Chapter 18
VEGF Signal Tranduction in Angiogenesis

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Abstract: The development of a number of novel tumor therapies targeting the function of vascular endothelial growth factors (VEGFs) and their receptors has promoted an interest in understanding signal transduction regulating angiogenesis, i.e. formation of new blood vessels. The VEGFRs regulate many if not all aspects of endothelial cell function during active angiogenesis, and mediate survival signals during endothelial cell quiescence. Most tumors produce VEGF as a consequence of the hypoxic tumor microenvironment, leading to persistent stimulation of angiogenesis necessary for an expansion of the tumor as well as tumor spread through the circulation. Increased understanding of VEGFR signal transduction properties may allow development of fine-tuned therapy, targeting pathways critical in formation of new tumor vessels while preserving pathways required for survival of endothelial cells in normal vessels.

Introduction to Vascular Endothelial Growth Factor (VEGF) Signal Transduction

The term "VEGF" denotes both the prototype family member now named VEGF-A, and the family of five structurally related, homodimeric polypeptides of 40 kDa; VEGF-A, -B, -C, -D and placenta growth factor, PlGF. VEGF-A is alternatively spliced to generate VEGF-A121, VEGF-A145, VEGF-A165 and VEGF-A189 (indicating the number of amino acid residues in the human splice variants; mouse variants are each one amino acid shorter) endowed with different biological properties [1]. A newly discovered splice variant, denoted VEGF-A165b, contains exon 8b, encoding a unique stretch of 5-amino acid residues. VEGF-A165b binds to VEGFR2 with high affinity but fails to transduce biological responses and may be an antagonist of VEGF-A165 [2]. VEGF-like proteins from the ORF virus family, denoted VEGF-E, cause contagious pustular dermatitis in sheep and goats and is transmissible to humans by direct contact [3, 4]. Snake venom-derived VEGF-like proteins (denoted VEGF-F) have unique structural features [5]. The mammalian VEGFs bind to different extents to three receptor tyrosine kinases, VEGF receptor-1, -2 and -3 [6].

The VEGF receptors are transmembrane glycoproteins with an extracellular ligand-binding domain, which in VEGFR1 and VEGFR2 is organized in 7 immunoglobulin-like loops. In VEGFR3, one of the loops is replaced by a disulfide bridge. The intracellular domain of each receptor is endowed with a ligand-activated kinase domain, which is split in two parts by the insertion of a "kinase insert" sequence of 70 amino acid residues.

In addition to the full-length receptor tyrosine kinase, VEGFR1 occurs as a soluble splice variant composed of the extracellular domain only [7]. The full-length form is expressed on a number of different cell types, including monocytes/macrophages and vascular endothelial cells (ECs) [8]. The soluble splice variant is highly expressed during gestation and has been associated with pre-eclampsia [9]. Deletion of the *vegfr1* gene leads to embryonic lethality at embryonic day (E) 11.5 due to excessive proliferation of ECs [10]. Thus, VEGFR1 is thought to serve as a negative regulator of VEGFR2, in part through the soluble variant which acts as a trap for VEGF-A. It cannot be excluded that the activated VEGFR1 kinase domain induces negative regulatory signaling in the target endothelial cell [11, 12]. However, priming cells by activation of VEGFR1 has been shown to enhance subsequent signal transduction via VEGFR2 [13]. The full length VEGFR1 mediates migration of hematopoietic precursors and monocytes (for a review, see

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[14]). Altogether, the contribution of VEGFR1 to signal transduction in endothelial cells either directly, or indirectly via VEGFR2, remains unclear.

VEGFR2 is implicated in most if not all aspects of vascular endothelial cell biology. A number of signal transduction pathways induced downstream of VEGFR2 have been identified (see below). During development, VEGFR2 is the first specific endothelial marker to be expressed on hematopoietic/endothelial progenitors [15]. Subsequently, expression of VEGFR2 is turned off in hematopoeitic cells. Expression of VEGFR2 on ECs declines during the third trimester, but is induced again in conjunction with active angiogenesis [16, 17]. Inactivation of the *vegfr2* gene leads to embryonic death at mouse E 8.5–9.5, due to lack of proper differentiation and/or migration of ECs [18].

VEGFR3 is found primarily on lymphatic endothelial cells, and is critical for lymphatic EC development and function [19]. VEGFR3 may also be expressed on fenestrated capillaries, tumor ECs and on monocytes/macrophages [20, 21]. Mice deficient for VEGFR3 die at E9.5 due to defective remodeling and maturation of the primitive blood vascular plexus into larger vessels [22]. VEGFR3 is the only VEGFR for which naturally occurring mutations have been described [23].

