis & angiogenesis	3	P35968
VEGFR3	*FLT4*	RY	1363	152.7	↑MAPK, MAPK8, JNK, and Akt pathways; phosphorylates Shc1, PI3K, and FAK1.	Lymphangiogenesis	3	P35916
PDGFRα	*PDGFRA*	RY	1089	122.7	Phosphorylates PIK3R1, PLCγ, and PTPN1; ↑MAPK, Akt, JAK-STAT pathways.	Plays a role in the development and maintenance of numerous organ systems	3	P16234
PDGFRβ	*PDGFRB*	RY	1106	123.9	Phosphorylates PLCγ, PI3K, Ship2, Ras-GAP, Cbl, Shc1 and Nck1; ↑MAPK, Akt pathways.	Same a PDGFRA	2	P09619
PKA	*PRKACG*	S/T	351	40.4	There are 4 PKA catalytic subunit and 4 PKA regulatory subunit genes. Following the generation of cAMP by adenylate cyclase, PKA becomes activated and mediates the phosphorylation of hundreds of target proteins and functions as a second messenger.	Cell growth, differentiation, and proliferation	1	P22612
PKB/Akt	*AKT1*	S/T	480	55.7	There are 3 *AKT* genes (*AKT1/2/3*) that signal by the mTOR pathway. The activation of PI3K leads to the generation of PIP_3, recruitment of Akt to the plasma membrane, phosphorylation by PDK1 and mTORC2, & the activation of Akt; Akt1/2/3 mediate the phosphorylation of hundreds of proteins.	Cell growth, differentiation, and proliferation	2	P31749
PKC	*PRKCA*	S/T	672	76.8	There are 4 *PRKC* genes (*PRKCA/B/D/E*). They are activated by Ca^{2+} and DAG and catalyze the phosphorylation of hundreds of proteins.	Cell growth, differentiation, and proliferation	2	P17252
PKD	*PRKD1*	S/T	912	101.7	There are 3 *PRKD* genes (*PRK1/2/3*). These enzymes catalyze the phosphorylation of numerous substrates including Akt & JNK1 (MAPK8).	Cell growth, differentiation, migration, and proliferation	6	Q15139
PI3K	*PIK3CA*	PI3K	1068	124.3	There are 3 genes encoding PIK3C catalytic subunits (*PI3KCA/B/D*) and six encoding the regulatory subunits (PI3KR1/2/3/4/5/6). Catalyzes the conversion of PIP_2 to PIP_3.	Cell growth, differentiation, migration, and proliferation following Akt activation	5	P42336
FAK1	*PTK2*	NRY	1052	119.2	Regulates PI3K, Akt, and MAPK pathways; interacts with Src.	Regulation of cell adhesion, migration, survival, proliferation, & focal adhesions.	7	Q05347
FGFR1	*FGFR1*	RY	822	91.9	There are 4 genes (*FGFR1/2/3/4*) that encode these kinases; ↑MAPK and Akt pathways.	Cell growth, differentiation, migration, and proliferation	21	P11362
Kit	*KIT*	RY	976	109.9	Phosphorylates PIK3R1, PLCγ, SH2B2/APS and Cbl; ↑Akt and MAPK modules.	Cell survival and proliferation, hematopoiesis, stem cell maintenance.	3	P10721
c-Met	*MET*	RY	1390	155.5	Upstream of Ras-ERK MAPK, PI3 kinase/Akt, & PLCγ/PKC pathways.	Hepatocyte growth factor receptor regulates cell proliferation and survival	3	P08581
Src	*SRC*	NRY	536	59.8	Non-receptor protein-tyrosine kinase that interacts with upstream c-Met, FGFRs, PDGFRs, and downstream FAK, Akt, and MAP kinases.	Participates in cell adhesion, cell cycle progression, apoptosis, migration, and transformation	3	P12931
TAK1	*NR2C2*	RY	596	65.4	TGFβ-activated kinase is upstream of MAP kinases, AMPK1, Rab1, JNK, and p38	Regulates transcription	1	P49116
Tie1	*TIE1*	RY	1138	125.1	Interacts with angiopoietin−1/2 &Tie2, ↑PI3K-Akt	Regulates vasculogenesis & angiogenesis	3	P35590
Tie2	*TIE2*	RY	1124	125.8	Interacts with angiopoietin−1/2 & Tie1, ↑ PI3k, MAPK, FAK	Regulates vasculogenesis & angiogenesis	3	Q02763
EphA1	*EPHA1*	RY	976	108.1	RhoA, Rac, Ilk, FAK	Cell proliferation, angiogenesis	3	P21709
EphA2	*EPHA2*	RY	976	108.2	FAK, PI3K/Akt, Nck1, Rac1. Vav2/3	Cell adhesion, migration, proliferation	2	P29317
EphA3	*EPHA3*	RY	983	110.1	Nck1, ADAM10	Cell adhesion, migration	2	P29320
EphA4	*EPHA4*	RY	986	109.9	Rac, Rap1/2, Rho, PLCγ, PI3K/Akt, STAT5B, Src family kinases	Cell morphology, adhesion, CNS angiogenesis	2	P54764
EphA5	*EPH5A*	RY	1037	114.8	pATM, Cdc42, PI3K, Rac1, STAT3	CNS development	3	P54756
EphA6	*EPHA6*	RY	1036	116.4	Abl1, Alk2, Odin, c-Met, SHIP2, Vav3	Cell migration, axon guidance, angiogenesis	3	Q9UF33
EphA7	*EPHA7*	RY	998	112.1	TNFR1, PI3K/Akt	CNS development	3	Q15375
EphA8	*EPHA8*	RY	1005	111.0	MAPK, Odin, Fyn, PI3K, TIAM1	Cell adhesion, migration, axon guidance	2	P29322
EphA10[b]	*EPHA10*	RY	1008	109.7	MAPK, PI3K, FAK, JNK, NF-κB	Cell migration, axon guidance	3	Q5JZY3
EphB1	*EPHB1*	RY	984	109.9	ADAM10, Crk, E-cadherin, Grb10, MAPK, JNK, Nik, Paxcillin, Src	Cell migration, adhesion, angiogenesis	3	P54762
EphB2	*EPHB2*	RY	1055	117.5	Abl, Arg, Nck, PAK	Axon guidance	3	P29323
EphB3	*EPHB3*	RY	998	110.3	Af6, Crk, Fyn, MLLT4, RasGAP, Vav2	Axon guidance, angiogenesis	1	P54753
EphB4	*EPHB4*	RY	987	108.3	Cav1, Rho, Src, Tie2	Cell adhesion, migration, angiogenesis	4	P54760
EphB6[b]	*EPHB6*	RY	1021	110.7	Abl, Clusterin, Dynactin, Src, ↓ JNK activation	Cell adhesion, migration	3	O15197

[a] Data from www.uniprot.org.
[b] Kinase impaired.

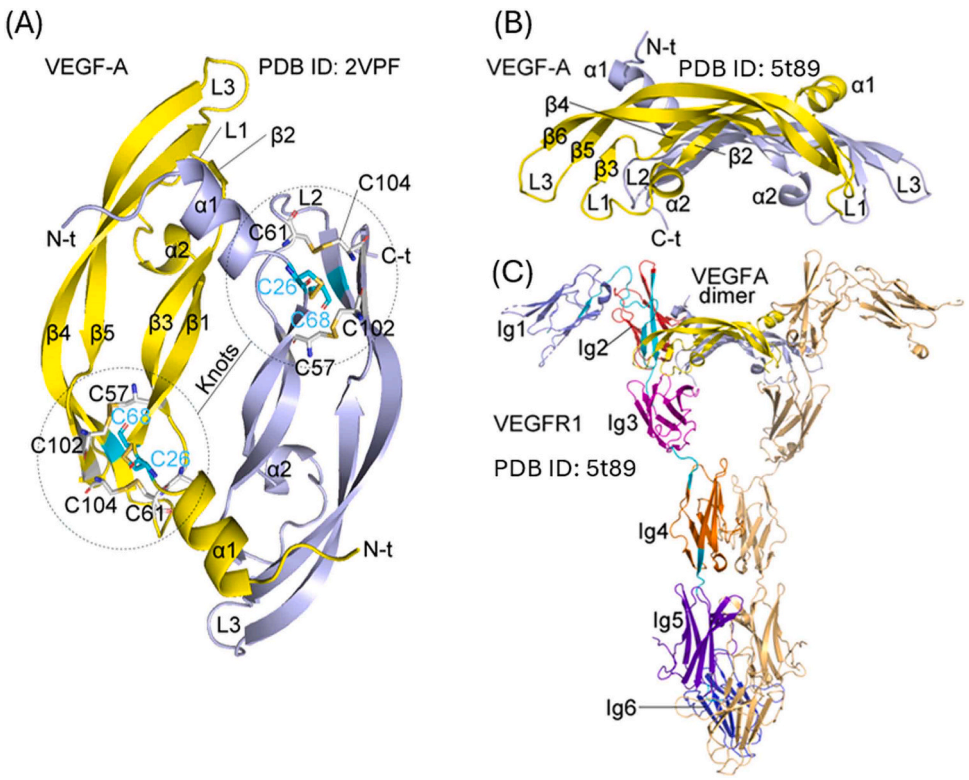

Fig. 2. Interaction of VEGF-A with VEGFR1. (A) VEGF-A and the cysteine knot. (B) VEGF-A. (C) VEGF-A bound to VEGFR1. VEGF-A consists of protomer A (yellow) and protomer B (light blue). Ig, immunoglobulin domain; C-t, carboxyterminus; L, loop; N-t, amino-terminus.

cancer cells express various isoforms of PlGF. *PlGF*-null mice are viable and fertile, but they exhibit diminished vascularization of the corpus luteum, retina, following ischemia (reduced blood supply), and healing wounds [45]. PlGF augments VEGF signaling and PlGF expression may obviate anti-VEGF-A based therapies.

3. VEGF receptors

3.1. *VEGFR1 (Flt-1)*

VEGFR1 (Flt-1, fms-like tyrosyl kinase-1, where fms refers to feline McDonough sarcoma virus) interacts with VEGF-A, PlGF, and VEGF-B (Table 1) [46–48]. VEGFR1 (MW = 210 kDa) has numerous actions that are dependent upon the developmental stage and the disposition and location of the endothelial cells that contain the receptor [49]. Using hybridization *in situ*, Quinn et al. demonstrated that VEGFR1 is expressed in embryonic cells from which the endothelium is derived including the early yolk sac mesenchyme [50]. Furthermore, these investigators reported that VEGFR1 is expressed in endothelial cells in adult mice. It is expressed in many organs including the developing brain, the perineural vascular plexus, the heart, aorta, cardinal veins, liver, lung, intestine, and limb mesenchyme. However, expression is restricted to the vascular endothelium in all organs. VEGFR1 has a higher avidity for VEGF-A than VEGFR2 (≈ 10 pM vs. 75–750 pM) [46, 49–51]. Unlike VEGFR2, VEGF-A stimulated VEGFR1 has weak tyrosine kinase phosphorylation activity [46]. Although activation of VEGFR1 lacks a direct cellular proliferative response, the receptor plays a role in monocyte migration, localization, and activation – processes that are important in the immune response [52].

The human *VEGFR1* gene contains 30 exons and is located at chromosome 13q12. Alternative splicing of VEGFR1 mRNA produces a freely diffusible soluble circulating receptor isoform (sVEGFR1) that binds to and inhibits the action of VEGF-A [53]. After signal peptide cleavage, sVEGFR1 encompasses the first six of seven extracellular immunoglobulin domains and contains 661 amino acid residues. The production and release of excessive sVEGFR1 by the human placenta into the circulation of the mother leads to the hypertension and proteinuria of preeclampsia [54,55]. Park et al. reported that PlGF binds to HUVEC samples, which express both VEGFR1 and VEGFR2, and displaces only a fraction of bound ^{125}I-VEGF-A165 [56]. This result is consistent with the notion that PlGF binds only to VEGFR1. Park et al. suggested that VEGFR1 binds to and inhibits VEGF-A action, acting as a decoy by preventing VEGF-A binding to VEGFR2 [56].

The VEGF receptors consist of an extracellular segment containing about 750 amino acid residues, a helical transmembrane segment with about 20 residues, and an intracellular domain containing about 500 residues [57]. The extracellular portion of each receptor contains a signal peptide containing about 25 amino acid residues followed by seven immunoglobulin-like (Ig) domains (Fig. 3A). The intracellular portion contains a juxtamembrane component with about 40 amino acid residues, a protein kinase domain containing about 350 residues followed by a C-terminal tail with about 25 residues. The protein kinase domain is split and it contains an insert with about 70 amino acid residues.

Protein kinases such as those of the VEGFRs contain a small N-terminal lobe and a large C-terminal lobe [11,57]. The small and large lobes form a cleft that serves as a docking site for ATP/ADP. A hinge region connects the two lobes. Most small molecule protein kinase inhibitors make contact with many of the residues of the ATP-binding pocket within the cleft. A K/E/D/D (Lys/Glu/Asp/Asp) tetrad plays a pivotal role in the catalytic activity of protein kinases. The *K* of the tetrad represents a β3-strand lysine that forms a salt bridge with an αC-glutamate (E) to form an αC_{in} structure. Furthermore, an HRD-D of the catalytic-loop (the first *D* of the K/E/*D*/D tetrad) functions as a Lowry-Brönsted base (proton acceptor) that abstracts a proton from the protein substrate –OH. The second *D* of the K/E/D/*D* tetrad is the first residue of the protein-substrate-binding activation segment (AS). This component of all protein kinases starts with DFG and ends with APE or a

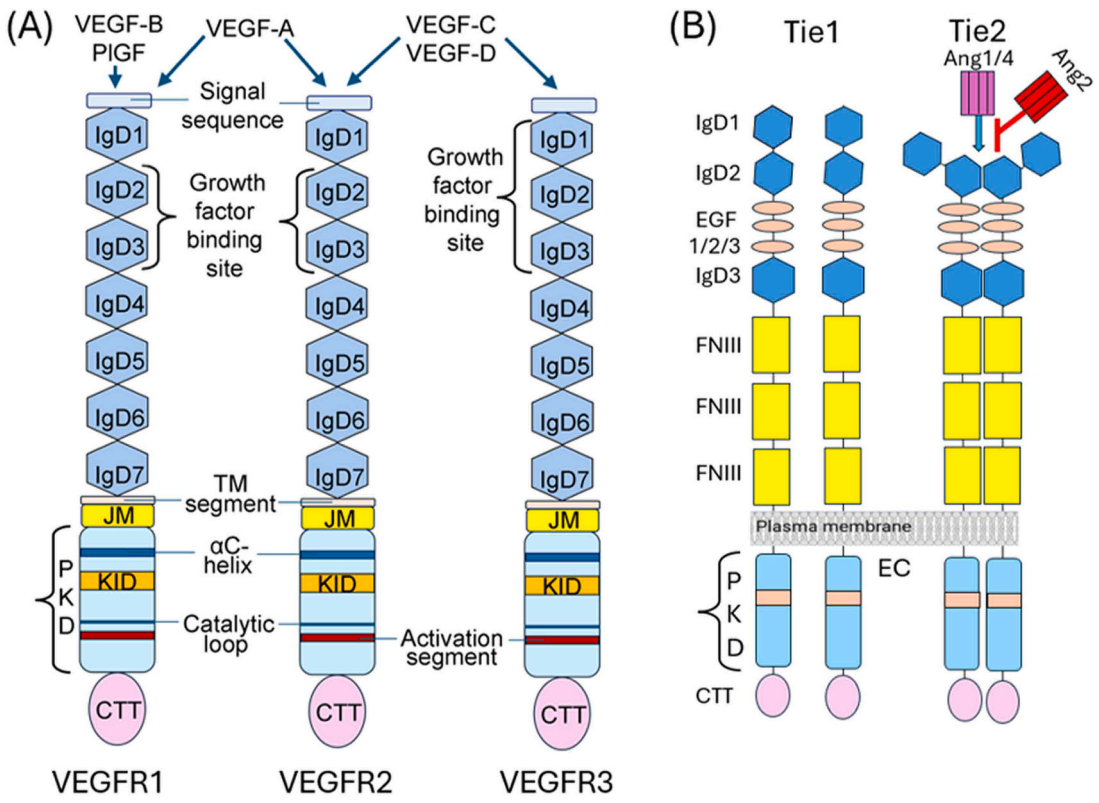

Fig. 3. Architecture of the (A) three VEGFRs and (B) the orphan Tie1 receptor and the Tie2 receptor. Ang2 blocks the binding of Ang1/4. CTT, C-terminal tail; IgD, immunoglobulin domain; KID, kinase insert domain; JM, juxtamembrane; PKD, Protein kinase domain with an insert; TM, transmembrane. Relative lengths are not to scale.

similar triad such as PPE.

Protein kinases contain a quartet of four amino acids that make up an R-spine (regulatory spine) and an octet of eight amino acids along with the adenine base of ATP that make up a C-spine (catalytic spine) [11, 57]. These spines produce a stable, but flexible, catalytically active ensemble. The C-spine positions ATP and the R-spine positions the protein substrate for catalysis. Protein kinases contain three residues that strengthen and stabilize the regulatory spine, which are labeled as Sh1, Sh2, and Sh3 where Sh refers to shell. Many small molecule therapeutic steady-state ATP-competitive protein kinase inhibitors interact with the R-spine (RS2/3), the C-spine (CS6/7/8), and shell (Sh1/2) residues. The important catalytic residues and the key structural spine and shell residues of the VEGF receptors are listed in Tables 3 and 4, respectively.

The interaction of the VEGF growth factors with their protein-tyrosine kinase receptors involves van der Waals, hydrophobic, and electrostatic interactions involving receptor Ig2 and Ig3 domains (Fig. 2C) [58]. Markovic-Mueller et al. reported that VEGF-A residues interacting with the Ig2 domain of VEGFR1 are part of the N-terminal α1-helix of ligand protomer A (M17, F18, Y21, Q22, and Y25) and of strands β2 (I46, K48), β4 (Q79, M81, I83), and β5 (Q89, I91) of protomer B [58]. The interface between the dimeric growth factor and Ig2 is chiefly hydrophobic and that with Ig3 is hydrophilic consisting of a number of electrostatic interactions. Ig3 contains an overall positive charge that comes in contact with the VEGF-A. Several charged residues from all three loops (L1, L2, and L3) of VEGF-A interact with Ig3. The most prominent residue is E64 in L2 that engages five residues located in the receptor Ig3 domain (N259, R261, R280, Q284, and N290) with hydrogen-bond and salt-bridge interactions. VEGF-A L2 residue D63 forms salt bridges with R224 located in the VEGFR1 Ig2-Ig3 linker. Moreover, E44 in L1 and K84 in L3 form hydrogen bonds with Q263 of Ig3. Loops 1 and 3 also contain hydrophobic residues including P40, I43, and P85 that form a complementary surface with Ig3 of VEGFR1. Loop1 I43 projects into the hydrophobic pocket on the receptor Ig3 surface formed by V262, M264, V278, and F292. Owing to the low resolution of their structure, some of the above-described interactions are tentative. VEGF-A binds to VEGFR2 Ig2 and Ig3 in a similar fashion (PDB ID: 3v2a) [59]; VEGF-A does not activate VEGFR3. The X-ray structure of VEGF-C to VEGFR3 indicates that the ligand binds only to the Ig2 domain of the receptor (PDB ID: 4bsk) [60].

Waltenberger et al. reported that the level of autophosphorylation of VEGFR1 in response to VEGF-A is minimal [46]. The initiation of signal transduction and receptor protein-tyrosine kinase activation requires the trans-autophosphorylation of protein-tyrosine residues [57]. Most receptor protein-tyrosine kinases undergo activation segment trans-autophosphorylation that increases catalytic activity. In contrast, VEGFR1 fails to undergo meaningful activation segment trans-autophosphorylation and activation [61]. However, six residues in the carboxyterminal tail of VEGFR1 (Y1169/1213/1242/1309/1327/1333) have been identified as potential phosphorylation sites (Fig. 4) [61–63]. Olsen et al. reported that pY1169 is implicated in the interaction and activation of phospholipase Cγ (PLCγ) leading to the activation of the MAP kinase signal transduction cascade [62]. Elucidating the signal transduction mechanisms promoted by VEGFR1 has been difficult owing to the low levels of autophosphorylation under physiological conditions.

Although PlGF and VEGF-A activate VEGFR1, the tyrosine phosphorylation sites differ. For example, Autiero et al. found that human PlGF-152 stimulates only Y1309 phosphorylation whereas human VEGF-A165 stimulates Y1213 phosphorylation as determined by mass spectrometry in cells expressing only mouse VEGFR1 receptors [63]. Although PlGF-152 and VEGF-A165 both interact with VEGFR1, these results show that they stimulate this receptor differently. Although VEGF-A165 modestly stimulates VEGFR1 phosphorylation, it fails to

Table 3
Composition and important residues of the human VEGF and Tie receptors[a].

	VEGFR1	VEGFR2	VEGFR3	Tie1	Tie2	Inferred function
Signal sequence	1–26	1–19	1–24	1–21	1–22	
Extracellular domain	27–758	20–764	25–775	22–759	23–748	
Domain 1	Ig1 32–123	46–110	30–127	Ig1 43–105	44–123	
Domain 2	Ig2 151–214	141–207	151–213	EGF1 214–256	210–252	Binds growth factors
Domain 3	Ig3 230–327	224–320	219–326	EGF2 258–303	254–299	Binds growth factors
Domain 4	Ig4 335–421	328–414	331–415	EGF3 305–345	301–431	
Domain 5	Ig5 428–553	421–548	422–552	FN1 446–545	447–541	
Domain 6	Ig6 556–654	551–660	555–617	FN2 548–642	545–636	
Domain 7	Ig7 61–747	667–753	678–764	FN3 646–739	641–735	
Domain 8				Ig2 372–426	Ig2 350–540	
Transmembrane segment	759–780	765–789	776–797	760–784	749–769	
JM segment	781–826	790–833	798–844	785–838	770–823	
Protein kinase domain	827–1158	834–1162	845–1173	839–1118	824–1096	
Glycine-rich loop	GRGAFG, 834–839	GRGAFG, 841–846	GYGAFG, 852–857	GEGNFG, 846–859	GEGNFG, 831–836	Anchors ATP β-phosphate
β3-K of K/E/D/D	861	868	879	870	855	Forms ion pair with ATP α- and β-phosphates
αC-E, E of K/E/D/D	878	885	896	887	872	Forms ion pair with β3-K
Hinge residues	EYCKYG, 910–915	EFCKFG, 915–920	EFCKYG, 928–933	EYAPYG, 918–923	EYAPHG, 903–908	Connects N- and C-lobes
Catalytic loop, HRDLAARN	1019–1027	1026–1033	1035–1042	977–984	962–999	Plays both structural and catalytic functions
Catalytic loop HRD, First D of K/E/D/D	1022	1028	1037	974	964	Catalytic base (abstracts proton)
Catalytic loop N, HRDLAARN	1027	1033	1042	984	969	Chelates Mg^{2+}(2)
AS DFG, Second D of K/E/D/D	1040–1042	1046–1048	1055–1057	997	982	Chelates Mg^{2+}(1)
AS	1040–1069	1046–1075	1055–1084	997–1023	982–1008	Positions protein substrate
AS tyrosines	1048[a], 1053[a]	1054, 1059	1063, 1068	1007	992	Stabilizes the AS after phosphorylation
AS End	APE 1067–1069	1073–1075	1082–1084	AIE, 1021–1023	1006–1008	Interacts with the αHI loop and stabilizes the AS
C-terminal tail	1159–1338	1163–1356	1174–1298	1119–1138	1097–1124	Contains phosphorylation sites used for protein docking
No. of residues	1338	1356	1298/1369	1138	1124	
Molecular Wt[b] (kDa)	150,735	151,527	145,599/ 153,462	125,090	125,830	
Swiss-Prot accession no.	P17948	P35968	P35916/ Q16067	P35590	QO2736	

[a] Abbreviations: AS, activation segment; EGF, epidermal growth factor-like domain; FN, fibronectin III domain; Ig immunoglobulin; JM, juxtamembrane
[b] Molecular weight of the unprocessed precursor

Table 4
Spine and shell residues of human VEGFR1/2/3 and Tie1/2.

	Symbol	KLIFS No.[a]	VEGFR1	VEGFR2	VEGFR3	Tie1	Tie2
Regulatory spine							
β4-strand (N-lobe)	RS4	38	L894	L901	L912	L309	L888
C-helix (N-lobe)	RS3	28	L822	L889	L900	L891	L876
Activation loop F of DFG (C-lobe)	RS2	82	F1041	F1047	F1055	F998	F983
Catalytic loop His/Tyr (C-lobe)	RS1	68	H1020	H1026	H1035	H977	H962
F-helix (C-lobe)	RS0	None	D1081	D1087	D1096	D1035	D1020
R-shell							
Two residues upstream from the gatekeeper	Sh3	43	V907	V914	V925	I915	L900
Gatekeeper, end of β5-strand	Sh2	45	V909	V916	V927	I917	I902
αC-β4 loop	Sh1	36	V892	V899	V910	I901	I886
Catalytic spine							
β3-AxK motif (N-lobe)	CS8	15	A859	A666	A977	A868	A853
β2-strand (N-lobe)	CS7	11	V841	V848	V859	V853	V838
β7-strand (C-lobe)	CS6	77	L1029	L1035	L1044	L986	L971
β7-strand (C-lobe)	CS5	78	L1030	L1036	L1045	V987	V972
β7-strand (C-lobe)	CS4	76	I1028	I1034	I1043	V985	V970
D-helix (C-lobe)	CS3	53	L917	L924	L935	L925	L910
F-helix (C-lobe)	CS2	None	L1088	L1094	L1103	L1042	L1027
F-helix (C-lobe)	CS1	None	I1092	I1098	I1007	L1046	I1031

[a] klifs.net, Kinase–Ligand Interaction Fingerprints and Structures. It systematically annotates the 3D structures of protein kinases and how small molecules interact with their catalytic domains.

modify the gene expression profile of primary mouse capillary endothelial cells. On the other hand, PlGF produces changes in the expression of more than 50 of these genes.

Although PlGF and VEGF-A165 interact with VEGFR1, they exert different biological effects indicating that each factor activates VEGFR1 in a dissimilar fashion. Autiero et al. proposed that the mechanism accountable for these differences may be due to the ability of these factors to induce different conformational changes in VEGFR1 [63].

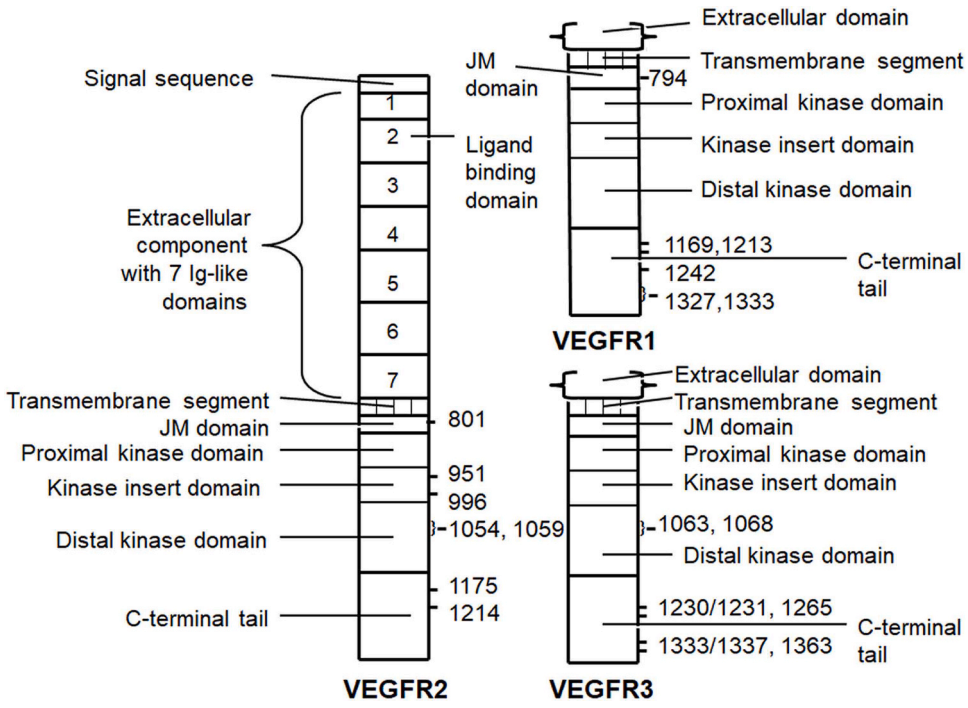

Fig. 4. VEGFR1/2/3 tyrosine phosphorylation sites correspond to the numbers on the right side of each receptor. JM, juxtamembrane.

However, the X-ray crystal structures of PlGF or VEGF-A bound to the second immunoglobulin domain of human VEGFR1 fail to reveal any differences in conformation [64,65]. The discovery of the mechanism responsible for the disparate autophosphorylation findings of the same receptor in response to stimulation by two different factors promises to add new insight into protein-protein signaling interactions.

3.2. *VEGFR2 (Flk-1/KDR)*

VEGFR2 (KDR/Flk-1, Kinase Domain-containing Receptor/Fetal liver kinase-1) binds to smaller forms of VEGF-A (110–165 amino acid residues) as well as the fully processed forms of VEGF-C and VEGF-D (Table 1). VEGFR2, which has a molecular weight of about 210 kDa [46], is the predominant mediator of VEGF-stimulated vascular permeability and endothelial cell migration, proliferation, and survival [66–71]. Although VEGFR2 has lower affinity for VEGF-A than VEGFR1, the former exhibits robust protein-tyrosine kinase activity in response to its stimulatory ligands. VEGF-A induces the formation of VEGFR2 homodimers that promote receptor trans-autophosphorylation and activation. Autophosphorylation of Y801 in the juxtamembrane segment occurs first and promotes additional phosphorylation. Autophosphorylation of tyrosine residues within the activation segment of the kinase domain (Y1054, Y1059) stimulates catalytic activity while autophosphorylation of tyrosine residues elsewhere produces docking sites for modular phosphotyrosine binding (PTB) and Src homology 2 (SH2) domains that recognize phosphotyrosine in sequence-specific contexts and lead to downstream signal transduction.

Takahashi et al. discovered that Y1175 and Y1214 are two major phosphorylation sites in VEGFR2 (Fig. 4) [67]. VEGFR2 phosphorylation promotes PLCγ binding and activation that in turn leads to protein kinase C activation. To determine which residue interacts with PLCγ, Takahashi et al. found that the Y1175F mutant receptor failed to phosphorylate and activate PLCγ in response to VEGF-A treatment whereas wild type and Y1214F mutants were effective. They also found that the VEGF-A induced phosphorylation of MAP kinase is decreased in the Y1175F mutant but not in the wild type or Y1214F mutant. Moreover, they discovered that tyrosine 1175 is essential for VEGF-A induced proliferation of VEGFR2-expressing aortic endothelial cells. Sjöberg et al. reported that PLCγ binding to mouse pY1173 VEGFR2 (corresponding to human pY1175) leads to the activation of eNOS and Src [72]. These findings underscore the importance of Y1175 in VEGFR2 signaling (Table 5).

Holmqvist et al. reported that Shb is an adaptor protein that is involved in signaling pathways involving several growth factor receptors including VEGFR2 (Table 6) [73]. Shb contains a proline-rich N-terminus. a central PTB domain, four central tyrosine phosphorylation sites, and a C-terminal SH2 domain (Uniprot ID: Q15464). Holmqvist et al. reported that Shb is phosphorylated and its SH2 domain binds directly to pY1175 in the C-terminal tail of VEGFR2 following VEGF-A stimulation of human aortic endothelial cells [73]. Based upon small interfering RNA (siRNA) methodology directed against Shb, they discovered that Shb is required for VEGF-A mediated stress fiber formation, cell migration, and activation of PI3K.

Autiero et al. examined the interaction of VEGFR1 and VEGFR2 in immortalized capillary endothelial cells prepared from *PlGF*-deficient mice; such endothelial cells respond to exogenous PlGF while those prepared from wild type mice are unresponsive to PlGF [74]. They found that PlGF (which activates VEGFR1 only) fails to stimulate the phosphorylation of VEGFR2 whereas VEGF-E (a protein encoded by the Orf virus and stimulates VEGFR2 only) produces a four-fold increase in VEGFR2 phosphorylation when compared with unstimulated specimens from the *PLGF*-deficient mice. On the other hand, a combination of VEGF-E and PlGF produces a 13-fold increase in VEGFR2 phosphorylation. These investigators proposed that VEGFR2 is transactivated by VEGFR1 through an intermolecular reaction involving VEGFR1 and VEGFR2 homodimer pairs. Transactivation by homodimer pairs is in contrast to the usual paradigm where it is generally presumed that transactivation occurs between singular heterodimers.

Treatment of Sf9 insect cells expressing VEGFR1 and VEGFR2 with human PlGF-152 increased VEGFR2 phosphorylation by 150 % [74]. Its phosphorylation failed to increase following PlGF-152 treatment when such cells expressed only VEGFR2. When kinase-dead VEGFR2 was co-transfected with VEGFR1, PlGF-152 stimulated VEGFR2 phosphorylation. However, this phosphorylation failed to occur when cells expressed a kinase-dead mutant of VEGFR1 and wild type VEGFR2. These experiments provide evidence that VEGFR2 is transactivated by

Table 5
Receptor phosphorylation sites. [a]

Receptor	Class[b]	Phosphorylated residues	Selected receptor interactions
VEGFR1	RY	Y914/1053/1169/1213/1242/1309/1329/1333	PLCγ & pY1169/1213; PI3K and SHP2 with pY1213; Cbl, Crk Nck1 with pY1333.
VEGFR2	RY	Y951/1054/1059/1175/1214	TSAd with pY951; Src binds to pY1054 and pY1059; Grb2, Nck1, PLCγ and Shb with pY1175; Nck2 and Fyn with pY1214. Grb2 is upstream from the MAPK and Akt pathways.
VEGFR3	RY	Y830/833/853/1063/1068/1230/1231/1265/1333/1337/1363	Crk with pY1063/1337; Grb2 with pY1230/1231/1337 leading to MAPK and Akt pathway activation.
Nrp1	Nrp	pY899/921; pS894/921	GIPC1 (synectin) is involved in the trafficking of integrins during cell migration and angiogenesis; it also interacts with VEGFR2, Rab11, Rac1, RhoA
Nrp2	Nrp	S901	VEGFR2; P120RasGAP; GSK3β; Rab11, Rac1, RhoA
PlxA1	Plx	Y1608; S1619/1632/1635	Crk, Rac1, Rnd1, RhoD, FARP2, VEGFR2
PlxA2	Plx	Y1605, S1612/1630, T1633	RhoD, Rnd1, Fyn
PlxA3	Plx	S1596/1607/1610; T1590	Fyn, RhoD, Rnd1
PlxA4	Plx	Y1606, S1630/1633, T1628	FARP2, FGFR1, Rac1, Rnd1, VEGFR2
PlxB1	Plx	S1535/1902	Rac1, Rnd1, RhoA, SHP2, ErbB2, c-Met
PlxB2	Plx	S1236/1244/1570	Rac1, RhoA, Rnd3, ErbB2, c-MET, Ron (receptor for MSP)
PlxB3	Plx	S1589, T1587	Rin, RhoA, RhoGDIα (which interacts with Rac1)
PlxC1	Plx	S978	ADCY4 (adenylate cyclase 4), cofilin, LIM kinase, Rap1B, Rap2A
PlxD1	Plx	Y1367/1371/1673, S1316	GIPC1, ErbB3, c-Met, Sh3BP1 (which interacts with Rac1)
Tie1	RY	Y1007/1027	αVβ3 and α5β1 integrins, PI3K, Tie2
Tie2	RY	Y860/992/1102/1108	Dok2, Grb2/7/14, PI3K/Akt, Shc1, SHP2, SOS1, Tie1
EphA1	RY	Y599/605/781/930	RhoA, Rac, ILK, FAK
EphA2	RY	Y575/588/594/628/694/735/772/921/930/960	FAK, Nck1, PI3K/Akt, Rac1, Vav2/3
EphA3	RY	Y596/602/701/779/937	Nck1, ADAM10
EphA4	RY	Y596/602/779/928	Rac1, Rap1/2, Rho, PLCγ, PI3K/Akt, STAT5B, Src family kinases
EphA5	RY	Y650/656/833/982	pATM, Cdc42, PI3K, Rac1, STAT3
EphA6	RY	Y606/612/831/978	Abl1, Alk2, Odin, c-Met, SHIP2, Vav3
EphA7	RY	Y608/614/791/940	TNFR1, PI3K/Akt
EphA8	RY	Y616/839	MAPK, Odin, PI3K, Fyn, TIAM1
EphA10	RY	Y644/645/651/669	MAPK, PI3K/Akt, FAK, JNK, NF-κB
EphB1	RY	Y594/600/928	ADAM10, Crk, E-cadherin, Grb10, MAPK, JNK, Nik, Paxillin, Src
EphB2	RY	Y577/584/596/602/780	Abl, Arg, Nck, PAK
EphB3	RY	Y600/608/614/792	AF6, Crk, Fyn, MLLT4, RasGAP, Vav2
EphB4	RY	Y574/581/590/596/614/774/906/987	Cav1, Tie2, Rho, Src
EphB6	RY	None	Abl, Clusterin, Dynactin, JNK, Src

[a] Data from www.uniprot.org.
[b] BP, binding protein; GDI, guanine nucleotide dissociation inhibitor; Nrp, neuropilin; Plx, plexin receptor; RY, receptor protein-tyrosine kinase.

VEGFR1. Furthermore, these findings provide evidence for the trans-phosphorylation of VEGFR1 and VEGFR2 and suggest that cross-talk between receptor signaling pathways occurs readily.

Autiero et al. examined the extent of VEGFR1 and VEGFR2 interactions in immortalized mouse capillary endothelial cells [74]. In the absence of any growth factors, VEGFR1 was consistently found in anti-VEGFR2 immunoprecipitates indicating that these receptors spontaneously form complexes. They found that human heterodimeric VEGF-A/PlGF increases VEGFR1/VEGFR2 complex formation by about 140 %. VEGF-A/PlGF bind to both VEGFR1 and VEGFR2. Although VEGFR1 and VEGFR2 form complexes in the absence of activating ligands, increased complex formation results only from stimulation by ligands that bind to both receptors. These studies show that VEGFR1 and VEGFR2 function in a cooperative fashion.

Ebos et al. described a soluble and circulating form of VEGFR2 reminiscent of sVEGFR1 [75]. As noted above, sVEGFR1 is implicated in the pathogenesis of preeclampsia [54,55]. sVEGFR2, a truncated form of the full-length VEGFR2 receptor, acts as an antagonist of lymphangiogenesis by binding to VEGF-C and preventing its interaction with VEGFR3. Iguchi et al. reported that crosstalk between endothelial cell VEGFR2 and cardiac myocyte EGFR family signaling pathways coordinates cardiac myocyte hypertrophy and angiogenesis and participates in physiological cardiac growth [76]. They summarized a multicenter prospective study demonstrating that low sVEGFR2 values were independently associated with cardiovascular death among patients with congestive heart failure.