Regulation of VEGF/VEGFR Expression

Hypoxia, i.e. low oxygen tension, is an important regulator of physiological and pathological angiogenesis. In hypoxia, the transcription factor hypoxia-inducible factor (HIF)-1 accumulates, allowing increased transcription of a multitude of genes through binding of HIF-1 to the hypoxia-responsive element (HRE). HIF-1 may also be induced by a number of other stimuli, such as growth factors, under normoxic conditions [24]. Such activation involves several different signal transduction pathways, including the phosphoinositide 3′ kinase (PI3K), extracellular regulated kinase (Erk) 1/2 and PKC pathways, which act through increasing HIF-1 translation or through regulatory phosphorylation [24, 25] (see chapters 15 & 16 for detailed discussion). The VEGFR2 promoter appears to lack a classical HRE, but has been shown to be regulated by the related HIF-2 [26].

Members of the Ets (E26 transforming sequence in avian erythroblastosis virus) family of transcription factors are expressed in endothelial cells and modify expression of several genes implicated in angiogenesis and inflammation; for example, Ets-1 regulates the expression of VEGFR1 and VEGFR2. Transcriptional activity by Ets is regulated e.g. by Erk1/2-mediated serine phosphorylation as well as through a number of other mechanisms (for a review, see (27)).

Activation of VEGFRs

Binding of VEGF leads to dimerization of receptor molecules followed by activation of the intrinsic tyrosine kinase. The VEGFRs have been shown to form both homo- and heterodimers in vitro [28–30]. The activated receptor molecules in the dimers transphosphorylate each other, on tyrosine residues. An initial phosphorylation on positive regulatory tyrosine residue(s) in the kinase activation loop precedes full activation of the kinase. This is followed by phosphorylation on other tyrosine residues in the intracellular domain of the receptor to create binding sites for signaling intermediates, thereby initiating signaling cascades.

Numerous tyrosine phosphorylation sites have been identified on VEGFR1 [31], VEGFR2 [32–34], and VEGFR3 [28] (Fig. 18.1). It is noteworthy that VEGFR1 lacks phosphorylation on positive regulatory tyrosine residues [31], due to replacement of a conserved residue in the activation loop, from Asp to Asn at position 1050 [35]. This may explain why VEGFR1 kinase activity is difficult to induce. Positive regulatory tyrosine phosphorylation is found both in VEGFR2 and VEGFR3. Interestingly, for all three VEGF receptors, certain tyrosine phosphorylation sites are used selectively. Thus, for VEGFR1, phosphorylation site usage is dictated by the particular activating VEGF ligand, such as PlGF [13] and VEGF-A [31], which have been shown to induce different phosphorylation site patterns. For VEGFR2, the Y951 phosphorylation site, located in the insert region between the two parts of the kinase domain, is used primarily when the receptor is expressed in endothelial cells engaged in active angiogenesis [34]. Thirdly, C-terminal sites in VEGFR3 are phosphorylated in VEGFR3 homodimers, but not when VEGFR3 is heterodimerized with VEGFR2 [28]. The implication of these findings is that VEGFR tyrosine phosphorylation is both highly dynamic and tightly regulated, in agreement with the versatility of these receptors in endothelial biology.

Trimeric Gαq/Gα11 proteins have been implicated as important regulators of VEGFR2 signaling. Thus, antisense-mediated suppression of Gq/11 expression completely attenuated VEGFR2 tyrosine phosphorylation and signal transduction through a mechanism involving direct association between the receptor and the trimeric G-proteins [36].

Down-regulation of VEGFR Activity

How are VEGFRs turned off in order to halt signal transduction? One important mechanism involves dephosphorylation by phosphotyrosine phosphatases (PTPs; [37]). One interesting example is the receptor type PTP denoted vascular endothelial (VE)-PTP (also denoted PTP receptor type B; PTPRB), which is required for maintenance and remodeling of blood vessels during development [38]. VEGFRs do not appear to be direct substrates for VE-PTP, however. The broadly expressed transmembrane PTP denoted density-enhanced phosphatase-1 (DEP1)/CD148, has been implicated in regulation of endothelial cell junctional integrity and silencing of VEGFR2 in dense cells [39]. Moreover, the Src Homology-2 (SH2) domain-containing PTP SHP-2, which is a ubiquitously expressed

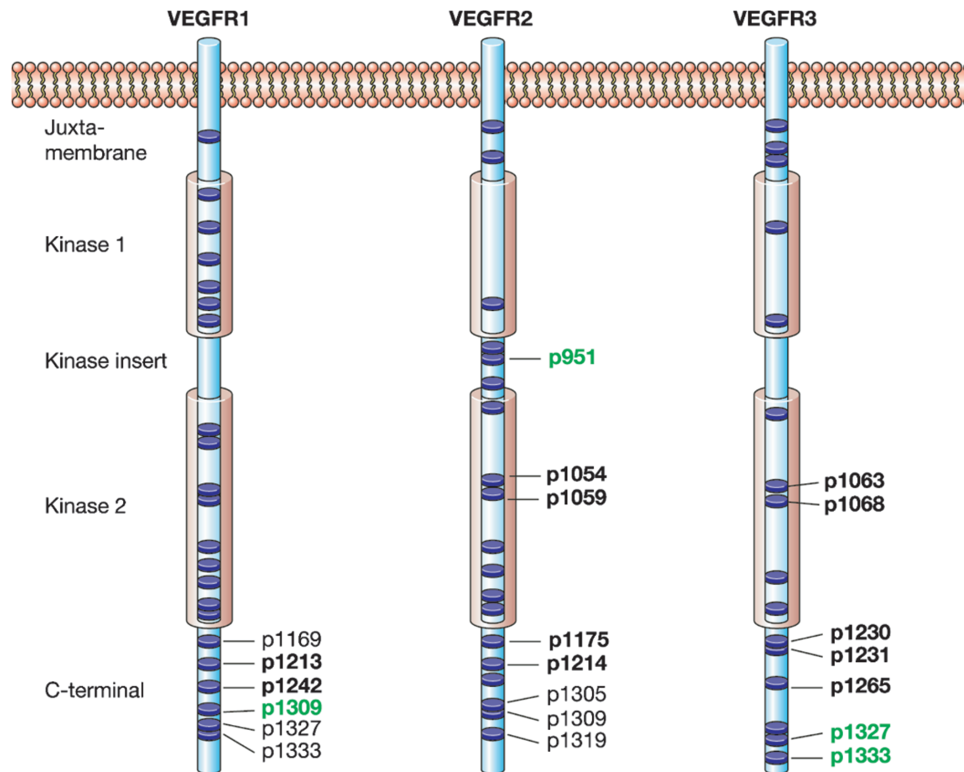

Fig. 18.1. Tyrosine phosphorylation sites in VEGFRs. Activated VEGFRs are represented as single intracellular domains (excised from dimerized receptor complexes). Tyrosine residues are indicated as *blue ovals*. Tyrosine phosphorylation sites are indicated as *p* (phospho) followed by *numbers* which show the position of the residue in the amino acid sequence. *Figures* in *green* indicate that phosphorylation sites are used selectively, i.e. p1309 is phosphorylated when VEGFR1 is activated by PlGF but not by VEGF, 951 is phosphorylated when VEGFR2 is expressed in endothelial cells devoid of a pericyte coat and 1327/1333 are phosphorylated when VEGFR3 is homodimerized and not when it is heterodimerized with VEGFR2 (see text description).

cytoplasmic PTP, has been shown to antagonize VEGF-function, possibly by direct dephosphorylation of VEGFR2 [40].

Another mechanism for clearance of activated VEGFRs involves rapid internalization and/or degradation, which have been studied in different in vitro EC models. Internalization of VEGFR2 expressed in human umbilical vein endothelial cells involves protein kinase C (PKC)-dependent serine phosphorylation on a C-terminal residue [41]. There are several reports that VEGFR2 is recycled to the cell surface from an endosomal compartment when endothelial cells are exposed to VEGF [42, 43]. Ubiquitination of VEGFR2 which at least in vitro involves activation of the ubiquitin ligase Cbl [44], leads to efficient degradation of receptors in the lysosome [42].

VEGF Co-receptors

A co-receptor is here defined as a molecular entity that binds to the ligand as well as to the receptor and thereby modulates the downstream signal transduction. Co-receptors may lack intrinsic enzymatic activity and do not necessarily signal independently of the receptor tyrosine kinase. There are at least two co-receptors for the VEGF/VEGFRs, heparan sulfate (HS) proteoglycans (HSPGs) and neuropilins (NRPs) that fit this definition (Fig. 18.2). HSPGs are composed of a protein backbone modified by attachment of repeated units of sulfated glucosaminoglycans (GAGs) that form long linear sugar chains. GAG sulfation confers a net negative charge, which allows binding to many different growth modulatory factors [45]. In cells lacking heparan sulfate completely, or express defective heparan sulfate with reduced degree of sulfation, there is no response to VEGF even though VEGF receptors are expressed [46]. The mode of presentation of HSPGs (on both endothelial cells and perivascular cells, or only on perivascular cells) determines the level and longevity of receptor activation [47].

Neuropilins (1 and 2) are transmembrane molecules with a short cytoplasmic tail, which lacks intrinsic enzymatic activity [48]. Neuropilins were first identified as negative regulators of neuronal axon guidance through binding of members of the class 3 Semaphorin (Sema) family [49, 50]. Binding of Sema to NRPs allows coupling to plexins, which have established roles in regulating Rho-family GTPases [51]. Neuropilin-1