3.3. *VEGFR3 (Flt-4)*

VEGFR3 (Flt-4, Fms-like tyrosine kinase 4), which has a molecular weight of about 170 kDa, is the third member of this receptor family [77,78]. VEGFR3 interacts with VEGF-C/D. VEGFR3 plays a pivotal role in the development of the primary capillary plexus in the embryo and contributes to lymphangiogenesis and angiogenesis in the adult. This receptor occurs in embryonic vascular endothelial cells where its expression subsides during development and is subsequently confined to lymphatic vessels after their formation [79]. Inactivating mutations in the catalytic loop of the VEGFR3 kinase domain leads to hereditary lymphedema (Milroy disease) that is characterized by the long-term and disfiguring swelling of the extremities owing to diseased cutaneous lymphatic vessels. During growth factor maturation, VEGFR3 undergoes proteolytic cleavage in the sixth immunoglobulin domain and the two segments of the original chain remain linked by a disulfide bridge [77].

Dixelius et al. examined the phosphorylation status mediated by VEGFR3 in VEGF-C–treated aortic endothelial cells overexpressing VEGFR3, transiently transfected human HEK293T cells expressing VEGFR3, or human primary lymphatic endothelial cells expressing VEGFR2/3 [80]. Following growth factor treatment, the cells were lysed, immunoprecipitated with anti-VEGFR3, and phosphorylation was performed with [γ-^{32}P]ATP in the immunocomplex. They identified five tyrosine phosphorylation sites (Y1230/1231/1265/1337/1363) in the C-terminal tail of human VEGFR3 (Table 5). pY1337 serves as the binding site for Grb2 and Shc, which occur at the beginning of the MAP kinase signal transduction module [77]. Using human primary lymphatic endothelial cells, these investigators found that, following VEGF-C (but not VEGF-A) treatment, VEGFR2 co-immunoprecipitated with VEGFR3 using anti-VEGFR3. Moreover, VEGFR3 residues Y1337/1363 were not autophosphorylated in the VEGFR2/3 immunocomplex. These results indicate that the interaction of the two receptors influenced the pattern of transphosphorylation and signal transduction by blunting the phosphorylation of the Grb2 and Shc binding sites.

Alam et al. examined the phosphorylation catalyzed by VEGFR3 in VEGF-A, VEGF-C, and VEGF-D–stimulated transiently transfected human HEK293T cells expressing VEGFR2, VEGFR3, or both receptors [81]. After various treatments, the cells were lysed, immunoprecipitated with anti-VEGFR2 or anti-VEGFR3. The immunoprecipitates were

Table 6
Properties of angiogenesis pathway adaptors[a].

Adaptor	Gene name	# Residues	MW (KDa)	Interactions	Signaling	Function	Domains	Isoforms	Uniprot ID
Cbl	*CBL*	906	99.6	Kit, Flt1, FGFR1/2, PDGFRα/β, CSF–1R, EphA8 and VEGFR2	Negative regulator of many pathways	E3 ubiquitin ligase	SH2-like, EF-hand-like, Cbl-PTB	5	P22681
Crk	*CRK*	304	33.8	GEF	Regulates cell adhesion & integrin activation	GEF activates Rap1	2 SH3, 1 SH2	2	P46108
Dok2	*DOK2*	412	45.4	Ras GAP, EGFR, Tie2	Cell proliferation	Scaffold	PH, PTB	3	O60496
FARP2	*FARP2*	1054	119.9	PlxA1, Rac1, RhoA/C	Regulates cytoskeleton	GEF	FERM, DH, PH1/2	3	O94887
Grb2	*GRB2*	217	25.2	SOS1	Connects RTKs to MAPK via SOS1	SOS1-Ras-Raf	2 SH3, 1 SH2	2	P62993
IQGAP1	*IQGAP1*	1657	189.3	Rac1, Cdc42	Connects RTKs to MAPK via Rac1 & Cdc42	Leads to Ras-Raf activation	IQ1/2/3/4	1	P46940
Nck1	*NCK1*	377	42.9	VEGFR2, PDGFRβ	Cell adhesion and migration	Links RTKs to signaling modules	SH2, 3 SH3	2	P16333
Nck2	*NCK2*	380	42.9	VEGFR2, PDGFRβ	Cell adhesion and migration	Links RTKs to signaling modules	SH2, 3 SH3	3	O43639
Paxillin	*PXN*	591	64.5	FAK, Src, vinculin, Crk, tubulin,	Regulates the assembly and turnover of the extracellular matrix	FAK and Src binds to active VEGFR2 and attracts paxillin	LIM zinc-binding 1/2/3/4	4	P49023
P120RasGAP	*RASA1*	1047	116.4	Ras upstream of MAPK pathway, Nck1	Regulation of cell growth, differentiation, & survival	Inactivates Ras	2 SH2, SH3, PH, C2, Ras-GAP	4	P20936
PLCγ	*PLCG1*	1290	148.5	PDGFRα/β, FGFR1/2/3/4	Actin reorganization & cell migration	Hydrolyzes PIP_2 to DAG & PIP_3 & activates PKC	2 SH2, SH3, 2 PH, PLC Y box	2	P19174
Rab11A	*RAB11A*	216	24.4	Myosin Vb	Intracellular membrane trafficking	GTPase	GTP-binding, switch 1/2	2	P62491
Rac1	*RAC1*	192	21.5	EGFR, K-Ras, Rab, Rho, c-Met, PI3K, PKC, PlxA1/A3	Cell proliferation & migration	GTPase	GTP-binding, switch 1/2	2	P63000
RhoA	*RHOA*	193	21.8	GEFs, GAPs, ROCK, PKN1/2, PLCγ, AKAP	Cytoskeletal organization	GTPase	Effector, switch 2	7	P61586
RhoC	*RHOC*	193	22.0	ROCK, IQGAP1, FAK, Akt, Pyk2, MRK	Cytoskeletal organization	GTPase	Effector, switch 2	7	P08134
Rin1	*RIN1*	783	84.1	Ras and Rab interactor 1	Cell migration and endosomal trafficking	Blocks association of Ras and Raf	SH2, Ras associating and Ras binding regions	2	Q13671
Rnd1	*RND1*	232	26.0	RhoA antagonist by blocking RhoGAP; PlxA2	Regulates cytoskeleton	Limits angiogenesis	α-helical & β-sheet	2	Q92730
Sck	*SHC2*	582	61.9	Grb2, VEGFR2	Cell growth, differentition, survival	MAPK, Atk activation	SH2, PTB	1	98077
SH3BP1	*SH3BP1*	701	75.7	PI3K, PlxD1, Abl, Src, Grb2, Rac1	Organization of the cytoskeleton	GAP	Rho-GAP, SH3	4	Q9Y3L3
Shb	*SHB*	509	55.0	FGFR1, PDGFRα/β, VEGFR2, Src, PI3K, Grb2	Cell growth, differentition, survival	MAPK, Atk activation	SH2, PTB	2	Q15464
Shc	*SHC1*	583	62.8	Grb2/SOS, PTKs	Cell growth, differentition, survival	MAPK, Atk activation	SH2, PTB, CH1	7	P29353
SOS	*SOS1*	1333	152.5	Ras, Rac, Rho, EGFR	Cell growth, differentition, survival	GEF, linked to MAPK, Atk activation	DH, PH	2	Q07889
SHIP2	*PTPN11*	593	68.0	Flt1/3/4, Kit, Grb2, PDGFRα, JAK-STAT pathway, MAPK pathway	Cell proliferation, migration, apoptosis	Protein phosphatase	2 SH2, phosphatase	3	Q06124
TSAd	*SH2D2A*	389	42.9	VEGFR2, Nck, ErbB1/2, PI3K, Src	Cell migration, transcriptional regulation	Regulates PTKs, Lck, Tck	SH2, 2 SH3	4	Q9NP31

[a]Data from www.uniprot.org.
[b]CH1, calponin homology domain-1; DH, Dbl homology domain; PH, pleckstrin homology domain; PLC, phospholipase C; PTB, phosphotyrosine binding; SH, Src homology;

probed with anti-VEGFR2, anti-VEGFR3, or antiphosphotyrosine antibodies. These experiments afforded information on the phosphorylation state that occurs intracellularly. In contrast to the findings of Dixelius et al. [80], Alam et al. found that VEGF-C failed to increase the phosphorylation of VEGFR3 expressed in HEK293T cells [81]. These investigators reported that VEGF-A and VEGF-D led to (i) the formation of a VEGFR2/VEGFR3 complex detected by immunoprecipitation and to (ii) the increased VEGFR2 phosphorylation. When a kinase-dead mutant of VEGFR2 and wild type VEGFR3 were co-expressed in the HEK293T cells, VEGF-A and VEGF-C led to increased VEGFR2 phosphorylation. These authors concluded that VEGFR3 interaction with VEGFR2 is required to catalyze growth factor-stimulated substrate phosphorylation.

Dixelius et al. found that VEGF-C induced VEGFR3 phosphorylation in immunoprecipitates derived from cells expressing only this receptor [80] while Alam et al. found that VEGF-A, VEGF-C, or VEGF-D failed to induce VEGFR3 phosphorylation in such cells [81]. This difference may be related to the cellular vs. immunocomplex phosphorylation

methodology. Although both groups found that VEGF-C induced the heterodimerization of VEGFR2 and VEGFR3, only Alam et al. found that VEGF-A induced heterodimerization [80,81]. 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.

3.4. Neuropilin-1 (Nrp1), neuropilin-2 (Nrp2), semaphorins, and plexins

3.4.1. Properties and expression

Based upon chemical cross-linking studies with disuccinimidyl suberate, Soker et al. identified a VEGF-A receptor in HUVEC samples that differed from VEGFR1/2 (VEGFR3 was unknown at the time) [82]. These investigators purified this receptor from human MDA-MB-231 breast cancer cells and showed that it is identical to neuropilin-1 (Nrp1), a surprising result at the time owing to the initial characterization of Nrp1as a neuronal recognition and cell adhesion molecule [83, 84]. Chen at al. subsequently characterized the related neuropilin-2 [84]. Numerous findings demonstrated that these receptors play a crucial role in tumor progression as well as axon guidance [85–87]. The neuropilins, which occur in numerous neoplasms, promote tumor growth by enhancing angiogenesis or by directly affecting tumor cells *per se*.

Neuropilins are transmembrane nonprotein-tyrosine kinase co-receptors for both the VEGF family and the semaphorin family [87, 88]. Although semaphorins were originally characterized as repulsive axon guidance regulators that collapse axonal growth cones and repel ganglionic axons during neurogenesis, these glycoprotein signaling molecules have diverse functions (Table 7). They regulate, *inter alia,* apoptosis, immune cell function, heart and blood vessel development, bone homeostasis, cancer cell progression, and adipogenesis. Semaphorins are a family of (i) membrane-bound, (ii) secreted, and (iii) proteolysis-released proteins characterized by a conserved extracellular Sema domain, a PSI domain, an immunoglobulin (Ig)-like domain, and thrombospondin repeats (Fig. 5A). PSI is the acronym for Plexins, Semaphorins, and Integrins and thrombospondin is an antiangiogenic protein characterized by its release from thrombin-treated platelets [89]. The Sema domain, with a seven-bladed β-propeller structure, is crucial for receptor binding and is found in both semaphorins and their plexin receptors (Fig. 6A).

Neuropilins operate as co-receptors with (i) large (≈ 250 kDa) transmembrane plexins that transduce semaphorin signaling and as co-receptors with (ii) VEGFR1/2/3 that mediate VEGF family signaling [87, 88,90]. The neuropilins are unique because they function as co-receptors in cell signaling initiated by two entirely different families of protein ligands (semaphorins and vascular endothelial growth factors) in combination with two different classes of co-receptors (plexins and VEGF receptors). The VEGF receptors are protein-tyrosine kinases while the mechanism of action of the semaphorin-plexin receptors is less clear. Plexins are large transmembrane semaphorin receptors with an average molecular weight of about 1.88 kDa (Table 8). They have a signal peptide of about 19 residues, an extracellular domain with about 1200 residues, and single-pass helical transmembrane segment of about 20 residues, and an intracellular domain containing about 1200

Table 7
Properties of semaphorins.[a]

Ligand[b]	# Residues	MW (kDa)	Receptors	Interactions	Tumor suppressor or promotor	Signaling	Secreted	Isoforms	Uniprot ID
Sema 3 A	771	88.9	Nrp1/PlxA1/A2/A4	ErbB2	Suppressor	Variable	Yes	3	Q14563
Sema 3B	745	83.1	Nrp1/2PlxA2/A4	PDGFα	Suppressor	↓PI3K/Akt	Yes	3	Q13214
Sema 3 C	751	85.2	Nrp1/2/PlxD1	ErbB1/2, PDGFα	Promotor	Wnt	Yes	2	Q99985
Sema 3D	777	89.6	Nrp1/2Plx	ErbB2, PDGFα	Suppressor	Rho/ROCK	Yes	1	O95025
Sema 3E	775	89.2	PlxD1	Src/ErbB2	Both	Rho/Notch	Yes	2	O15041
Sema 3 F	785	88.4	Nrp1/2/PlxA1/A3	PDGFRα	Suppressor	↓PI3K/mTOR, MAPK	Yes	2	Q13275
Sema 4 A	761	83.6	Nrp1/PlxB1/D1	Rab3b	Both	↓PI3K/Akt/mTOR	No	2	Q9H351
Sema 4B	837	92.8	Nrp1/PlxB2	Rac	Both	↓PI3K/Akt/mTOR	No	2	Q9NPR2
Sema 4 C	833	92.6	PlxB1/B2	ErbB2, c-Met, Rab family	Promotor	Variable	No	2	Q9C0C4
Sema 4D	862	96.2	PlxB1/2	Sema 4B/4D/4 G, PDGFRα	Promotor	Src, PI3K/Akt	No	2	Q92854
Sema 4E	?	?	?	?	?	?	?	?	?
Sema 4 F	770	83.5	?	PSD95	Both	TGFβ, Rho	No	2	O95754
Sema 4 G	838	91.5	PlxB2	PDGFRα	Both	?	No	1	Q9NTN9
Sema 5 A	1074	120.6	PlxA1/B3	c-Met	Both	?	No	1	Q13591
Sema 5B	1151	125.9	PlxA1	?	Promotor	?	No	1	Q9P238
Sema 6 A	1030	114.4	PlxA2/A4	VEGFR2	Promotor	Wnt/Rho	No	1	Q9H2E6
Sema 6B	888	95.3	PlxA2	VEGFR2, FGFR1	Both	Wnt, Notch, JAK/STAT	No	2	Q9H3T3
Sema 6 C	930	99.7	Nrp1/2PlxA1	FAK	Both	↓Akt	No	3	Q9H3T2
Sema 6D	1073	119.9	PlxA1	VEGFR2, ErbB1/2	Both	Notch, PI3K	No	8	Q8NFY4
Sema 6E	?	?	?	?	Suppressor	?	No	?	?
Sema 6 F	?	?	A1/A4	c-Met, ErbB2 VEGFR2	Both	PI3K	No	?	?
Sema 6 G	?	?	?	?	Suppressor	?	No	?	?
Sema 7 A	666	74.8	PlxC1	FAK	Promotor	Integrin β1 PI3K	No	2	O75326

[a] Data from www.uniprot.org.
[b] Sema, semaphorin.

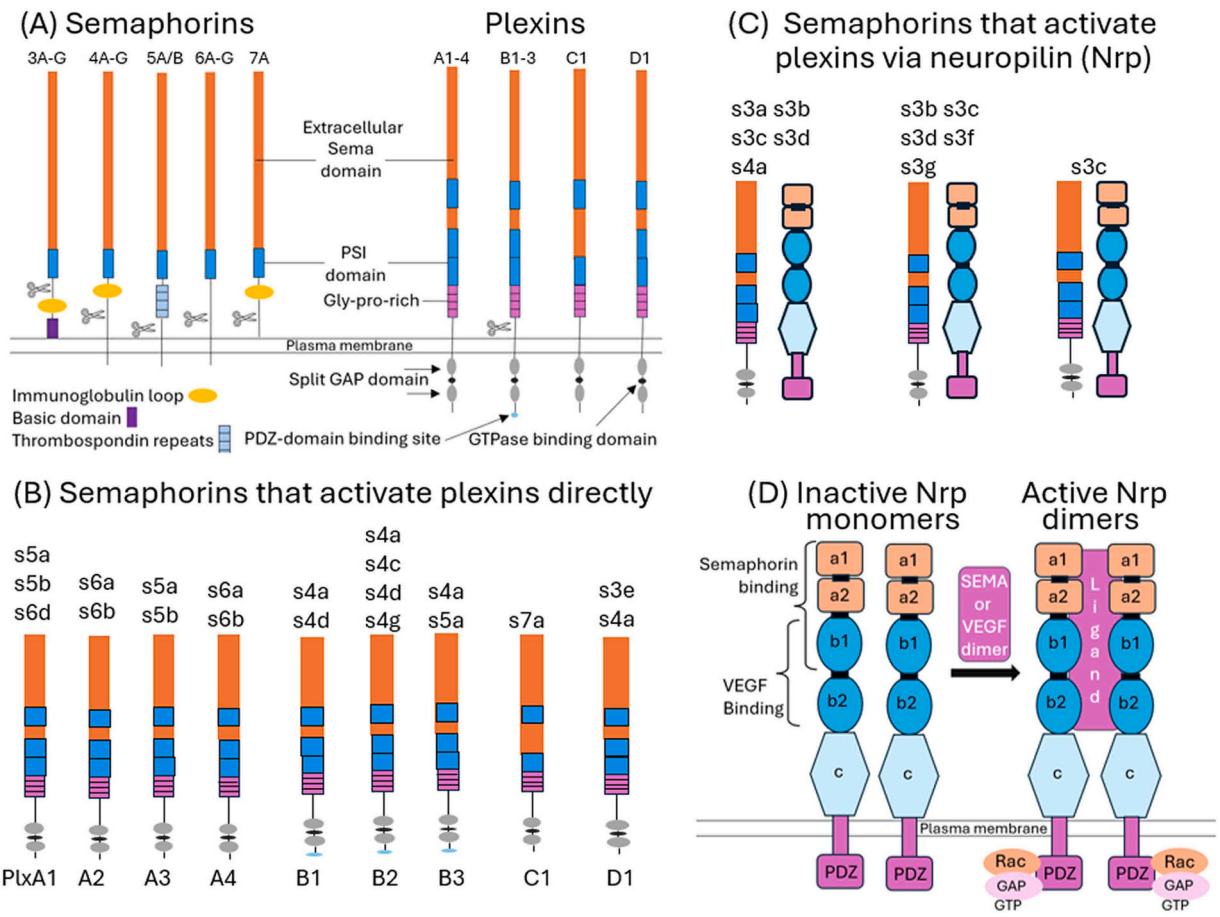

Fig. 5. (A) Architecture of semaphorins and their plexin receptors. (B) and (C) semaphorins that activate plexin receptors directly and indirectly, respectively. (D) Architecture of neuropilins. The scissors indicate the approximate location of proteolysis that releases a soluble extracellular fragment. The intracellular segments interact with various adapters. Relative lengths are not to scale. GAP, GTPase activating protein; PDZ, Postsynaptic density 95, Disk large, Zona occludens-1 domain; PSI, plexins, semaphorins, integrins; s5a, Sema 5 A.

residues. The extracellular domain contains seven disulfide bridges, nine N-linked glycosylation sites, a 500-residue Sema domain, a PSI domain, and four proline-rich motifs (Fig. 5A). The proline-rich segments facilitate conformational changes that enable interactions with co-receptors. The intracellular domain contains a split GTPase activating domain (GAP). Neuropilins function as receptors for VEGF isoforms independent of VEGFR1/2/3. Of the seven semaphorin classes [86,91], neuropilins interact with specified representatives of class III semaphorins (Table 1) [92–96].

Tanaka et al. determined the X-ray crystal structure of the extracellular Sema domain of the Sema 6D ligand interacting with the extracellular Sema domain of the PlexA1 receptor (Fig. 6A) [97]. The ligand binds to its receptor via hydrogen and electrostatic bonds, van der Waals contacts, and hydrophobic interactions. They found that M110 of Sema 6D interacted hydrophobically with PlxA1 residue F411, and M110 made van der Waals contact with PlxA1 residues S398, P399, L400, and Q409. Ligand K111 interacted hydrophobically with F411, made van der Waals contact with I396 and N397, and it made a salt-bridge with D410 of PlxA1, and ligand G112 made van der Waals contact with PlxA1 N379. Continuing toward the C-terminus of the ligand, L163 and A164 interact hydrophobically with PlxA1 F224, and ligand residue A189 interacts hydrophobically with F224. Ligand L192 interacts hydrophobically with PlxA1 residues L393 and F411while making van der Waals contact with D410 and Q413. Ligand residues Y199 and A207 make van der Waals contact with PlexA2 residue E223. Ligand residue I211 makes van der Waals contact with PlxA2 S233, and ligand K212 hydrogen bonds with PlxA1 F224. Ligand residue Y213 makes van der Waals contact with PlxA1 K391, S233, and D234. Ligand residues D214 and S215 hydrogen bond with PlxA1 S233 and D234, respectively. Ligand residue Q267 hydrogen bonds with PlxA1 Q102 and makes van der Waals contact with Y96, and ligand residue R268 hydrogen bonds with PlexA1 Q102 and makes van der Waals contact with Y96 and Y197. Ligand residue K394 interacts electrostatically with E196.

Sema 6D ligand residues L192, Y213, and R268 and receptor residues Y197, F224, L393, I396, P399, and F411 are especially important in forming the interface between the ligand and the PlxA1 receptor [97]. Sema 6D readily forms a homodimer, which then interacts with the receptor. Residues ^{286}VPGDS^{290} of one ligand protomer interact with ligand residues ^{321}NSIPG^{325} of the other protomer and vice versa, thereby forming a homodimer. Each ligand protomer interacts with a PlxA1 receptor to form a PlxA1-Sema 6D-Sema 6D-PlxA1 tetramer. Together these data indicate that protein-protein interactions are rather intricate.

Neuropilins are *N*-linked and *O*-linked glycoproteins with molecular weights ranging from 120 to 140 kDa [84]. The *NRP1* gene is located at 10p12 and the *NRP2* gene is located at 2q34. The neuropilins contain a large extracellular component (≈ 870 amino acid residues), a transmembrane segment (≈ 23 residues), and a short (≈ 40 residues) intracellular portion [98] Although the intracellular domain is too small to function as a catalyst, it is likely that it serves as a docking site for downstream adaptor molecules such as Rac and synectin, alone or in combination with VEGFR co-receptors. Fig. 5D depicts the architecture and functional components of the neuropilins.

Nrp1 and Nrp2 occur in sympathetic and sensory neurons (www.un

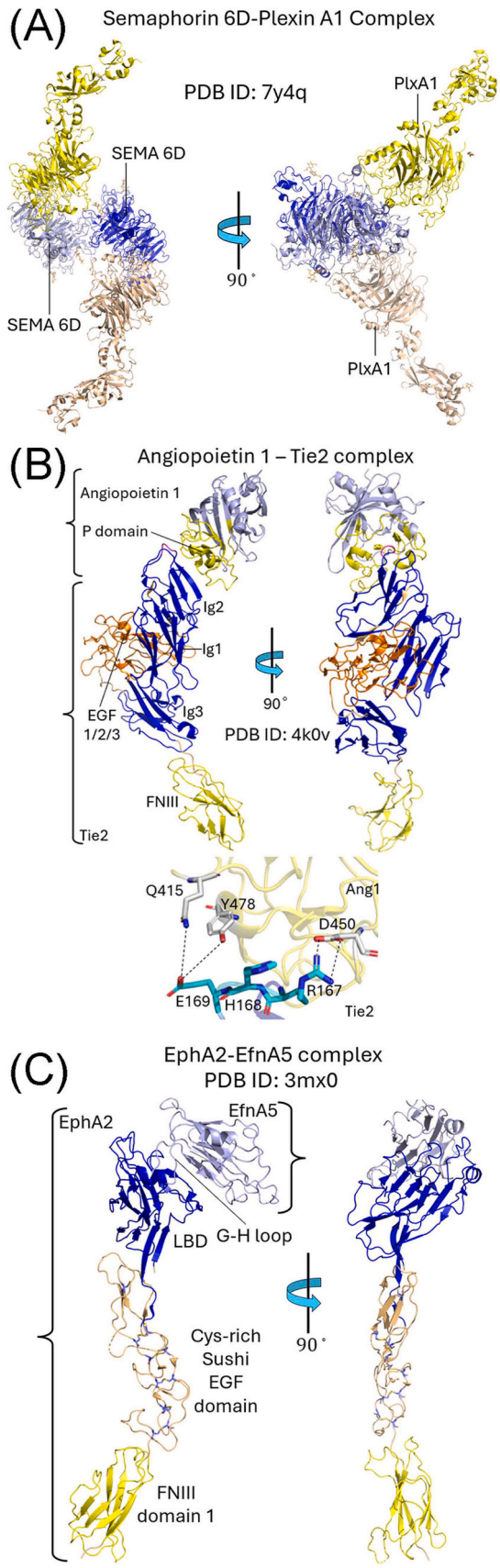

Fig. 6. (A) Semaphorin 6D-plexin A1 complex. (B) Angiopoietin 1-Tie2 complex. (C) EphA2-EfnA5 complex. EGF, epidermal growth factor-like; FNIII, fibronectin 3-like; LBD, ligand binding domain.

iprot.org) [99]. Both neuropilins participate in postsynaptic development and organization. Although they received their name from their initial neuronal localization, they are expressed with partially overlapping patterns in a wide variety of adult human tissues and tumor samples including those from breast, colorectum, lung, prostate, and melanoma [100]. Cultured HUVEC samples express both Nrp1 and Nrp2 with the latter predominating [86].

Kärpänen et al. examined the mode of interaction of VEGF-C and VEGF-D with the neuropilins [38]. As previously noted, the growth factor signal peptide is cleaved from the prepro-protein to yield a pro-protein. The generation of the mature growth factor requires two additional proteolytic cleavages to produce the N- and C-termini of the mature growth factors [29,30,37]. These investigators prepared soluble neuropilin-Ig (immunoglobulin) fusion proteins and discovered that the partially processed, but not mature, VEGF-C binds to Nrp1 whereas both bind to Nrp2 (Table 1). They reported that unprocessed, but not mature, VEGF-D binds to both Nrp1-Ig and Nrp2-Ig fusion proteins. These workers found that VEGF-C binds to the b1b2 segments of both neuropilins as well as it binds to the extracellular segments of the a1a2-b1b2 sector. VEGF-C failed to bind to an a1a2-b2 construct, which argues that the b1 domain participates in this binding interaction (Fig. 5D). These authors showed that Sema 3 F competes with the binding of VEGF-C to both the Nrp1-Ig and Nrp2-Ig fusion proteins.

Kärpänen et al. reported that aortic endothelial cells designed to express VEGFR3 and Nrp2 exhibited VEGF-C– induced internalization of a Nrp2-VEGFR3 complex [38]. VEGF-C–induced internalization of VEGFR3 occurred in cells expressing VEGFR3 alone, but a growth factor induced internalization of Nrp2 failed to occur in cells expressing Nrp2 alone. Hence, VEGF-C–induced Nrp2 endocytosis is contingent on VEGFR3. Furthermore, they observed VEGF-D–induced internalization and co-localization of VEGFR3 and Nrp2 in endocytic vesicles prepared from blood vascular or lymphatic endothelial cells. Although VEGF-A165 binds to Nrp2, this factor failed to affect the internalization of Nrp2 in lymphatic endothelial cells. These investigators suggested that the mechanism by which Nrp2 transmits VEGF-C and VEGF-D signaling probably involves its interaction with VEGFR3 [38].

Favier et al. treated aortic endothelial cells that were designed to express both human Nrp2 and VEGFR2 with VEGF-C and they performed immunoprecipitation studies [101]. Although there was modest association of Nrp2 and VEGFR2 in the absence of VEGF-C, they reported that VEGF-C greatly increased the complex formation. Furthermore, they reported that Nrp2 decreased the concentration of both VEGF-A and VEGF-C required to induce VEGFR2 autophosphorylation in aortic endothelial cells. These investigators transfected human microvascular endothelial cells with Nrp2 cDNA leading to its overexpression. These cells ordinarily express Nrp2, VEGFR2, and VEGFR3. This protein expression profile 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 Nrp2 overexpression promoted enhanced cell survival and cell migration evoked by VEGF-A or VEGF-C, responses that were blocked by Sema 3 F. These findings are consistent with the observation that Sema 3 F blocks VEGF-A and VEGF-C interaction with Nrp2 [38] and consequently blocks responses to these growth-factors. Furthermore, Favier et al. reported that VEGF-A and VEGF-C induced VEGFR2 phosphorylation in untransfected human microvascular endothelial cells and that treatment of these cells with a small interfering RNA targeting Nrp2 antagonized ligand-stimulated VEGFR2 phosphorylation and activation [101]. Evidence exists for the formation VEGFR1-Nrp1, VEGFR2-Nrp1, VEGFR1-Nrp2, VEGFR2-Nrp2, and VEGFR3-Nrp2 complexes [38,86,101]. The formation of VEGFR3-Nrp1 complexes is likely.

3.4.2. Neuropilins, semaphorins, and tumors

Neuropilin-1 and neuropilin-2 are found in numerous neoplasms (Table 9) and the relative expression of these two receptors differs. For example, Nrp1 expression in prostate and breast cancers exceeds that of

Table 8
Properties of plexins.[a]

Plexin	Residues	MW (KDa)	Ligands[b]	Interactions	Tumor suppressor or promotor	Signaling	Isoforms	Uniprot ID
A1	1896	211.1	S3a/c/f/6d	Nrp1/2	Both	VEGFR2, Rap, Rac1/2/3, Akt, JAK/STAT	1	Q9UIW2
A2	1894	211.1	S3a/6a/PlxA4	Nrp1/2	Promotor	Akt, p38MAPK, Rac1/2, Fyn, Rap1B	2	O75051
A3	1871	207.7	S3a/f	Nrp1/2	Both	Fyn, Rnd1, RhoD	1	P51805
A4	1894	121.5	S3a	Nrp1/2	Promotor	Rac1, FARP2, Rnd1	4	Q9HCM2
B1	2135	232.3	S4a/d	Nrp1/2	Both	RhoA, Rac, Src, c-Met, PI3K, ErbB2	3	O43157
B2	1838	205.1	S4c/d/g	Nrp1/2	Both	RhoA, Rac1/2/3, c-Met	1	O15031
B3	1909	206.8	S5a	Nrp1/2	Both	Rac1/2, Rho, c-Met	2	Q9ULL4
C1	1568	175.7	S7a	Nrp1	Suppressor	PI3K, FAK, Il−6/STAT3, integrin β1	1	O60486
D1	1925	212.0	S3c/3e/4a	Nrp1/2	Both	Rac1, Rap1, PI3K	2	Q9Y4D7

[a] Data from www.uniprot.org.
[b] S3a, Semaphorin 3a.

Nrp2 while Nrp2 expression in melanoma and glioblastoma exceeds that of Nrp1 [85]. In experiments to learn the potential function of Nrp1 in tumors, Miao et al. overexpressed it in AT2.1 (Dunning rat prostate carcinoma) cells using a tetracycline-inducible promoter [102]. They reported that increased expression of Nrp1 promoted VEGF-A165 binding to these cells in culture. Following injection of the AT2.1-Nrp1 cells into rats, the tumor size increased several fold following Nrp1 induction by doxycycline when compared with the control samples. The larger tumors with induced Nrp1 expression displayed endothelial cell proliferation and increased microvessel content. These experiments show that Nrp1 expression in tumors stimulates angiogenesis and tumor progression.

Parikh et al. studied the role of Nrp1in human colon tumor progression and growth [103]. They found that Nrp1 mRNA and protein were expressed in human colon adenocarcinoma samples, but not in adjacent normal colon mucosa. These investigators established a human colon carcinoma cell line (KM12SM/LM2) that was engineered to stably overexpress Nrp1 and found that subcutaneous xenografts in athymic nude mice displayed increased tumor angiogenesis and growth when compared with cells not overexpressing Nrp1. These investigators also found that the cells overexpressing Nrp1 exhibited a two-fold increase in cell migration in response to VEGF-A165 stimulation. Using cultured human colon carcinoma HT29 cells, these authors found that EGF and IGF-1 (insulin-like growth factor-1), but not Il-1β or TGFα, increased Nrp1 mRNA and protein expression. The EGF response was blocked by an anti-EGF receptor antibody. These investigators thus discovered a potential mechanism for growth factor-induced tumor progression by augmenting Nrp1 expression.

In experiments with the specific aim to determine the identity of signal transduction pathways, Parikh et al. reported that wortmannin, an irreversible PI3K3-kinase inhibitor, and U0126, a MEK1/2 inhibitor, diminished both basal and EGF-stimulated Nrp1 mRNA expression in HT29 cells [103]. Antagonism of basal expression points to an autocrine mechanism for activating these pathways. Because the colon cell lines expressed Nrp1, but not VEGFR2, these authors hypothesized that VEGF-A165 may bind simultaneously to Nrp1 on tumor cells and VEGFR2 on adjacent endothelial cells thereby activating endothelial cells and providing a juxtacrine mechanism for Nrp1 induction of angiogenesis and tumor progression.

Table 9
Neuropilin expression in selected human neoplasms and tumor cell lines.

Tumor type/origin
Astrocytoma
Bladder
Breast
Colorectum
Esophagus
Gall bladder
Glioma
Melanoma
Neuroblastoma
Non-small cell lung cancer
Pancreas
Prostate
Stomach
Small cell lung cancer

[a]Adapted from Ref. [2].

Wey et al. engineered human pancreatic carcinoma cells (FG) to stably overexpress Nrp1 [104]. Nrp1 overexpression decreased detachment-induced apoptosis (anoikis) and decreased sensitivity to (i) gemcitabine and (ii) 5-fluorouracil, which are cytotoxic drugs that are used to treat pancreatic and other malignancies. They found that Nrp1 overexpression increased unstimulated Erk 1/2 phosphorylation six-fold and Jun N-terminal kinase (JNK) phosphorylation four-fold. These investigators suggest that activation of Erk or JNK signaling may explain the chemoresistance observed with these two cytotoxic drugs.

Using a different human pancreatic cancer cell line (PANC-1), Wey et al. found that, in contrast to the FG cells, Nrp1 expression increased the sensitivity of cells to gemcitabine and 5-flourouracil [104]. Based upon experiments with the FG cells, these investigators surmised that the expression of Nrp1 in pancreatic cancer cells may one feature that leads to their notoriously widespread chemoresistance [104,105] while blocking Nrp1 signal transduction may foster increased sensitivity to cytotoxic agents [86,104]. However, the disparate results obtained with FG cells (Nrp1 expression leads to decreased sensitivity to cytotoxic agents) and PANC-1 cells (Nrp1 expression leads to increased sensitivity) accentuate the importance of the setting in which Nrp1 signaling occurs, and additional experiments will be required to determine the source of these inconsistencies.

Barr et al. studied the role of Nrp1 in VEGF-mediated survival of human MDA-MB-231 breast cancer cells [106]. They found that these cells express Nrp1 and Nrp2, but neither VEGFR1 nor VEGFR2. Furthermore, these cells constitutively express VEGF-A. Treatment of these cultured cells with an Nrp1 peptide antagonist, which corresponded to the sequence of exon 7 of VEGF-A165, produced apoptosis. These authors thus demonstrated that Nrp1 plays a crucial role in pro-survival signaling by VEGF-A in this breast cancer cell line and that Nrp1 blockade induces tumor cell apoptosis. Based upon confocal microscopic observations, these authors also demonstrated that anti-Nrp1 bound to both co-cultured tumor and HUVEC samples while anti-VEGFR2 bound only to the endothelial cells. Human Sema 3B inhibited tumor cell growth and induced apoptosis in the MDA-MB-231 human breast cancer cell line [2]. This result was reversed by VEGF-A165 but not VEGF-A121. This finding is consistent with the experiments demonstrating that Sema 3B and VEGF-165, but not VEGF-A121, bind to Nrp1 (Table 1). VEGF-A, which is widely expressed, possesses the ability to act as a pro-survival factor in a variety of cells containing Nrp1 but lacking VEGFR1 and VEGFR2.

In addition to its role in apoptotic signaling, neuropilin signaling underlies breast cancer cell migration. Nasarre et al. reported that VEGF-A165 promotes, while Sema 3 F abrogates, cell spreading and membrane ruffling in MCF7 and C100 human breast cancer cell lines [107]. The MCF7 cell line expresses Nrp1 but not Nrp2, and Sema 3 F inhibition of

cell spreading was blocked by anti-Nrp1. In contrast, the C100 cell line expresses Nrp2 and lower levels of Nrp1, and Sema 3 F signaling was inhibited by anti-Nrp2. The VEGF-A165 induced membrane ruffling was inhibited by Sema 3 F, which is consistent with the notion that Sema 3 F competes with VEGF-A165 for binding to the neuropilins.

In a subsequent study, Nasarre et al. developed an assay to determine whether motile human C100 breast cancer cells would migrate to or from Sema 3F-containing zones [108], a paradigm previously used to study nerve growth cone guidance. They reported that C100 cells migrated away from Sema 3 F, and this migration was blocked by anti-Nrp1 antibodies. In less motile MCF7 human breast cancer cells, Sema 3 F induced the loss of cellular contacts with the partial delocalization of E-cadherin and β-catenin. Moreover, MCF7 cell proliferation decreased in response to Sema 3 F. These investigators suggested that semaphorins maintain cellular boundaries, and loss of this activity increased cell migration during tumor progression. Loss of Sema 3 F expression because of chromosomal deletion (see below) is hypothesized to enhance cell migration and contribute to tumor spread and metastasis.

Sema 3B and Sema 3 F were originally identified from a recurrent 3p21.3 homozygous deletion in small cell lung cancer cell lines indicating that these might represent tumor suppressors [109–111]. Kusy et al. produced human lung cancer cell lines (NCI-H157 and NCI-H460) that stably express Sema 3 F [112]. These were implanted into the lungs of athymic nude rats to produce pulmonary tumors. Such cancer cell lines are generally more tumorigenic when implanted in their orthotopic, or usual, location than when they are when injected into an ectopic location such as the skin [112]. The tumorigenicity of H157 cells producing Sema 3 F was diminished when compared with cells lacking Sema 3 F. In contradistinction, the tumorigenicity of the H460 cell line was not diminished in the Sema 3 F producing cells. All these cell lines express comparable levels of PlxA1, PlxA3, PlxB1, and PlxB2, VEGFR1, and VEGFR2. The sensitive H157 cells express Nrp2 whereas the resistant H460 cells lack this receptor while both the sensitive and insensitive cells express Nrp1. The authors thus ascribed the sensitivity of the H157 cells to Sema 3 F to the expression of Nrp2. Overall, these experiments suggest that Sema 3 F has antitumor activity in Nrp2-expressing cells in this lung cancer paradigm.

Kessler et al. examined the role of Sema 3 F on angiogenesis in HUVEC samples [113]. They found that it inhibits VEGF-A165 and bFGF stimulated HUVEC proliferation. Sema 3 F antagonizes the interaction of VEGF-A165 with Nrp2, but it does not inhibit the binding of bFGF to its receptors. The mechanism of inhibition of the latter by Sema 3 F is thus unclear. Sema 3 F also inhibited VEGF-A165 and bFGF stimulation of angiogenesis *in vivo*. Overexpression of Sema 3 F in tumorigenic human embryonic kidney (HEK293) cells inhibits their tumor-forming ability in athymic nude mice, but Sema 3 F fails to alter their proliferation in cell culture. These findings suggest that Sema 3 F restrains tumor growth by antagonizing tumor angiogenesis, but not tumor cell proliferation.