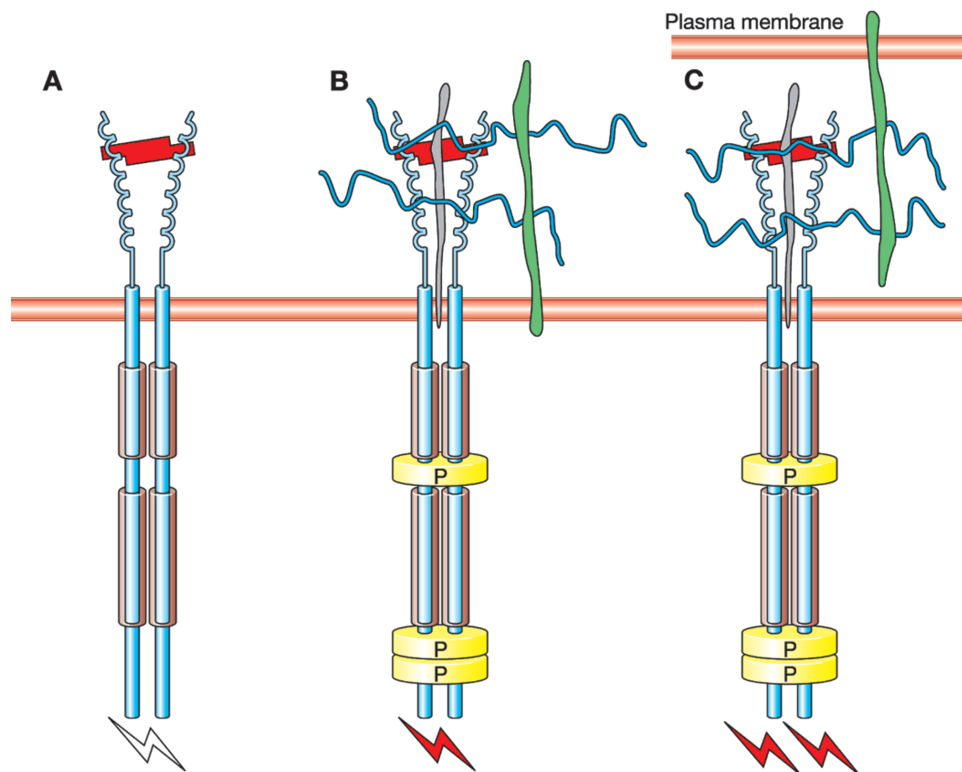

Fig. 18.2. VEGF co-receptor function. **A** VEGFR2 is unable to transduce signals leading to establishment of cellular responses in cells devoid of coreceptors HSPG and NRP1. **B** HRPG (GAG side chains indicate in *blue* and protein part in *green*) or NRP1 (indicated in *grey*) expressed on endothelial cells are engaged in the VEGF/VEGFR signaling complex and may affect signaling quantitatively (by stabilizing the complex) and qualitatively (by allowing transduction of signaling pathway not induced in the absence of coreceptors. **C** Presentation of HSPGs in trans, e.g., on pericytes, leads to further stabilization of the VEGF/VEGFR signaling complex, and prolonged signal transduction (*red activity arrows*).

was subsequently shown to bind exon-7-containing VEGF-A isoforms such as VEGF-A165 [52]. Neuropilin-1 -/- mice die at embryonic day (E)10.5–12.5 due to defects in vascular and neuronal development [53]. It is noteworthy that NRP-1 may be modified by chondroitin and heparan sulfation [54], potentially allowing binding of VEGF to the protein core as well as to the HS-side chains. Through these interactions, NRPs become an integral part of the VEGF/VEGFR signaling complex and may thereby enhance the activity of the VEGFR kinase. Whereas neuropilin-1 is engaged in VEGFR2 signaling, neuropilin-2 interacts with the VEGF-C/VEGFR3 signaling complex in lymphendothelial cells [55]. Gene targeting of neuropilin-2 leads to severe reduction of small lymphatic vessels and capillaries [56, 57].

VEGFR Signal Transduction Pathways

The Phospholipase Cγ (PLCγ) Pathway

Within the family of phospholipases (PLCs), PLCγ1 and 2 are equipped with Src homology 2 (SH2) domains, which confer binding to activated growth factor receptors [58]. PLCγ2 is preferentially expressed in hematopoietic cells, whereas PLCγ1 is ubiquitously expressed and will henceforth be referred to as “PLCγ”. PLCγ is a substrate for all three VEGF receptors. Upon binding to phosphorylated Y1173 (1175 in the human) in the VEGFR2 C-terminal tail, PLCγ becomes tyrosine phosphorylated and thereby activated [59] (Fig. 18.3). PLCγ hydrolyzes phosphatidylinositol 4,5 bisphosphate (PI-4,5-P2), a plasma membrane lipid. The hydrolysis results in generation of inositol 1,4,5-P3 and diacylglycerol (DAG), which leads to release of Ca^{2+} from intracellular stores and activation of PKC, respectively. VEGF-induced PLCγ activation has been shown to lead to Ras-independent activation of Erk1/2, via PKC [59]. This pathway has been implicated in VEGF-driven proliferation of endothelial cells in vitro [60]. A knockin mutation replacing Tyr1173 with Phe, thereby removing the PLCγ-binding site, leads to embryonic lethality [61], and overall features that are similar to those observed for the complete receptor knockout [18]. The Y1175 phosphorylation site on VEGFR2 is also a binding site for the adaptor molecules Shb [62] and Sck [63]. It is noteworthy that inactivation of the