Gagnon et al. reported that Nrp1, like VEGFR1 and VEGFR2, exists as a soluble isoform that contains the extracellular ligand binding a1a2 and b1b2 domains but lacks the downstream c, transmembrane, and cytoplasmic components [114]. Soluble Nrp1, like the membrane isoform, binds to VEGF-A165 but not VEGF-A121. The soluble and membrane isoforms are expressed differently. The membrane isoform is associated with blood vessels whereas the soluble form has a more widespread distribution including hepatocytes and proximal and distal renal tubules. Gagnon et al. prepared Dunning rat prostate carcinoma cells that expressed soluble Nrp1 and found that expressing and nonexpressing cells have identical growth rates in culture. Following subcutaneous injection into rats, however, they found that the tumors expressing soluble Nrp1 contained large hemorrhagic centers and exhibited widespread apoptosis but modest cell proliferation. In contradistinction, the Nrp1 nonexpressing tumor cells were solid, vascular, and exhibited sparse apoptosis and extensive proliferation [114]. Soluble Nrp1 presumably binds to VEGF-A and decreases its signaling, which impairs tumor cell vascularization and migration.

In summary, there is considerable evidence that Nrp1 and Nrp2 participate in tumor progression. As co-receptors with VEGFR1/2/3, the neuropilins function in conjunction with receptor protein-tyrosine kinase cascades. As co-receptors for the semaphorins, the neuropilins interact with plexins where the nature of signal transduction involves the regulation of numerous GTPases that affect the cytoskeleton and cell migration. It is likely that the neuropilin-plexin complexes interact with additional downstream components. Furthermore, activated neuropilins *per se* initiate a signaling cascade independently of the VEGF receptors or the semaphorin-plexin receptors. The nature of neuropilin signal transduction is undergoing further study. Whether Nrp2 (like Nrp1) participates in pro-survival signaling and whether the neuropilins participate in pro-survival signaling under physiological conditions as opposed to tumorigenic disorders remains to be determined.

4. The Tie protein-tyrosine kinases and their angiopoietin ligands

After the initial discovery of the VEGF-VEGFR system in the 1980s, the Tie receptors and their angiopoietin ligands were identified as a second endothelial cell-specific receptor protein-tyrosine kinase system [115,116]. The Tie acronym represents Tyrosine kinase with Ig and EGF homology domains, which describes the initial structural features of the extracellular domain of the Tie receptors. Both Tie1 and Tie2 possess two N-terminal Ig domains, three EGF domains, a third Ig domain, three fibronectin-like domains, a single-pass helical transmembrane segment, and a split protein kinase domain with an insert that is followed by a carboxyterminal tail (Uniprot IDs: P35590, Q02763; Fig. 3B). Tie1 was discovered in 1992 and its gene is located at 1p34.2; Tie2 was discovered in 1996 and its gene is located at 9p21.2. Tie1 is expressed in developing and mature blood and lymphatic vessels and in some hematopoietic cells [117]. Tie1 is an orphan receptor with no known specific ligand; it can nevertheless be activated by angiopoietins via its interaction with Tie2. Tie2 is expressed in the endothelium of all tissues and organs including angioblasts, sprouting vasculature, the quiescent vasculature of adults, and healing wounds. Tie2 is also expressed in some macrophages and various neoplasms. The immunoglobulin-like and EGF-like domains interact with its growth factors. Both Tie1 and Tie2 possess potential intracellular tyrosine phosphorylation sites. Its ligands are considered next. Important catalytic Tie residues are listed in Table 3 and the structural spine and shell residues are given in Table 4.

The ligands or growth factors for Tie2 include angiopoietin 1/2/4 (Ang1/2/4, Ang3 is a mouse-specific ligand closely related to human Ang4) [117]. Ang1 was discovered in 1996 and its gene is located at 8q23.1; Ang2 was discovered in 1997 and its gene is located at 8p23.1; Ang4 was discovered in 2000 and its gene is located at 19p13.2. Ang1 contains an N-terminal superclustering domain that allows it to form higher order oligomers and multimers [118]. This is followed by a coiled-coil domain that promotes oligomerization and a short linker domain that interacts with the extracellular matrix. The C-terminal FLD (fibrinogen-like domain) is responsible for its interaction with Tie2. Ang2 and Ang4 have similar structures. Ang1 is distributed throughout the normal adult vascular system and is expressed in mesenchymal cells, pericytes, fibroblasts, and megakaryocytes. It binds to Tie2 in a paracrine fashion and maintains vascular stability by its effects on endothelial cell-endothelial cell junctions and it promotes endothelial cell survival. Ang2 is expressed in low levels in most normal adult tissues and was found to be stored in the Weibel-Palade bodies of arterial endothelial cells [119]. This subcellular structure was found subsequently to contain von Willibrand factor, P-selectin, and Il-8. Ang2 is a competitive Tie2 antagonist of Ang1 [118]. However, this action is context-dependent and Ang2 can also function as an agonist. Ang4 plays multiple roles in humans primarily affecting lipid metabolism and angiogenesis. It is often overexpressed in various cancers and its upregulation can promote tumor growth, metastasis, and angiogenesis. It is

the least studied angiopoietin.

The initial assembly of the vasculature requires the VEGF family and its receptors [120]. In contrast, the Ang-Tie system is required for later stages of vascular development as vessels remodel and acquire pericytes and smooth muscle cells. *TIE2*$^{-/-}$ mouse embryos die between E9.5 and E12.5 owing to diminished amounts of peri-endothelial support cells while *TIE1*$^{-/-}$ embryos die after E13.5 because of defective microvessel integrity, severe edema, and hemorrhages resulting from an abnormal vasculature. The phenotypes of Ang1 and Tie2-deficient mice are quite similar. Ang2 deficiency in mice primarily affects postnatal angiogenesis and lymphatic development and function. These phenotypes underscore the critical role of Ang2 in vascular stability, permeability, and remodeling in both the blood and lymphatic systems during mid-gestation. Basal Ang1-Tie2 signaling maintains the quiescent endothelial phenotype, which involves the movement and clustering of Tie2 at inter-endothelial cell-cell junctions. Following endothelial cell activation, Ang2 is released from Weigel-Palade granules and abrogates Ang1-Tie2 signaling. Ang-Tie signaling controls vascular permeability, inflammation, and pathological angiogenic responses in adult tissues involving, *inter alia,* tumorigenesis and ocular disorders.

Yu et al. determined the X-ray crystal structure of Ang1 bound to the Ig2 domain of Tie2 (Fig. 6B) [121]. The receptor-binding portion of Ang1 is a coiled region that is fibrinogen-like and interfaces with the receptor via van der Waals, electrostatic, and hydrophobic interactions. Based upon fibrinogen nomenclature, the Ang1 binding component consists of A, B, and P domains and it is the P domain (residues 285–498) that interacts with Tie2. This region contains little secondary structure and consists of extended coiled sections. Ligand residues Q415 and Y478 make polar contact with Tie2 residue E169, and T450 forms salt bridges with Tie2 residue R167 (Fig. 6B). Ang1 residue M436 makes van der Waals contact with E151 of Tie2 and Ang1 L471 makes van der Waals contact with I162 and interacts hydrophobically with F161 of the Tie2 Ig2 domain. Ligand residue C437 makes van der Waals contact with kinase Ig2 residue G195. Ang1 residue M442 interacts hydrophobically with kinase residue I194 and ligand residue D450 forms a salt bridge with kinase residue R167. A451 of Ang1 interacts hydrophobically with kinase E151, and C452 forms a polar bond with D152. Ligand residues K470 and L471 interact hydrophobically with F161 of the kinase ectodomain. Ang1 residues H477, Y478, and S204 make van der Waals contact with Tie2 residues S164, P166, and I162, respectively. Furthermore, ligand residue S203 interacts hydrophobically with kinase residue H163. These interactions were obtained from PDB ID file 4k0v, but the residue numbers were derived from the uniprot database entry (https://www.uniprot.org/).

The interaction of Ang1 with Tie2 leads to the reciprocal trans-phosphorylation and activation of the Tie2 homodimer [117]. Auto-phosphorylation occurs sequentially. Y992 within the protein-kinase activation loop is phosphorylated first, followed by Y1108 and then with Y1102 (both within the C-terminal tail), and then with other residues. pY1108 interacts with Dok2, pY1102 binds to Shc1, Grb2 and Grb7, pY1106 interacts with Grb14 and SHIP2, and pY1101 leads to the activation of PI3K3R1. The interaction of the phosphorylated protein kinase domain with these proteins promotes growth factor-mediated receptor signal transduction. The Akt pathway is typically stimulated by the binding of a ligand to a receptor on the cell membrane, leading to the activation of PI3K and subsequent production of PIP_3 or phosphatidylinositol (3,4,5)-trisphosphate. PIP_3 recruits Akt to the cell membrane, where it is phosphorylated and activated by PDK1 (phosphoinositide-dependent protein kinase-1) and mTORC2 (mechanistic target of rapamycin complex-2). Activation of Akt promotes the activation of survivin, eNOS, transcription factor FOXO1, and the inhibition of caspase-9 and Bad [122].

Angiopoietins represent an unusual example of soluble ligands that engage receptor oligomers on the same cell *in cis* or bridge their receptors across cell–cell contacts *in trans* [115]. The activation of Tie2 by Ang1 *in trans* vs. *in cis* activates somewhat different signaling pathways. Ang1 binding to Tie2 in the endothelial cell extracellular matrix regions of contact represents the *cis* association mode, which preferentially activates the Erk1/2 and Dok pathways. In interacting endothelial cells, both Ang1 and Ang2 induce Tie2 translocation to cell–cell junctions, but Ang2 is capable of only weak Tie2 activation. Tie2 association may involve distinct ligand-binding modes *in trans* vs. those *in cis*. Tie1 interacts with Tie2 in endothelial cell-endothelial cell junctions and can regulate the context-dependent differences in Tie2 signaling during angiogenesis. The interplay between Tie1 and Tie2 is complex and can both promote and antagonize tumor angiogenesis. While Tie1 can limit excessive vessel growth and improve vessel maturity, tumors can exploit Tie2 and its ligands to promote their own growth and survival.

Besides its expression in endothelial cells, Tie2 occurs in a subset of tumor-associated macrophages that play an important role in tumor angiogenesis and metastasis [123]. These cells migrate toward and bind to sprouting blood vessels in response to hypoxia and cell death after vascular regression. Such proangiogenic perivascular macrophages promote vascular anastomoses and the formation of functional vessels. These cells are attracted to tumors to promote vascular reconstruction leading to metastasis. The Tie2-containing macrophages promote tumor cell intravasation allowing the neoplastic cells to enter the circulation and disseminate to secondary niches. This intravasation results from a reduction in the cohesion of endothelial junctions with the concomitant induced transient secretion of VEGF-A, a process that causes localized and transient vascular opening. In addition to the primary tumor, Tie2-positive macrophages are found in metastatic sites. Numerous neoplastic cells also produce Tie2 including those of the thyroid, ovary, stomach, brain (glioblastoma), and melanoma.

5. Ephrin receptors (Eph) and ephrin ligands (Efn)

The human erythropoietin-producing hepatocellular (Eph) receptors make up 14 of the 58 members of the receptor protein-tyrosine kinase family of proteins, thus making it the largest family of this class [124]. The ephrin receptors are classified into two subgroups – EphAs and EphBs – based upon their amino acid sequences. The ephrin ligands and their receptors are expressed in abutting cells leading to the formation and activation of an intricate signaling paradigm at cell-cell interfaces. The EphA subgroup consists of nine receptors (EphA1–8 and EphA10) and the EphB subgroup consists of five members (EphB1–4 and EphB6) (Table 10). The EphA10 and EphB6 receptors are kinase impaired. Ephrin ligands for these receptors include five type A factors (EfnA1–5) and three type B factors (EfnB1–3) (Table 11). The former activate the A class of ephrin receptors and the latter activate the B class of ephrin receptors. However, EfnA5 can interact with EphB2 and EfnB ligands can interact with EphA4. Important ephrin A receptor catalytic residues and spine residues are given in Tables 12 and 13, and those for ephrin B receptors are provided in Tables 14 and 15, respectively.

The ephrin family of ligands and receptors regulate numerous biological processes during embryonic development and extending throughout life [125]. The ephrin-ephrin receptor signaling pathways control the cellular cytoskeleton and regulate responses that involve cellular migration and changes in cellular morphology. This receptor family regulates axon guidance, organ segmentation, epithelial cell dispersal, and cell patterning. Ephrins and their receptors are important mediators of cell-cell interactions involved in regulating cell attachment, shape, and mobility. In adults, moreover, the ephrin-ephrin receptor group participates in the regulation of neuronal plasticity, cytoskeletal functioning, angiogenesis, and wound healing. The nature of ephrin-ephrin receptor signaling is context-dependent; in some cases, a given complex is stimulatory and in other situations, the same complex is inhibitory. The ephrin system can promote oncogenesis or it can function as a tumor suppressor. Cancers that have been linked to the ephrin system include those of the breast, brain, colon, kidney, lung, ovary, prostate, and stomach [126].

Although the ephrin family of receptors and ligands have been

Table 10
Properties of human ephrin receptor protein-tyrosine kinases.[a,b]

Kinase	Gene name	Type	# Residues	MW (kDa)	Ligand and signaling	Function	↑ in neoplasms	↓ in neoplasms	↑↓in neoplasms	Isoforms	Uniprot ID
EphA1	*EPHA1*	RY	976	108.1	Binds EfnA1, FAK, ILK, RhoA, Rac	Cell attachment to the ECM	HCC, PrC, BLCa, BRC, RCC, GC.		CRC	3	P21709
EphA2	*EPHA2*	RY	976	108.2	Binds EfnA1, FAK, Nck1, PI3K/Akt, Rac1, Vav2/3	Cell migration & adhesion	CRC, OV, GB, ESCa, PaC		BRC, CRC, NSCLC	2	P29317
EphA3	*EPHA3*	RY	983	110.1	Binds EfnA5, ADAM10, Nck1	Cell adhesion, cytoskeletal organization, cell migration	GB, GC	CRC, SCLC		2	P29320
EphA4	*EPHA4*	RY	986	109.9	Binds EfnA1/B3, Rac1, Rap1/2, Rho, PLCγ, PI3K, STAT5, Src	CNS development and plasticity	CRC, PaC, RC, GB, GC, NSCLC, RCC, MN		BRC	2	P54764
EphA5	*EPH5A*	RY	1037	114.8	Binds EfnA5, pATM, Cdc42, PI3K, Rac1, STAT3	Synaptic plasticity, pancreatic β-cell function	PTC, NSCLC, ENM	PrC, OV, BLCa,		3	P54756
EphA6	*EPHA6*	RY	1036	116.4	Binds EfnA1/3/4, Abl1, Alk2, c-Met, Vav3	Bidirectional signaling	BRC, PrC	GB		3	Q9UF33
EphA7	*EPHA7*	RY	998	112.1	Binds EfnA5, MAP2K1/2, MAPK1/3, TNFR1, PI3K	Brain development modulating cell-cell adhesion and repulsion	GB, HCC, NSCLC, LNG, PaC	CRC, PrC, ESCa, CC	GC	3	Q15375
EphA8	*EPHA8*	RY	1005	111.0	Binds EfnA2/3/5, Fyn, MAP kinases, Odin, TIAM1	CNS development and neurite outgrowth	OV, BLCa, GC, TSCC		CRC	2	P29322
EphA10[c]	*EPHA10*	RY	1008	109.7	A scaffold protein that binds to EfnA3/4/5 and signals via ERK1/2, FAK, JNK, NANOG, OCT4	Neuronal mobility and attachment	BRC, NSCLC, PaC, GC		BLCa	3	Q5JZY3
EphB1	*EPHB1*	RY	984	109.9	Binds EfnB1/2/3, MAPK/ERK, Nik, JNK, Src	Regulate cell migration and adhesion in the CNS	NSCLC, BLCa, GC, PrC, ESCa	GB, AML	CRC, OV,	3	P54762
EphB2	*EPHB2*	RY	1055	117.5	Binds EfnB1/2, Abl, Arg, Nck, PAK	Axon guidance and CNS development	MB, CC, HCC, PaC, HNC,	PrC	CRC, GC	3	P29323
EphB3	*EPHB3*	RY	998	110.3	Binds EfnB1/2, AF6, Crk, Fyn, MLLT4, RasGAP, Vav2	Cell migration and positioning, nerve development	PTC, NSCLC, ESCa	GC, GB	CRC	1	P54753
EphB4	*EPHB4*	RY	987	108.3	Binds EfnB2, Cav1, Tie2, Rho, Src	Cell adhesion and migration, heart morphogenesis, angiogenesis and blood vessel remodeling	OV, ESCa, HNC, PrC, GC, BLCa		CRC, PaC, BRC, NSCLC	4	P54760
EphB6[c]	*EPHB6*	RY	1021	110.7	Binds EfnB1/2, Abl, Clusterin, Dynactin, Src, ↓JNK	Cell adhesion and migration	CRC, TSCC	NSCLC, BRC		3	O15197

[a] Data from www.uniprot.org, Refs. [129,130,133,134].
[b] BLCa, bladder cancer; BRC, breast cancer; CRC, colorectal cancer; CC, cervical cancer; ECM, extracellular matrix; ENM, endometrial cancer; ESCa, esophageal cancer; GB, glioblastoma; GC, gastric cancer; HCC, hepatocellular carcinoma; HNC, head and neck cancer; LNG, laryngeal cancer; MB, medulloblastoma; MN, melanoma; MW, molecular weight; NSCLC, non-small cell lung cancer; OV, ovarian cancer; PaC, pancreatic cancer; PrC, prostate cancer; PTC, papillary thyroid cancer; RC, rectal cancer; RCC, renal cell carcinoma; RY, receptor protein-tyrosine kinase; SCLC, small cell lung cancer; TSCC, tongue squamous cell carcinoma
[c] Kinase impaired.

known for more than 35 years, our knowledge of their interactions and mechanisms of action has lagged behind that of the VEGF, angiopoietin, and other families [127]. One reason for this is that the various ephrins interact with each other and with numerous combinations of receptors thereby leading to a multitude of different signaling complexes making it difficult to determine the function of a single ligand or single receptor. With 14 ephrin receptors and eight ligands, the number of combinations taken two, three, four, or more at a time amounts to millions of possible interactions. The EphA and EphB receptor protein-tyrosine kinases have similar extracellular and intracellular structures. Contrariwise, the structures of the ephrin-A and -B ligands differ from each other. Ephrin type A factors are located extracellularly and bind to the plasma membrane by a glycosyl phosphatidylinositol anchor, and ephrin type B factors are transmembrane proteins with an intracellular PDZ domain. The P of PDZ refers to the Post Synaptic Density-95 protein, D corresponds to Disc large homolog-1 (a scaffold protein), and Z signifies ZO-1 (zona occludens-1 protein of tight junctions). Both type A and B ephrins have an extracellular receptor-binding domain (RBD) at their N-terminus as indicated in Fig. 6C. Alternative splicing of EfnA1/4/5 mRNAs produce secreted forms of these factors.

As observed in the VEGF and angiopoietin families, the interaction of ephrin ligands with their receptors involves van der Waals, hydrophobic, and electrostatic interactions [128]. In the case of the interaction of EfnA5 with EphA2 (PDB ID: 3mx0), the side chain of ligand β3-strand residue Y57 forms a hydrogen bond with the side chain of D53 of the EphA2 kinase Ig2 domain. Ligand β5-strand residue F97 interacts hydrophobically and ligand β5-strand residues K98 and R99 make van der Waals contact with the kinase Ig2 residue I58. Ligand β6-strand residues K113, F114, and S115 make van der Waals contact with Q56 of the kinase Ig2 domain and ligand β6-strand residue K117 and G-H loop residue Q119 form salt bridges with D53 of the kinase ectodomain. Ligand residue F121 of the G-H loop interacts hydrophobically with

Table 11
Selected angiogenic growth factors. [a]

Factor	Gene	# Residues	Mw (KDa)	Effectors	Function	Iso-forms	Uniprot ID
VEGF-A	*VEGFA*	395	43.6	Flt1/VEGFR1, KDR/VEGFR2, Nrp1	Active in angiogenesis, vasculogenesis & endothelial cell growth	17	P15692
VEGF-B	*VEGFB*	207	21.6	Same as VEGF-A	Metabolic support, neuroprotection, inhibits pathological angiogenesis	2	P49765
VEGF-C	*VEGFC*	419	46.9	VEGFR2/3, Nrp2	Angiogenesis and lymphangiogenesis	1	P49767
VEGF-D	*VEGFD*	354	40.4	VEGFR2/3	Angiogenesis, lymphangiogenesis & endothelial cell growth	1	O43915
PlGF	*PGF*	221	24.8	Flt1/VEGFR1, Nrp1/2	Angiogenesis and endothelial cell growth, proliferation & migration	4	P49763
PDGFA	*PDGFA*	211	24.0	PDGFRα/β	Embryonic development & wound healing	2	P04085
PDGFB	*PDGFB*	241	27.3	PDGFRα/β	Embryonic development, wound healing, blood vessel formation	2	P01127
PDGFC	*PDGFC*	345	39.0	PDGFRα/β	Embryonic development, wound healing, angiogenesis	4	Q9NRA1
PDGFD	*PDGFD*	370	42.8	PDGFRα/β	Embryonic development & wound healing	2	Q9GZP0
FGF1	*FGF1*	155	17.5	FGFR1/2/3/4 are upstream from MAPK & Akt	There are 18 fibroblast growth factors that bind to FGFR1/2/3/4; FGF1 is known as acidic FGF	2	P05230
FGF2	*FGF2*	288	30.8	Same as FGF1	FGF2 is known as basic FGF	4	P09038
Kit ligand	*KITLG*	273	30.9	Kit is upstream from MAPK, Akt, JAK-STAT	Growth factor for various cell types	3	P21583
HGF	*HGF*	728	83.1	c-Met	Growth factor for various cell types	6	P14210
Ang1	*ANGPT1*	498	57.5	Tie1/2, FAK, MAPK	Vasculogenesis & angiogenesis	2	Q15389
Ang2	*ANGPT2*	496	56.9	Tie2	Vasculogenesis, angiogenesis & lymphangiogenesis	3	O15123
Ang4	*ANGPT4*	503	56.8	Tie2	Vasculogenesis & angiogenesis	2	Q9Y264
EfnA1	*EFNA1*	205	23.8	Eph receptors	Cell adhesion, migration, angiogenesis	2	P20827
EfnA2	*EFNA2*	213	23.9	Eph receptors	Cell adhesion, migration, angiogenesis in the CNS	1	O43921
EfnA3	*EFNA3*	238	26.4	Eph receptors	Cell adhesion, migration, vasculogenesis, angiogenesis	2	P52797
EfnA4	*EFNA4*	201	22.4	Eph receptors	Neuronal, vascular, and epithelial development	3	P52798
EfnA5	*EFNA5*	228	26.3	Eph receptors	Axonal development, luminogensis	1	P52803
EfnB1	*EFNB1*	346	38.0	Eph receptors	Cell adhesion, vascular development	1	P98172
EfnB2	*EFNB2*	333	36.9	Eph receptors	Heart morphogenesis & angiogenesis	1	P52799
EfnB3	*EFNB3*	340	35.8	Eph receptors	Neuronal and vascular development		Q15768

[a] Data from www.uniprot.org.

Table 12
Composition and important residues of the human Ephrin A receptors.

Receptor component[a]	EphA1	EphA2	EphA3	EphA4	EphA5	EphA6	EphA7	EphA8	EphA10
Signal sequence	1–25	1–23	1–20	1–19	1–24	1–22	1–27	1–27	1–33
Extracellular domain	26–543	24–537	21–541	20–547	25–573	23–550	28–555	28–542	34–565
LBD	27–209	28–206	29–207	30–209	60–238	34–212	32–210	31–209	35–216
FN1	332–445	328–432	436–531	328–439	357–467	331–441	331–441	328–438	340–452
FN2	447–538	438–529	621–882	621–882	468–562	442–537	633–894	439–534	456–554
Transmembrane segment	544–572	538–558	542–565	548–565	574–594	551–571	556–576	543–563	566–586
JM segment	573–623	559–612	565–620	566–620	595–674	572–630	577–632	564–634	566–586
Protein kinase domain	624–884	613–875	621–882	621–882	675–936	631–944	633–894	635–896	645–900
Glycine-rich loop	GEGEFG, 631–636	GAGEFG, 620–625	GAGGFG, 628–633	GVGEFG, 628–633	GAGGFG, 682–687	GAGGFG, 638–643	GAGEFG, 640–645	GSGDSG, 642–647	GGGRFG, 652–657
β3-K of K/E/D/D	656	646	653	653	707	663	663	667	H677
αC-E, E of K/E/D/D	673	663	670	670	724	680	682	684	694
Hinge residues	EFMFNG, 703–708	EYMFNG, 693–698	EYMENG, 700–705	EYMENG, 700–705	EYMENG.754–759	EYMENG, 752–757	EFMENG, 712–717	EYMENG, 714–719	EYMSHG, 724–729
Catalytic loop, HRDLAARN	747–754	737–744	744–751	744–751	798–805	796–803	756–763	758–765	768–775, HRGLAARH
Catalytic loop HRD, First D of K/E/D/D	749	739	746	746	800	798	758	762	G770
Catalytic loop N, HRDLAARN	754	744	751	751	805	803	763	765	H775
AS DFG, Second D of K/E/D/D	767	757	764	764	818	816	726	778	G770
AS	767–796	757–784	764–793	764–794	818–848	816–846	776–806	778–808	788–816
AS tyrosines	781	772	779	779	833	831	791	793	801
AS End	APE, 794–796	APE, 784–786	SPE, 792–794	APE, 792–794	APE, 846–848	APE, 844–846	APE, 804–806	APE, 806–808	APE, 814–816
C-terminal tail	885–976	876–976	883–983	883–986	849–1037	847–1036	895–998	897–1005	901–1008
SAM	913–976	904–968	911–975	911–975	965–1029	961–1025	923–987	930–994	933–977
PDZ	974–976	974–976	981–983	984–986	1035–1037	1034–1036	996–998	1003–1005	1006–1008
No. of residues	976	976	983	986	1037	1036	998	1005	1008
Molecular Wt[b] (kDa)	108.1	108.2	110.1	109.9	114.8	116.4	112.1	111.0	109.7
Swiss-Prot accession no.	P21709	P29317	P29320	P54764	P54756	Q9UF33	Q15375	P29322	Q5JZY3

[a] AS, activation segment; FN, fibronectin III domain; JM, juxtamembrane; LBD, ligand binding domain; PDZ, PSD95/DLG/ZO-1 for post synaptic density protein-95/tumor suppressor DLGA/tight junction protein ZO-1; SAM, sterile alpha motif.
[b] Molecular weight of the unprocessed precursor.

Table 13
Spine and shell residues of human Ephrin A receptor family.

	Symbol	KLIFS No.[a]	EphA1	EphA2	EphA3	EphA4	EphA5	EphA6	EphA7	EphA8	EphA10
Regulatory spine											
β4-strand (N-lobe)	RS4	38	V691	L678	L685	L685	L739	V698	V700	L699	V712
C-helix (N-lobe)	RS3	28	M677	M667	M674	M674	M728	M684	M686	M688	L698
Activation loop F of DFG (C-lobe)	RS2	82	F768	F758	F765	F765	F819	F817	F777	F779	F789
Catalytic loop H (C-lobe)	RS1	68	H747	H737	H744	H744	H798	H796	H756	H758	H768
F-helix (C-lobe)	RS0	None	D808	D799	D806	D806	D860	D858	D818	D820	D828
R-shell											
Two residues upstream from the gatekeeper	Sh3	43	I700	I690	I697	I697	I751	I749	I709	I711	I721
Gatekeeper, end of β5-strand	Sh2	45	T702	T692	T699	T699	T753	V751	I711	T713	T723
αC-β4 loop	Sh1	36	L686	I676	I683	I683	I737	I693?	V695	I697	V707?
Catalytic spine											
β3-AxK motif (N-lobe)	CS8	15	A654	A644	A651	A651	A705	A661	A663	A665	A675
β2-strand (N-lobe)	CS7	11	V638	V627	V635	V635	V689	V645	V647	V649	L659
β7-strand (C-lobe)	CS6	77	L756	L746	L753	L753	L807	L805	L765	L767	L777
β7-strand (C-lobe)	CS5	78	V757	V747	I754	V754	I808	V806	V766	V768	V778
β7-strand (C-lobe)	CS4	76	I755	I745	I752	I752	I806	I804	I764	V766	V776
D-helix (C-lobe)	CS3	53	L710	L700	L707	L707	L761	L859	L719	L721	L731
F-helix (C-lobe)	CS2	None	V815	V806	V813	V813	V867	V865	V825	V827	I835
F-helix (C-lobe)	CS1	None	V819	V810	V817	V817	V871	V869	V829	V831	V839

[a] klifs.net, Kinase–Ligand Interaction Fingerprints and Structures.

Table 14
Composition and important residues of the human Ephrin B receptors.

Receptor component[a]	EphB1	EphB2	EphB3	EphB4	EphB6
Signal sequence	1–17	1–18	1–33	1–15	1–31
Extracellular domain	18–540	19–543	34–559	16–531	32–594
LBD	19–201	20–202	39–217	17–202	33–237
FN1	322–432	324–434	339–451	323–432	369–486
FN2	433–528	435–530	452–545	438–529	487–582
Transmembrane segment	541–563	544–564	560–580	540–560	595–615
JM segment	564–618	565–620	581–632	561–614	616–669
Protein kinase domain	619–882	621–884	633–896	615–899	670–919
Glycine-rich loop	GAGGFG, 626–631	GAGEFG, 628–632	GAGEFG, 640–645	GAGEFG, 622–627	GTGSFG, 677–682
β3-K of K/E/D/D	651	653	665	647	Q702
αC-E, E of K/E/D/D	668	670	682	664	R719
Hinge residues	EFMENG, 698–703	EFMENG, 700–705	EFMENC, 712–717	EFMENG, 694–699	EFMELG, 749–754
Catalytic loop, HRDLAARN	742–780	744–751	756–763	738–745	793–800, HRSLSAHS
Catalytic loop HRD, First D of K/E/D/D	744	746	758	740	S795
Catalytic loop Asn, HRDLAARN	749	751	763	745	S800
AS DFG, Second D of K/E/D/D	762	764	776	758	R813
AS	762–794	764–796	776–808	758–790	813–831
AS tyrosines	Y778	Y780	Y792	None	None
AS End	APE, 792–794	APE, 794–796	APE, 806–808	APE, 788–790	APE, 829–831
C-terminal tail	883–984	885–1055	897–998	900–987	920–1021
SAM	911–975	913–977	925–989	907–971	948–1012
PDZ	982–984	984–986	996–998	985–987	1019–1021
No. of residues	984	1055	998	987	1021
Molecular Wt[b] (kDa)	109.9	117.5	110.3	108.3	110.7
Swiss-Prot accession no.	P54762	P29323	P54753	P54760	O15197

[a] Abbreviations: AS, activation segment; FN, fibronectin III domain; JM, juxtamembrane; LBD, ligand binding domain; PDZ, PSD95/DLG/ZO-1 for post synaptic density protein-95/tumor suppressor DLGA/tight junction protein ZO-1; SAM, sterile alpha motif.
[b] Molecular weight of the unprocessed precursor.

R103, F108, and C188 of the kinase Ig2 domain and ligand G-H loop residue T122 forms a salt bridge with kinase residue R102 while interacting hydrophobically with kinase residue F156. Ligand residue P123 of the G-H loop makes van der Waals contact with EphA2 Ig2 residues V69, C70, T101, C188, V189, and A190. Ligand G-H loop residue F124 interacts hydrophobically with kinase Ig2 residues M66, S68, T101, A190, and L192; ligand G-H loop residue S125 makes van der Waals contact with EphA2 Ig2 residues D155, H160, and V161. Ligand residue L126 of the G-H loop interacts hydrophobically with ephrin receptor ectodomain residues N57, M59, I64, M66, and V161. Ligand G-H loop residue G127 makes van der Waals contact with kinase Ig2 residue N67 and the side chain of ligand residue E129 of the G-H loop forms a salt bridge with kinase Ig2 residue R103. The strands are numbered 1–8 from the N- to C-terminus of PDB ID 3mx0 and the loop residue nomenclature was taken from the report by Nikolov et al. [128]. The G-H loop residues play a predominant role in the interaction of the ligand with the EphA2 kinase domain (Fig. 6C).

Owing to their membrane-tethered structure, ephrin ligands and their receptors usually initiate intracellular signaling at sites of cell-cell contact and their predominant roles center on cell communication [124]. Both the ligands and receptors form distinct clusters within their corresponding plasma membranes thereby forming discrete signaling centers. Nearly all receptor protein-tyrosine kinases activate signaling pathways that regulate cell proliferation and differentiation. In contrast, the ephrin ligands and their receptors regulate cell adhesion, migration, and interaction with the extracellular matrix. Aberrant ephrin signaling can promote cell proliferation and differentiation under non-physiological conditions.

Table 15
Spine and shell residues of human Ephrin B receptor family.

	Symbol	KLIFS No.[a]	EphB1	EphB2	EphB3	EphB4	EphB6
Regulatory spine							
β4-strand (N-lobe)	RS4	38	L683	L685	L697	L679	V837
C-helix (N-lobe)	RS3	28	M672	M674	M686	M668	L723
Activation loop F of DFG (C-lobe)	RS2	82	F763	F765	F777	F759	L814
Catalytic loop H (C-lobe)	RS1	68	H742	H744	H756	H738	H793
F-helix (C-lobe)	RS0	None	D806	D808	D820	D802	D843
R-shell							
Two residues upstream from the gatekeeper	Sh3	43	I695	I697	I709	I691	V746
Gatekeeper, end of β5-strand	Sh2	45	T697	T699	T711	T693	T748
αC-β4 loop	Sh1	36	I681	I683	I695	I677	L792
Catalytic spine							
β3-AxK motif (N-lobe)	CS8	15	A749	A651	A663	A645	A700
β2-strand (N-lobe)	CS7	11	V633	V635	V747	V629	V684
β7-strand (C-lobe)	CS6	77	L751	L753	L765	L747	L802
β7-strand (C-lobe)	CS5	78	V752	V754	V766	V748	V803
β7-strand (C-lobe)	CS4	76	I750	I752	I764	I746	V801
D-helix (C-lobe)	CS3	53	L705	L707	L719	L701	L756
F-helix (C-lobe)	CS2	None	V817	V819	V827	V809	L850
F-helix (C-lobe)	CS1	None	V813	V815	V831	V813	V854

[a] klifs.net, Kinase–Ligand Interaction Fingerprints and Structures.

Also unique among receptor protein-tyrosine kinases is the ability of ephrin signaling to transduce signals bidirectionally [124,129,130]. As illustrated in Fig. 7, interaction between the ephrin ligand can promote signaling in the receptor-containing cell in the forward direction, in the ligand-containing cell in the reverse direction, or bidirectionally. The interaction of two ephrin and ephrin-receptor complexes *in trans* can promote augmented signaling in one direction while the interaction of the ephrin-ephrin receptor *in cis* abolishes signaling. Forward signaling by the ephrin system promotes cell proliferation, differentiation, migration, and adhesion. In contrast, reverse signaling regulates integrin-mediated cell adhesion and cell survival. Forward signaling is mediated by various GTPases and PI3K/Akt following activation of EphA or EphB receptors. Reverse signaling is mediated by Src and Src family kinases in conjunction with type B ephrin ligands. The

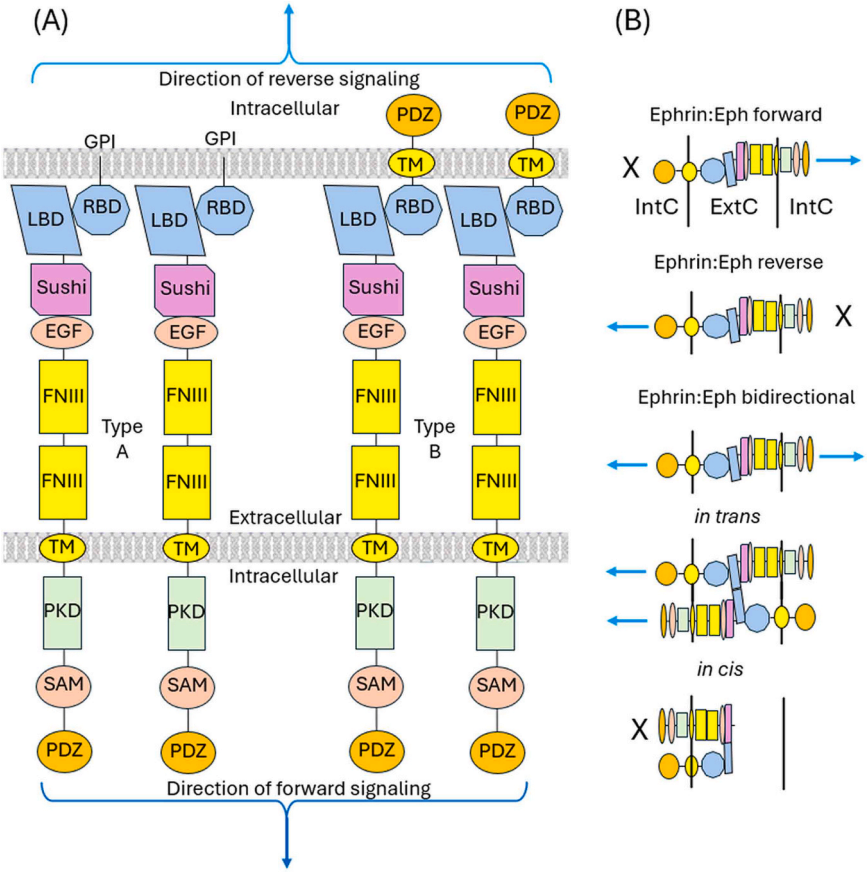

Fig. 7. Architecture of ephrin ligand and ephrin receptor and bidirectional signaling. EGF, epidermal growth factor-like; ExtC, extracellular; FN, fibronectin 3-like; GPI, glycosyl phosphatidyl inositol; IntC, intracellular; LBD, binding domain of the ligand; PDZ, Postsynaptic density 95, Disk large, Zona occludens-1 domain; PKD, protein kinase domain; RBD, receptor binding domain; TM, transmembrane.

nonreceptor protein-tyrosine kinases catalyze the phosphorylation of specific residues in the ligand thereby providing docking sites for Grb4 and other adaptor proteins. The adaptors interact with FAK and other proteins to alter cytoskeletal dynamics. The reverse signaling of type A ephrin ligands requires the participation and interaction with type B ephrin ligands.

Following the interaction of the ephrin ligand with its corresponding ephrin receptor, the pathways lead to the clustering, oligomerization, and trans-phosphorylation of tyrosine residues within the intracellular receptor protein-kinase domain [131]. The degree and extent of phosphorylation is dependent on the cellular context and the extent of ligand and receptor clustering. The strength of ephrin signaling is positively correlated with the extent of clustering. Ephrin-ephrin receptor signaling is attenuated by phosphatase-mediated receptor dephosphorylation and by endocytosis. The endocytoses can be unidirectional or bidirectional where the ephrin ligand-containing cell internalizes both the ligand and the ephrin receptor and vice versa [132].