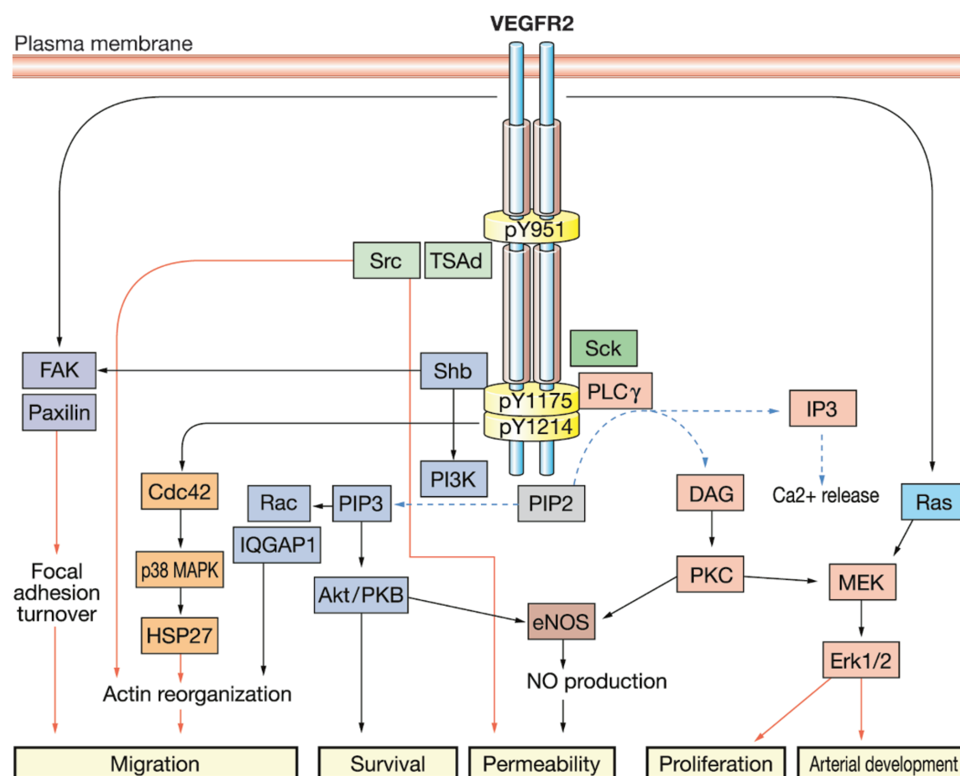

FIG. 18.3. Signal tranduction pathways induced by VEGFR2. Binding of signaling molecules to certain phosphorylation sites (*yellow ovals*) initiate signaling cascades leading to establishment of biological responses (*migration*, *survival*, etc). *Dashed arrows* indicate enzyme/substrate reactions involving PI-4,5-P2 (*PIP2*), PI 3,4,5-P3 (*PIP3*) or inositol 1,4,5-P3 (*IP3*). Both *Shb*, *Sck* and *PLCγ1* associate with *pY1175* whereas *TSAd* associates with *pY951*. *DAG* Diacylglycerol; *eNOS* endothelial nitric oxide synthase; *Erk* extracellular regulated kinase; *FAK* focal adhesion kinase; *HSP27* heat shock protein 27; *MAPK* mitogen activated protein kinase; *PI3K* phosphoinositide 3′kinase; *PKC* protein kinase C; *PLCγ*; phospholipase Cγ, SH2 and β-cells; *TSAd* T cell-specific adapter.

murine PLCγ1 gene is accompanied by defective vasculogenesis and erythropoiesis and early embryonic death [64]. Inactivation of the zebra fish PLCγ gene leads to loss in arterial specification [65].

The PI3K Pathway

VEGF is known to be important for endothelial cell survival. Under conditions where VEGF function is interrupted (e.g., by neutralization using VEGF-reactive antibodies, or VEGF receptor kinase inhibitors), fenestrated capillaries in endocrine organs regress [66]. One important mediator of VEGF-dependent survival is the PI3K pathway [67]. The PI3K family of lipid kinases phosphorylate the 3′-hydroxyl group of phosphatidylinositol and phosphoinositides (for a review, see [68]). PI3K is activated downstream of VEGFR2 and VEGFR3, but only indirectly and not through binding of the regulatory p85 subunit directly to tyrosine phosphorylation sites in the receptors (for a review, see [6]). Interestingly, Gille and colleagues identified a repressor sequence in the juxtamembrane of VEGFR1 which, when exchanged for the corresponding domain from VEGFR2, allowed activation of the PI3K pathway [69]. VEGFR2-dependent activation of PI3K occurs downstream of focal adhesion kinase (FAK) [70] or vascular endothelial (VE)-cadherin [71]. Furthermore, VEGF-induced activation of PI3K is inhibited by siRNA-mediated knockdown of the adaptor molecule Shb [62]. Accumulation of PI 3,4,5-P3 as a result of PI3K activation, in turn allows membrane recruitment of molecules equipped with a pleckstrin homology (PH) domain, notably the serine/threonine (Ser/Thr) kinase Akt (also known as protein kinase B; PKB). The activity of the membrane-localized Akt is stimulated through phosphorylation on Ser/Thr by phosphoinositide-dependent kinases (PDKs), and Target of rapamycin (Tor). Akt promotes endothelial cell survival via regulation of several downstream effectors, such as the the pro-apoptotic Bcl-2 related protein, BAD [72], and the forkhead family of transcription factors (for a review, see [68]).

PI3K-dependent activation of Akt leading to phosphorylation of endothelial nitric oxide synthase (eNOS) and generation of NO, has been implicated in VEGF-induced vascular permeability [73]. Expression in endothelial cells of constitutively

active Akt, modified by attachment of a myristyl group that confers constitutive plasma membrane-association, leads to formation of enlarged, hyperpermeable vessels resembling tumor blood vessels [74]. However, although these vessels expressing constitutive active Akt show features of chronic vascular permeability, they still respond to VEGF with increased acute permeability, indicating the complexity of this response.

PI3K also regulates the cytoskeleton by modulating the activity of small GTPases belonging to the Rho family. According to consensus, these are activated via PI-3,4, 5-P3-dependent recruitment of guanine nucleotide exchange factors (GEFs;[75]). However, certain aspects of this regulatory mechanism remain elusive, since the sequence of the PH domain of several of these GEFs do not conform to the consensus sequence for binding of PI3K products. Regulation of the actin cytoskeleton is critically involved in endothelial cell migration and formation of the vascular tube, by controlling filopodia, lamellipodia and actin stress fiber formation.

Interestingly, different aspects of endothelial cell biology seem to require a balanced activity of PLCγ1 versus PI3K. This includes arterial/vein specification (for a review, see [76]) and the formation and stability of the vascular tube [77]. Moreover, activation of eNOS by VEGFR1 and -2, have been shown to involve activation of PI3K and PLCγ, respectively [78]. These lipid modifiers compete for the same substrate, PI-4,5-P2, which may constitute a limiting factor, guiding the relative strengths of downstream signaling pathways.

The SRC Family of Cytoplasmic Tyrosine Kinases

Cytoplasmic Ser/Thr kinases of the Src family are known to be vital for EC function and involved in regulation of the cytoskeletal architecture, which is critical in EC migration and formation of the three-dimensional aspect of the vessel. It is not clear how Src kinases (here primarily indicating the ubiquitously expressed Src, Yes and Fyn kinases) are activated in response to VEGF treatment, e.g., via VEGFR2. One possible mechanism involves the adaptor VEGF receptor associated protein (VRAP; [79]), also denoted TSAd (T cell specific adapter), that binds to phosphorylated Y949/951 in VEGFR2, and which has been shown to associate with Src in a VEGF-regulated manner [34]. An important role for Src appears to be in regulation of endothelial cell junctions and, consequently, endothelial permeability [80]. Interestingly, although endothelial cells express the highly related Src, Fyn and Yes, only Src and Yes appear to be involved in regulation of VEGF-mediated permeability. Thus, gene inactivation of Src or Yes attenuates VEGF-mediated permeability without apparent blockade in other aspects of endothelial cell function [80, 81]. Src appears in complex with VEGFR2 and VE-cadherin, a main component of endothelial cell adherens junctions, and the stability of the complex is an important aspect in vascular leakage [71, 81, 82]. In vitro, VE-cadherin is a substrate for Src [83, 84]. It appears that VEGF causes disruption of the VEGFR2/VE-cadherin complex in a Src-dependent manner, causing release of VE-cadherin-associated β-catenin. Thereby, β-catenin signaling becomes integrated in VEGF-induced responses [81]. For further discussion on signal transduction in vascular permeability, see below.

Endothelial Cell Biology and Signal Transduction

Endothelial Cell Survival and Proliferation

VEGF is an important survival factor for endothelial cells, through activation of the Akt pathway (see above). To what extent VEGF induces EC proliferation in primary cells or in vivo, is not clear, although several different pathways for VEGF-regulated endothelial cell DNA synthesis have been suggested. By mutation of the PLCγ-binding site in VEGFR2, and by use of PLCγ neutralizing antibodies, it has been demonstrated that the PLCγ-MEK/Erk pathways is critical for VEGF-induced DNA synthesis [60]. These data have been corroborated by exchange of the VEGFR2 Y1173 residue in vivo, which leads to early embryonic lethality [61]. It is noteworthy that Y1173/1175 is a binding site also for other signal transduction molecules, moreover, at least in certain cell types, VEGF-induced DNA synthesis appears to involve activation of Ras [85, 86].

Actin Cytoskeleton, Migration and Formation of the Vascular Tube

Several different signal transduction pathways have been implicated in regulation of endothelial cell actin cytoskeleton and migration. The migratory response is a complex series of coordinated events involving mechanisms for orientation of the movement, as well as release and formation of new contacts with the underlying substrate. It is conceivable that different signaling pathways regulate different stages in cell migration. It is furthermore likely that pathways that have been implicated in regulation of the cell cytoskeleton and migration in many different cell types are important also in ECs. Thus, FAK and its substrate paxillin which are involved in focal adhesion turnover during cell migration [87] are known to be activated in VEGF-stimulated endothelial cells. Other pathways implicated in cell migration such as PI3K, Src and small GTPases (Rac and cdc42) are also regulated by VEGF during EC migration (see below).