The expression of several ephrin receptors and ligands have been reported in various neoplasms (Table 10) [129,130,133,134]. In lung cancers these include ephrin receptors (A1/2/3/4/5/7 and B3/4/6) and ephrin ligands (A3/B2/3). The levels of most of these are increased except for EphA3 and EphB6. In contrast, the levels of EphB4 are variable with studies demonstrating both increased and decreased expression in a context-dependent fashion. The levels of EphA2/4/10 and EphB2/4/6 have been examined in breast cancers with EphA2 and EphB4 being the most studied. The amounts of most of these are elevated except for EphB6, the expression of which was diminished. The EphA2 content in breast were context dependent with increases and decreases [134] demonstrating that EphB4 is linked to both pro- and anti-oncogenic roles. In the central nervous system, EphA2/3/4/5/7/8 and EphB2/4 were elevated in gliomas and Eph2A and EphB1/2 were increased in medulloblastomas. In prostate cancer, EphA2/3/4 levels were increased while EphA1 and EfnA1/5 were diminished. In colorectal neoplasms, EphB4 levels were increased and EphA6/7 and EphB1 levels were decreased. EphA2 levels were initially increased and then decreased in the advanced stages of colorectal cancers. Moreover, EphA3/4/8 and EphB4 have been reported to function as both tumor promoters and tumor suppressors in colorectal cancers [130]. EphA1/2/5, EfnA2/3/4, and EfnB1/2 levels were increased in hepatocellular carcinoma [124]. In summary, all ephrin receptors exhibited variable expression patterns in these six neoplasms.

Ephrin receptor activation occurs by ligand-induced trans phosphorylation of tyrosine residues in the juxtamembrane domain and then in the activation segment [134]. Under resting conditions, the juxtamembrane domain negatively regulates protein kinase activity. The phosphotyrosine residues attract various adaptor proteins bearing Src homology 2 (SH2) domains or phosphotyrosine-binding (PBD) domains. The SH2 domain of the regulatory subunit of PI3K binds to phosphorylated EphA and EphB leading to the activation of the PI3K catalytic domain. This leads to the conversion of PIP_2 to PIP_3 and the activation of the Akt protein-serine threonine kinases. The Akt isoforms promote cell growth, proliferation, metabolism, survival, and protein synthesis. SH2-contaning Vav2 and Vav3 GEFs interact with phosphorylated ephrin receptors leading to the activation of Rac1, RhoA, and Cdc42 and thereby regulating cytoskeletal dynamics, cell migration, proliferation, and gene transcription. EphA and EphB also activate the nonreceptor protein-tyrosine focal adhesion kinase (FAK) leading to the activation of RhoA, a small GTPase regulator of the cytoskeleton. Reverse signaling via ephrin A and ephrin B ligands participate in the regulation of cell adhesion, migration, the formation and patterning of blood vessels, and potentially influencing tumor growth, survival, and metastasis. The ligands regulate the activity of the nonreceptor Src and Src family protein-tyrosine kinases that mediate many of their downstream effects. The ephrin receptors act, *inter alia*, via Grb4, Rac1, and FAK.

6. Antiangiogenic drugs

6.1. Monoclonal antibodies and VEGF traps

Monoclonal antibody-based therapy is one of the most important modalities used for the treatment of various illnesses including solid tumors, hematological neoplasms, eye diseases, and immunological disorders [135]. The US FDA has approved more than 160 therapeutic antibodies and many more are under evaluation in clinical trials. Diseases that are candidates for the therapeutic inhibition of angiogenic signaling include cancer, diabetic macular edema, diabetic retinopathy, retinopathy of prematurity, retinal vein occlusion, endometriosis, neovascular age-related macular degeneration of the eye, rheumatoid arthritis, and psoriasis [4]. Of these conditions, the role of VEGF-A in tumor angiogenesis has received the greatest attention. Various approaches for blunting neoplastic growth and progression include abrogating VEGF signaling by using monoclonal antibodies targeting (i) VEGF isoforms, (ii) VEGF receptors, (iii) PDGF receptors, or by using small molecule inhibitors directed against VEGFR and other receptor protein-tyrosine kinases. Related strategies for attenuating pathological ocular angiogenesis with biologics include antibodies directed against angiopoietins [135].

Monoclonal antibodies are produced by a B cell hybridoma (a cell line derived by the fusion of a single normal B cell and an immortal B cell tumor line) [136]. César Milstein and Georges Köhler (a postdoctoral fellow with Milstein) received the Nobel Prize in Medicine or Physiology in 1984 for developing the hybridoma methodology (1975) for producing monoclonal antibodies [137]. There are several procedures for generating fully human monoclonal antibodies that rely on genetic engineering and cell culture techniques. These methods allow for the production of antibodies that consist of only human protein sequences, which reduces the risk of immune rejection compared to methods that used mouse-derived antibodies. These techniques include phage display, transgenic mice, and human single B-cell technologies [136].

Bevacizumab, which is a recombinant humanized mouse monoclonal antibody directed against human VEGF-A, is FDA approved for the treatment of metastatic colorectal cancer (initial approval in 2004), nonsquamous NSCLC, recurrent glioblastoma, metastatic renal cell carcinoma, persistent, recurrent, or metastatic cervical cancer, epithelial ovarian, fallopian tube, or primary potential peritoneal cancer, and hepatocellular carcinoma [4]. By sequestering VEGF-A, it prevents the growth factor from interacting with its receptors and blocks the activation of VEGFR signaling pathways that promote angiogenesis. Bevacizumab is not orally bioavailable and is given intravenously at 14-day intervals. Common side effects include headache, hypertension, nose bleeds, and rash. Hypertension is a common side effect of all direct and indirect systemic VEGFR inhibitors. Bevacizumab intravitreal injections are used off-label for the treatment of wet (neovascular) age-related macular degeneration, diabetic macular edema, and retinal vein occlusion.

Ramucirumab is an FDA approved (2014) humanized monoclonal antibody directed against the extracellular domain of VEGFR2 [138]. It is used for the treatment of NSCLC, gastric or gastro-esophageal adenocarcinoma, colorectal carcinoma, and hepatocellular carcinoma alone or in combination with other medications. The antibody binds to the extracellular domain 3 of VEGFR2 and blocks its interaction with VEGF-A/C/D thereby preventing receptor activation. Like bevacizumab, ramucirumab is given intravenously at 14-day intervals. Its major side effects include hypertension, proteinuria, and thrombocytopenia.

Monoclonal antibodies are used in the treatment of ocular diseases [4]. Faricimab (MW of 149 kDa) is a humanized bispecific antibody Fab fragment targeting VEGF-A and Ang2. The FDA approved this agent in 2021 and it is now used for the treatment of neovascular age-related macular degeneration, diabetic macular edema, and macular edema following retinal vein occlusion. The bispecific heterodimeric construct has different light chains in each of the Fab regions that bind to each of

its targets. Brolucizumab (MW 26 kDa) is a humanized monoclonal single-chain Fv monoclonal antibody fragment directed against the major isoforms of VEGF-A that was FDA-approved (2019) for the treatment of neovascular age-related macular degeneration and diabetic macular edema. Ranibizumab (MW 48 kDa) is a recombinant humanized IgG1- κ isotype monoclonal antibody fragment derived from bevacizumab which lacks an Fc region and targets VEGF-A. The drug, which was approved by the FDA in 2006, is used for the treatment of neovascular age-related macular degeneration, diabetic retinopathy, diabetic macular edema, and retinal vein occlusion. It has greater diffusion properties than bevacizumab owing to its lower molecular weight. These ocular drugs are given by intravitreal injection in monthly or bimonthly intervals with the exception of faricimab, which is given every 12–16 weeks.

Trebananib (AMG-386) is a peptide-Fc fusion protein (composed of the Fc portion of human immunoglobulin IgG1 acting as a carrier that is fused to a biologically active peptide) which inhibits angiogenesis by binding to the angiopoietins Ang1 and Ang2, and so preventing them from binding to Tie2 (www.guidetopharmacology.org). Unlike a typical antibody, it lacks the variable regions or light chains that bind to specific antigens. The medicinal was developed as a potential non-VEGF anti-angiogenesis agent for the treatment for recurrent epithelial ovarian cancer. It is or has been in 34 clinical trials. It was used for the management of various neoplasms including endometrial, ovarian, gastric, renal cell, hepatocellular, prostate and breast cancers. After promising initial results, it has faced setbacks and its FDA approval is unlikely. Zansecimab (LY3127804) is a humanized IgG4-κ monoclonal antibody constructed to block angiogenesis by targeting Ang2. It was developed for the treatment of solid tumors and for the treatment of pulmonary diseases. It was in clinical trials for the management of these disorders, but the studies were discontinued because it provided no clinical benefit.

Aflibercept is a recombinant fusion protein consisting of (i) the second extracellular domain (Ig2) of VEGFR1 and (ii) the third extracellular domain (Ig3) of VEGFR2 both fused to the Fc portion of human immunoglobulin G (Table 16) [138]. This dimeric glycoprotein (MW 115 kDa) acts as a soluble decoy receptor that binds VEGF-A, VEGFB, and PlGF thereby inhibiting the binding and activation of their corresponding VEGF receptors. Aflibercept inhibits choroidal neovascularization induced by overexpressed stimulatory growth factors. It is given by intravitreal injection in monthly or bimonthly intervals. Aflibercept is given intravenously at two-week intervals for the second-line treatment of colorectal cancer in combination with fluorouracil, leucovorin, and irinotecan [139]. Conbercept is similar to aflibercept except that it contains the third and fourth extracellular Ig domains of VEGFR2 and not just the third domain. It is not approved by the US FDA, but it is approved in China for the treatment of neovascular age-related macular degeneration and diabetic macular edema.

OPT-302 is a recombinant fusion protein consisting of three VEGFR3 extracellular Ig domains attached to the constant domain of human IgG1 [140]. It binds to and sequesters VEGF-C/D and prevents their binding to VEGFR2 and VEGFR3. It has undergone clinical trials alone and in combination with ranibizumab and with aflibercept. These trials have been completed or terminated, and it is unlikely that this agent will receive FDA approval. In contrast to these proteins, pegaptanib (MW 50 kDa) is a 28-base RNA molecule linked to polyethylene glycol that interacts with the heparin-binding domain of VEGF-A and prevents the binding of the latter to its receptors. Pegaptanib was previously FDA approved for the treatment of neovascular age-related macular degeneration, but this approval has been withdrawn.

6.2. *Small molecule drugs used for the treatment of neoplasms*

Because dysregulation of protein kinase activity occurs in many illnesses including autoimmune, dermatologic, inflammatory, and neoplastic diseases, this enzyme family has become one of the most important drug targets since the FDA approved imatinib for the treatment of chronic myelogenous leukemia in 2001 [141–145]. Protein kinases contain a small N-terminal lobe and a larger C-terminal lobe with a cleft in between that contains the ATP-binding site. Most small molecular kinase inhibitors are steady-state ATP inhibitors and bind directly to the ATP-binding site. Kinase antagonists that bind to their target enzyme elsewhere are classified as allosteric inhibitors. Owing to the conserved nature of the ATP-binding site, most small molecule kinase inhibitors target multiple protein kinases as indicated in Table 17.

Sorafenib (Fig. 8N) is FDA approved for the treatment of renal cell carcinoma, differentiated thyroid cancer, and hepatocellular carcinoma [143–145]. This agent blocks the activity of all three VEGF receptors and PDGFRβ, enzymes that play key roles in angiogenesis. It also blocks the activity of Raf, Kit, Flt3, and RET (Table 17). The pathogenesis of renal cell carcinomas is associated with angiogenesis [57]. Sunitinib is approved for the treatment of renal cell carcinoma, gastrointestinal stromal tumors, and pancreatic neuroendocrine tumors. It abrogates the activity of all three VEGFRs and both PDGF receptors; it also inhibits the activity of Kit, Flt3, RET, and colony stimulating factor-1 receptor. Pazopanib is FDA approved for the treatment of renal cell carcinoma and soft tissue sarcomas. It inhibits the activity of all three VEGFRs, both PDGF receptors, all four FGF receptors, EphA3/A5, and Src; note that these are among the chief activities associated with angiogenesis.

Table 16
FDA-approved drugs for the treatment of ocular diseases.[a,b]

Drug	Therapeutic indications	Primary targets[b]	Features	Trade name	Company	Year approved
Aflibercept	Neovascular age-related macular degeneration, diabetic retinopathy, diabetic macular edema, retinopathy of prematurity, macular edema following retinal vein occlusion	VEGF-A	Chimeric sVEGFR1/2	Eylea, Zaltrap	Bayer	2011
Bevacizumab	Used off-label for neovascular age-related macular degeneration	VEGF-A	Monospecific antibody	Avastin	Genentech	2004
Brolucizumab	Neovascular age-related macular degeneration, diabetic macular edema	VEGF-A	Monospecific single-chain antibody	Beovu	Novartis	2019
Conbercept	Neovascular age-related macular degeneration, diabetic macular edema	VEGF-A/B & PlGF	Chimeric sVEGFR1/2	Lumitin	Chengdu	2013
Faricimab	Neovascular age-related macular degeneration, diabetic macular edema, macular edema following retinal vein occlusion	VEGF-A & Ang2	Bispecific antibody	Vabysmo	Roche	2022
Ranibizumab	Neovascular age-related macular degeneration, diabetic retinopathy, diabetic macular edema, retinal vein occlusion, myopic choroidal neovascularization	VEGF-A	Monospecific antibody Fab fragment	Lucentis	Novartis	2006
Pegaptanib	Neovascular age-related macular degeneration (FDA approval withdrawn)	VEGF-A165	Aptamer, a single-strand nucleic acid	Macugen	OSI Pharm.	2004

[a] Data from Ref. [155] and www.accessdata.fda.gov/scripts/cder/daf/.
[b] Therapeutic antibody nomenclature conventions: - mab refers to a monoclonal antibody; - mumab refers to a human mab (e.g., ipilimumab), - zumab refers to a humanized mab (e.g., atezolizumab); -cixxmab is directed toward the cardiovascular system, (e.g., bevacizumab).

Pazopanib also blocks the activity of Kit, RET, Lck, Fms, and Itk.

Vandetanib was the first small molecule drug approved by the FDA (2011) for the management of medullary thyroid cancer (Fig. 8R) [138]. Although vandetanib inhibits the activity of key pro-angiogenic enzymes including the VEGFRs, Tie2, EphB6, Src, and Lck, its activity against medullary thyroid cancer may be more directly related to its inhibition of RET [152]. Axitinib is a second generation FDA approved drug that is prescribed as a single agent or as part of a combination therapy with avelumab or pembrolizumab for the treatment of renal cell carcinoma [153]. Axitinib targets all three VEGFRs, PDGFRβ, and Kit while the two monoclonal antibodies target the PD-1 receptor on T cells. Cabozantinib is an FDA approved drug used in the management of renal cell carcinoma, differentiated thyroid cancer, and hepatocellular carcinoma [154]. The drug is a multikinase blocker with activity against the VEGFRs and Tie2, which are important participants in angiogenesis. The drug also blocks the activity of RET, Kit, c-Met, Flt3, and ROS1.

Regorafenib is a fluorinated derivative of sorafenib that is used for the second-line therapy of colorectal and hepatocellular carcinomas and gastrointestinal stromal tumors [154]. This drug blocks the activity of several pro-angiogenic enzymes including VEGFR1/2/3, Tie2, Eph2A, PDGFRα/β, and FGFR1/2. It also diminishes the activity of Abl, B-Raf, and Kit. Nintedanib is used in the management of idiopathic pulmonary fibrosis and chronic fibrosing interstitial lung disease. The medicinal decreases the activity of the proangiogenic FGFR1/2/3, PDGFRs, and VEGFR1/2/3; it also blocks the activity of Flt3 receptor protein-tyrosine kinase. Nintedanib is or has been in more than 200 clinical trials, many of them targeting breast, non-small cell lung, renal cell, and colorectal cancers. Lenvatinib is approved by the FDA for the first-line treatment of differentiated thyroid cancer and hepatocellular carcinoma and for the treatment of renal cell carcinoma in combination with pembrolizumab [153]. It is approved for the second-line treatment of (i) RCC in combination with everolimus (an mTOR blocker) and (ii) endometrial carcinoma in combination with pembrolizumab. Lenvatinib blocks the activity of VEGFR1/2/3, FGFR1/2/3/4, and PDGFRα, which are pro-angiogenic proteins [57,151,155,158]. It also inhibits the activity of Kit and RET.

Fruquintinib is FDA approved for the second-line treatment of adults with metastatic colorectal cancer who received prior fluoropyrimidine-, oxaliplatin-, and irinotecan-based chemotherapy, an anti-VEGF therapy, and, if *RAS* wild-type and medically appropriate, an anti-EGFR therapy (Fig. 8D) [156,157]. This agent is a selective inhibitor of VEGFR1 (IC_{50} value of 33 nM), VEGFR2 (35 nM), and VEGFR3 (0.5 nM). It has been tested against more than 250 protein kinases and was found to have slight activity at a concentration of 1 μM against RET, FGFR1 and Kit. Tivozanib is approved for the third-line treatment of advanced renal cell carcinoma [57,154]. This medicinal inhibits the activity of VEGFR1/2/3, PDGFRβ, and Kit [159]. The IC_{50} values against the VEGF receptors are less than 0.25 nM making it a potent blocker. The daily dose of tivozanib is 1.34 mg per day and it is given for 21 days in each 28 day cycle; the 1.34 mg is the lowest daily dose for any of the FDA-approved protein kinase blockers [144]. Like all VEGFR antagonists, hypertension is a common adverse effect. Dasatinib and ponatinib are FDA approved protein kinase inhibitors that attenuate the activity of pro-angiogenic pathways (Table 17). However, they are approved for the treatment of Ph^+ leukemias and their therapeutic effect is not directly related to the blockade of angiogenesis.