Removal of the binding site for the adaptor molecule TSAd by mutation of the Y949/951 phosphorylation site in VEGFR2 to Phe, or siRNA-mediated down-regulation of TSAd, attenuates VEGF-induced actin reorganization and migration of endothelial cells [34]. Another pathway implicated in VEGF-induced actin reorganization and EC migration is dependent on binding of the adaptor molecule Shb to Y1173/1175 in VEGFR2. VEGF induces tyrosine phosphorylation of Shb in a Src-dependent manner, allowing further

downstream coupling to PI3K and FAK activation [62]. Endothelial cell motility appears furthermore to be regulated via a newly identified binding partner of phosphorylated VEGFR2, IQGAP1, which binds to and activates Rac1 [88]. Phosphorylation of Y1212/1214 in the C-terminal tail of VEGFR2 has been implicated in VEGF-induced actin remodelling via triggering of a signaling complex involving the adaptor Nck, the cytoplasmic tyrosine kinase Fyn and p21-activated kinase (PAK)-2, which in turn promotes activation of Cdc42 and p38 mitrogen associated protein kinase (MAPK) [89]. Cdc42 is a Rho-family GTPase regulating cytoskeletal remodelling [90], and p38MAPK has been implicated in neoangiogenesis [91], by phosphorylating Hsp27, a heat-shock protein involved in VEGF-induced actin reorganization and migration. Nck may also regulate the formation of focal adhesions [92]. Thus, VEGF induces a plethora of responses that all have the ability to cause cytoskeletal rearrangements and cell migration.

Arterial-Vein Specification

Arteries and veins are structurally and functionally distinct. Arteries, which have to tolerate large changes in blood pressure generated by the contractions of the heart muscle, develop a thicker outer coat with several layers of smooth muscle cells. In contrast, veins have a thin smooth muscle cell coat, which locally may fail to cover the vessel. The blood pressure as well the blood flow have been considered important in establishing such differences and, consequently, in arterial/vein specification [93]. Arteries and veins also differ with regard to gene expression patterns, such as the transmembrane ligand ephrin B2 and its receptor tyrosine kinase EphB4, which are expressed on arteries and veins, respectively [94]. Interestingly, a balance in the PI3K and PLCγ pathways appears to direct the development of arteries. Activation of Erk1/2 downstream of PLCγ is required for arterial development, and this pathway is opposed by activation of PI3K [95]. Erk activity may be essential in VEGF-mediated induction of the Notch signaling pathways, in an as yet unidentified circuit. Notch ligands and receptors are required for arterial development downstream of VEGF/VEGFR2 (for reviews, see refs. [76, 96]).

Signal Transduction in Vascular Permeability

VEGF-A was originally discovered as a vascular permeability factor (VPF) [97, 98]. VEGF-A is unique in its ability to induce permeability with unusual potency and kinetics without involvement of mast cell degranulation or endothelial cell damage. Pathological angiogenesis, such as in cancer, is often accompanied by vascular permeability, leading to formation of edema and ascites, and allowing the distant spread of metastases. These serious clinical complications have led to an intense interest in determining the signal transduction pathways regulating vascular permeability. Thus far, many different pathways have been implicated, possibly due to the involvement of more than one mechanism, or because different endothelial cell types are differently regulated. One principal mechanism appears to involve assembly of vesiculo-vacuolar organelles (VVOs) to form trans-endothelial pores, allowing passage of large molecules [99]. Another mechanism involves loosening of endothelial cell-cell junctions. Endothelial adherens junctions are crucial for the maintenance and regulation of normal microvascular function [100]. The major cell–cell adhesion molecule in adherens junctions (also denoted zonula adherens) is VE-cadherin. VE-cadherin gene inactivation allows formation of a primitive vascular plexus, but vascular remodeling is deficient leading to an early lethal phenotype [71]. Adherens junctions are destabilized by VEGFR2-induced activation of the cytoplasmic kinases Src and Yes, leading to dissociation of a VEGFR2/VE-cadherin complex (Fig. 18.4). This complex is central to the integrity of the junction. It has also been implicated in regulation of the signaling strength of VEGFR2, via receptor dephosphorylation by junctional phosphatases, which would be an underlying mechanism in contact-inhibition of growth [39]. A critical role for Src in permeability is indicated by the fact that Src blockade stabilizes the VEGFR2/VE-cadherin complex, reducing edema and tissue injury following myocardial infarction [81]. However, although VE-cadherin indeed is a substrate for Src, loosening of adherens junctions may not require tyrosine phosphorylation of VE-cadherin. Instead, VEGF has been shown to induce rapid endocytosis of VE-cadherin in a pathway involving Src-mediated tyrosine phosphorylation and activation of Vav2, which is a GEF for Rac. Via activation of the p21-activated kinase (PAK), Rac in turn induces serine phosphorylation of VE-cadherin, which triggers endocytosis [101].