Pexmetinib is an orally bioavailable small-molecule inhibitor of p38 MAP kinase (β isoforms) and Tie2 kinases [146]. Concomitant inhibition

Table 17
Selected FDA-approved anti-angiogenic small molecule protein-kinase inhibitors, their targets, and therapeutic indications.[a]

Drug	Code	Company	Trade name	Year approved	Targets	Therapeutic indications[b]
Axitinib	AG013736	Pfizer	Inlyta	2012	VEGFR1/2/3, PDGFRβ, Kit	Single agent and combination therapy for renal cell carcinoma (RCC)
Cabozantinib	BMS907351	Exelixis	Cometriq & Cabometyx	2012	RET, VEGFR1/2/3, Tie2, Kit, c-Met, Flt3, ROS1	Differentiated thyroid cancer, RCC, HCC
Dasatinib	BMS 354825	Bristol-Meyers Squib	Sprygel	2006	EphA2/A5/B4, Abl, EGFR, Src, Lck, Yes, Fyn, Kit, PDGFRβ	Ph^+ CML or ALL
Fruquintinib	HMPL013	Takeda	Fruzaqla	2023	VEGFR1/2/3	CRC
Lenvatinib	AK175809	Easai Co.	Lenvima	2015	VEGFR1/2/3, FGFR1/2/3/4, PDGFRα, Kit, RET	Differentiated thyroid cancer, HCC, RCC, endometrial carcinoma
Nintedanib	BIBF 1120	Boehringer	Ofev	2014	FGFR1/2/3, PDGFRs, VEGFRs, Flt3	Idiopathic pulmonary fibrosis
Pazopanib	GW786034	GSK	Votrient	2009	VEGFR1/2/3, PDGFRα/β, FGFR1/3, Kit, Lck, Fms, Itk,	RCC, soft tissue sarcomas
Pexmetinib	ARRY−615	Array BioPharma	N/A	N/A	p38, Tie2	Myelodysplastic syndromes, AML
Ponatinib	AP 24534	Ariad Pharm	Iclusig	2012	BCR-Abl, VEGFR1/2/3, Tie2, PDGFRα/β, FGFR1/2/3/4, EphA2/A5, Src family kinases, Kit, RET, Flt3	Ph^+ ALL, CML, $T315I^+$ CML
Rebastinib	DCC−2036	Deciphera	N/A	N/A	VEGFR2, Tie2, Lyn, Hck, Fgr, BCR-Abl	Breast cancer, CML, other solid tumors
Regorafenib	BAY 734506	Bayer	Stivarga	2012	VEGFR1/2/3, Tie2, Eph2A, BCR-Abl, B-Raf, Kit, PDGFRα/β, RET, FGFR1/2	Second-line treatment of CRC, HCC, GIST
Rivoceranib	YN968D1	LSK Bio (USA), Jiangsu Hengrui (China), HLB Life Sci (Korea)	N/A	N/A	VEGFR2	Gastric, hepatic, breast, thyroid, ovarian, colorectal carcinomas, GIST, gliomas, sarcomas
Sitravatinib	MGCD−516	Mirata Ther.	N/A	N/A	VEGFR1/2/3, EphA4, Axl, c-Met, Flt3, Kit, TRKA/B	NSCLC, RCC, liposarcomas
Sorafenib	BAY 439006	Bayer	Nexavar	2005	VEGFR1/2/3, B/C-Raf, Kit, Flt3, RET, PDGFRβ	HCC, RCC, differentiated thyroid cancer
Sunitinib	SU11248	Pfizer	Sutent	2006	VEGFR1/2/3, PDGFRα/β, Kit, Flt3, CSF−1R, RET	GIST, RCC, pancreatic neuroendocrine tumors
Tesevatinib	XL647	Exelixis/ Kadmon	N/A	N/A	ErbB1/2, VEGFR2/3, EphB4	NSCLC, polycystic kidney disease
Tivozanib	AV951	AVEO Pharma	Fotvida	2021	VEGFR1/2/3, PDGFRβ, Kit	Third-line treatment of RCC
Vandetanib	ZD6474	Sanofi	Zactima	2011	VEGFR1/2/3, Tie2, EphB6, RET, Brk, EGFR, Src, Lck, Abl1	Medullary thyroid cancer

[a] Data from Ref. [144,146–151].

[b] ALL, acute lymphoblastic leukemias; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; CRC, colorectal cancer; GIST, gastrointestinal stromal tumor; HCC, hepatocellular carcinoma; NSCLC, non-small cell lung cancer; Ph^+, Philadelphia chromosome positive; RCC, renal cell carcinoma.

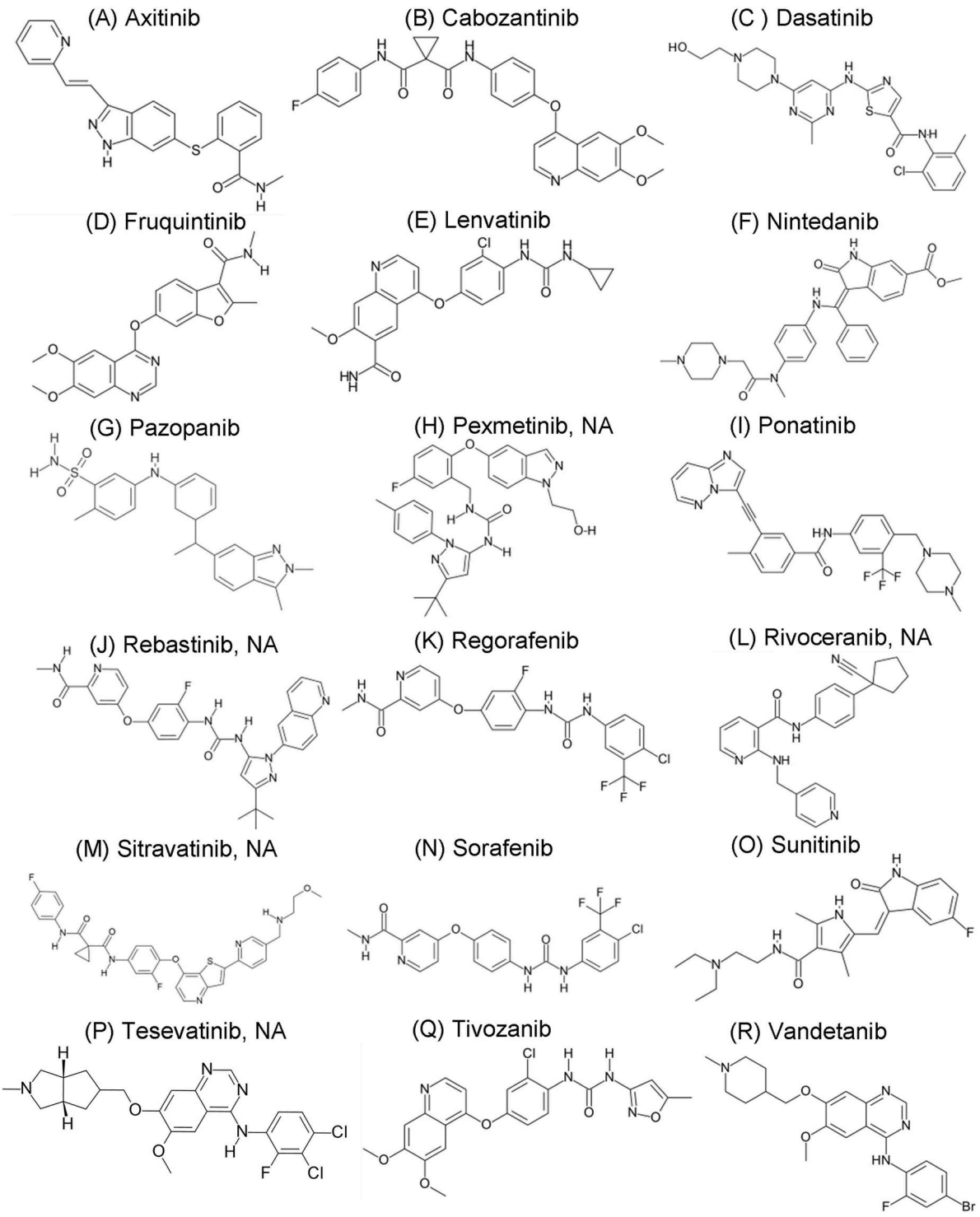

Fig. 8. Selected small molecule protein-tyrosine kinase blockers. NA, Not FDA Approved.

of these kinases is hypothesized to produce antineoplastic, antiinflammatory, and antiangiogenic responses that represent a novel treatment modality for myelodysplastic syndromes (MDS), RCC, melanomas, and other solid tumors, which are being studied in three clinical trials (clinicaltrials.gov). Pexmetinib is not currently approved by the FDA. Rebastinib (DCC-2036) is an orally bioavailable small-molecule inhibitor of multiple tyrosine kinases (Table 17) with potential antineoplastic activity [147]. The Tie2 and VEGFR2 receptor protein-tyrosine kinases regulate angiogenesis while the Src family kinases Abl, Lyn, and Hck regulate a variety of cellular responses including differentiation, division, adhesion, and the stress response. Rebastinib has been in four clinical trials for various solid tumors, breast cancer, and CML. It has not yet received FDA approval. Tesevatinib (Fig. 8P) is an orally effective multikinase blocker (ErbB1/2, VEGFR2/3, and EphB4) [149] that is or has been in seven clinical trials for polycystic kidney disease, glioblastoma, NSCLC, and HER2-positve breast cancer. All of these clinical trials have been completed or have been terminated, so it is unlikely that this medicine will be approved by the FDA.

Sitravatinib is an orally bioavailable multikinase inhibitor targeting VEGFR1/2/3, EphA4, c-Met, Flt3, Kit, and PDGFRβ (Table 17) [148]. It

is or has been in 34 clinical trials alone or as part of a combination therapy targeting various neoplasms including NSCLC, RCC, and liposarcomas. It has not been approved by the US FDA. Rivoceranib is a specific blocker of VEGFR2 with no significant effect against a panel of 269 other protein kinases [150]. The drug has been approved in China and Japan and it is or has been in 576 clinical trials against a variety of malignancies including gastric, hepatocellular, and breast cancers. It has not been approved by the US FDA. However, of the five unapproved drugs listed in this section, rivoceranib and sitravatinib are the most likely to reach such a milestone.

6.3. Small molecule drugs used for the treatment of eye diseases

Intravitreal injections are the standard of care for treating neovascular age-related macular degeneration, diabetic macular edema, and diabetic retinopathy [160]. However, frequent administration creates a significant treatment burden owing to the limited drug half-life and the chronic nature of these conditions. Patients generally find intravitreal injections burdensome, but they are willing to continue with injections if their vision stops deteriorating. Various treatment modalities are underway to decrease the frequency of intraocular injections including the use of reservoirs for intravitreal drug delivery, hydrogel delivery systems, and polymer-based delivery [161].

The Port Delivery System with ranibizumab (Susvimo) is a nondegradable refillable implant that is surgically placed in the pars plana and allows for the sustained release of the monoclonal antibody into the vitreous [161]. The implant is a small, rice-grain-sized device surgically implanted in the eye that continuously delivers the drug over months, reducing the need for frequent injections. It consists of a central drug reservoir with a self-sealing septum for refills, an extrascleral flange for anchoring, and a semipermeable titanium membrane on the distal end that releases the drug passively into the vitreous. This system is FDA approved for the second-line treatment of neovascular age-related macular degeneration, diabetic macular edema, and diabetic retinopathy. Clinical trials using the Port Delivery System with a combination of ranibizumab and faricimab are currently underway.

The EYP-1901 durasert implant (Duravyu), which consists of polymeric fluocinolone acetonide in conjunction with vorolanib, a drug that inhibits the activity of VEGFR1/2/3 and PDGFRα/β [162]. This treatment modality is in clinical trials against neovascular age-related macular degeneration. Data for this non-FDA approved treatment indicate that the implant effectively delivers the drug for six months. Hydrogels are multidimensional structures created through the chemical cross-linking of polymer chains. Axpaxli (OTX-TKI) is a bioresorbable intravitreal polyethylene glycol hydrogel implant that contains axitinib, which is a VEGFR1/2/3, PDGFRβ, and Kit antagonist (Table 17). This hydrogel implant gradually releases axitinib over a span of six to 12 months. Axpaxli is in five clinical trials for neovascular age-related macular degeneration and diabetic retinopathy (clinicaltrials.gov).

Another strategy for decreasing the interval between ocular injections involves the use of suprachoroidal injections [161]. The suprachoroidal space (SCS) is a thin potential space located between the sclera and choroid. The SCS allows for a less invasive access point to the posterior region of the eye for drug delivery owing to posterior pole fluid flow. Moreover, the relative insolubility of small-molecule suspensions can increase durability in the SCS [161]. Reliable access to suprachoroidal space involves microneedles that have been designed to penetrate the sclera to facilitate the delivery of therapeutic agents into the SCS. The intraocular pressure drives the injectate circumferentially and posteriorly toward the lower pressure posterior SCS to target chorioretinal cells. CLX-AX is a drug modality designed for suprachoroidal injections with axitinib as the active ingredient. It is currently undergoing clinical trials to establish the efficacy and safety of this agent. See Ref. [161] for a description of ocular drug delivery using the suprachoroidal space methodology.

Eye drops obviate the necessity of ocular injections and studies are underway to develop nanocrystallized eye drops for the treatment of ocular diseases with the use of KHK4951 [162]. This agent uses tivozanib, which antagonizes the activity of VEGFR1/2/3, PDGFRβ, and Kit as the active ingredient (Table 17). A nanocrystallized eye drop is a medication that uses nanotechnology to create active drug ingredients in tiny crystal forms. By reducing the size of the drug particles to the nanoscale (1–100 nanometers), these drops overcome the eye's natural barriers more effectively than traditional eye drops, which are usually composed of larger microsized particles. KHK4951, which is not FDA approved, is in three clinical trials for the management of neovascular age-related macular degeneration and diabetic macular edema (clinicaltrials.gov).

7. Epilogue

Vasculogenesis and angiogenesis are complex processes [2]. The present overview focuses on the role of the VEGF, angiopoietin-Tie, and the ephrin families. These represent proximal signaling modules that initiate and maintain the vasculature. Other primary regulators of angiogenesis include PDGFRα/β, FGFR1/2/3/4, the stem cell factor receptor (Kit), and the hepatocyte growth factor receptor (c-Met). Because angiogenesis entails cell growth, differentiation, division, and maintenance, many steps are involved. Thousands of chemical reactions are involved in vascular endothelial cell replication, and cell division is only one portion of the angiogenic response. Other modules and pathways involved in the complete angiogenesis process include PI3K, Hippo, Myc, Notch, TGFα/β, and Wnt.

The concentration of oxygen plays a pivotal role in regulating the expression of hundreds of genes [2]. Under hypoxic conditions, the expression of many proteins including enzymes of the glycolytic pathway, erythropoietin, and VEGF-A increases. Erythropoietin augments red blood cell production leading to increased oxygen transport. Greater VEGF-A production promotes angiogenesis, which in turn leads to amplified red cell and oxygen delivery to tissues and cells. The hypoxia-inducible transcription factor (HIF) family is central to the adaptive response to hypoxia. HIF is a heterodimer composed of HIF-1α and HIF-1β. Whereas HIF-1β expression and activity are unaffected by changes in oxygen concentration, the concentration of cellular HIF-1α is augmented during hypoxia leading, *inter alia*, to increased VEGF-A production. Greg L. Semenza, Peter J. Radcliffe, and William G. Kaelin Jr. received the Nobel Prize in Medicine or Physiology in 2019 for their independent work on HIF.

Judah Folkman was the first to hypothesize that abrogating angiogenesis represented a potential strategy for treating cancer [163]. This led to the isolation, cloning, and elucidation of the structure of VEGF-A [18]. Subsequently, the rest of the VEGF family of ligands, associated isoforms, and receptors were discovered and thus opened new frontiers for angiogenesis research [4,160]. Bevacizumab (Avastin), a monoclonal antibody that binds to VEGF-A, was the first angiogenesis inhibitor approved by the FDA (2004); it was initially approved for the treatment of colorectal cancer and has subsequently been approved for several other cancers [4]. Pegaptanib (Macugen) was approved by the FDA later in 2004 for the treatment of neovascular age-related macular degeneration. It was the first FDA-approved aptamer and the first anti-VEGF agent approved for treating this eye condition. Aptamers are short (10–30 nucleotides in length), single-stranded oligonucleotides (either DNA or RNA) that bind to specific molecular targets, in this case an RNA aptamer binding to VEGF-A165 [164]. Sorafenib (Nexavar), which inhibits VEGFR1/2/3 and other protein kinases (Table 17), was first approved by the FDA in 2005 for the treatment of advanced renal cell carcinoma. It was subsequently approved for the management of hepatocellular carcinoma in 2007 and for differentiated thyroid carcinoma in 2013 [144].

Neovascular or wet age-related macular degeneration, diabetic retinopathy, diabetic macular edema, and retinal vein occlusion represent major health and economic problems worldwide, and they are

usually treated with antiangiogenic therapies [160]. Excessive neovascularization and inappropriate vascular remodeling are common causes of visual loss in these disorders. Wet age-related macular degeneration accounts for about 10 % of total age-related macular degeneration cases and dry age-related macular degeneration accounts for the rest. The wet form is responsible for 80–90 % of age-related macular degeneration-associated legal blindness and is characterized by robust choroidal neovascularization. These pathological changes cause serious and often irreversible visual impairment. Clinical evidence indicates that VEGF-A is the key angiogenic driver of neovascularization. Hypoxia and inflammation are the main triggers for promoting VEGF-A expression in wet age-related macular degeneration. In human patients, VEGF-A levels positively correlate with retinal ischemia. Nearly all anti-VEGF family biologics developed for oncological indications have been tested in eye diseases. Despite the relative effectiveness of antiangiogenic ocular therapies, progression of wet age-related macular degeneration and the other retinopathies is common and additional treatment modalities and options are needed.

In a worldwide analysis extending from 2014 to 2024, Zhou et al. reported that angiogenesis inhibitors are linked to a myriad of different thromboembolic events [165]. In a retrospective study that evaluated more than 13,000 cases, a total of 34.9 % were classified as arterial thromboembolic episodes (e.g., aortic embolus), 26.5 % as venous events (e.g., pulmonary venous thrombosis), and 38.6 % as "other" episodes (e.g., angiogram abnormal). Their analysis found that the rank order of drugs covered in this review that are linked to thromboembolic events were aflibercept, ramucirumab, cabozantinib, sunitinib, lenvatinib, bevacizumab, pazopanib, axitinib, sorafenib, erdafitinib, regorafenib, vandetanib, and fruquintinib. Zhou et al. reported that olaparib (a poly-ADP ribose polymerase or PARP blocker) was associated with a higher incidence of thromboembolic episodes than the aforementioned drugs. The median time-to-onset following drug administration was 32 days, with 48.5 % of cases occurring within the first month and 12 % persisting beyond one year. The exact mechanism that angiogenesis inhibitors use to trigger thromboembolic events is unclear. Inhibiting VEGFs may expose pro-coagulant phospholipids on the vascular endothelial cell luminal membrane, ultimately producing thrombosis. Inhibition of VEGFs may increase the risk of thrombosis by increasing the hematocrit and blood viscosity following erythropoietin production. Additionally, inhibition of VEGFs may enhance the expression of pro-inflammatory cytokines, leading to tissue damage and the formation of thrombi.

Owing to the substantial number of human ephrin ligands (8) and ephrin receptors (14) which interact with each other, it has been difficult to delineate distinct signaling modules [129]. Future efforts to identify the best ephrin-ephrin receptor therapeutic targets and targeting strategies may require the use of integrated omics approaches which include genomics, transcriptomics, proteomics, and metabolomics. Examination of the entire ephrin system with its unique and shared functions promises to enable personalized therapies that take into account the distinctive properties of each relevant family member. These integrated approaches will promote the understanding of how the cellular context dictates the activities of ephrin ligands and receptors in cancer progression. Although there are several multikinase inhibitors that coincidentally block ephrin receptor protein-tyrosine kinase activity (dasatinib, ponatinib, regorafenib, sitravatinib, tesevatinib, vandetanib), it is difficult to determine the specific contribution of ephrin receptor inhibition on their efficacy. However, we have no FDA-approved drugs that specifically target the ephrin system. See Ref. [129] for a comprehensive review of the role of the ephrin family in cancer progression and Ref. [130] for a summary of the results of the few clinical trials specifically targeting the ephrin family.

Angiogenesis is required for tumor growth in the primary setting and for metastasis to distant sites [2]. Clinical observations indicate that cancer management with antiangiogenesis inhibitors is characterized by incremental survival benefits [4]. Management is currently plagued by a lack of validated biomarkers and a need for such markers that would allow for the selection of patients who are most likely to be responsive to treatment. There is simultaneously a need for the identification of reliable surrogate markers to monitor therapeutic benefits. More work is needed to optimize conventional therapies, targeted therapeutics, and immunotherapies – in combination or alone – to improve clinical benefits. Such studies have been performed for renal cell, lung, and breast carcinomas [153,166,167]. This work needs to be extended for these neoplasms as well as many other cancers. In addition to antiangiogenic therapies and nearly all cancer therapies, we have the problem of primary and secondary drug resistance [168], which occurs with both targeted antagonists as well as cytotoxic drugs. As noted by Winer et al. "Biologically, the cancer cell is notoriously wily; each time we throw an obstacle in its path, it finds an alternate route that then must be blocked" [169].

CRediT authorship contribution statement

Robert Roskoski: Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The author is unaware of any affiliations, memberships, or financial holdings that might be perceived as affecting the objectivity of this review.

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Data availability

No data was used for the research described in the article.

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