VEGF-induced vascular permeability also depends on nitric oxide (NO) production, which requires activation of eNOS either as a consequence of PLCγ activation and calcium influx, or through phosphorylation of eNOS by PKB/Akt [73, 102]. In agreement, targeted deletion of eNOS abrogates VEGF-induced vascular permeability [103].

Studies on different VEGF family members that preferentially bind to one or both of VEGFR1 and −2 indicate that vascular permeability involves both VEGFR receptors. A member of the snake venom VEGF-Fs, (*T. flavoviridis* svVEGF), is a potent inducer of vascular permeability [104]. *T.f.* svVEGF preferentially binds and activates VEGFR1, but also to some extent, VEGFR2. These results may suggest that cooperative action of VEGFR1 and VEGFR2 may be critical for generation of vascular permeability.

Changes in VEGF Signaling as a Consequence of Changes in the Endothelial Microenvironment

Targeted inactivation of the *vegf-a* gene has shown that the level of VEGF-A expression, and thus the amplitude or duration of signaling during vasculogenesis is critical, as deletion of only one allele leads to embryonic death [105, 106]. Interestingly,

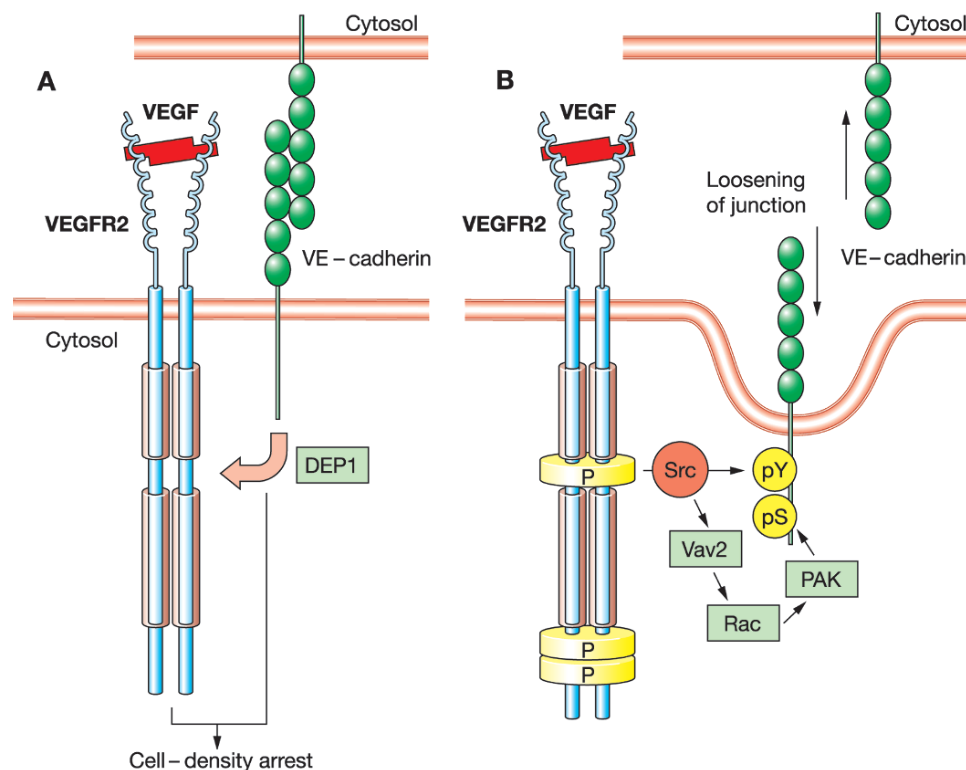

Fig. 18.4. Role of VE-cadherin and VEGFR2 interactions. **A** In dense cells, the density enhanced phosphatase (*DEP*)-1 is regulated via VE-cadherin, leading to dephosphorylation of VEGFR2 and attenuates endothelial cell proliferation. **B** Vascular permeability involves loosening of adherens junctions through disruption of the VEGFR2/VE-cadherin complex followed by Src-dependent tyrosine (*Y*) as well as *PAK* (p21 activated kinase)-dependent serine (*S*) phosphorylation of VE-cadherin.

different levels of VEGF may be required for induction of different VEGFR2-dependent processes [107]. Thus, tissues vascularized through vasculogenesis, such as spleen and lung, express higher levels of VEGF-A than tissues vascularized through angiogenesis such as brain [107]. Whether different levels of VEGF induce qualitatively distinct signals remains to be shown. Local changes in the microenvironment may lead to altered VEGF expression/signaling, possibly as part of an adaptive response. Such examples have been delineated by knockout studies. During embryonic development of mice lacking expression of either the platelet-derived growth factor-B or the PDGF-β receptor as a result of gene targeting, endothelial cells fail to attract pericytes in several organs, including the brain [108]. VEGF-A becomes up-regulated in these embryos, leading to the appearance of endothelial cells with an altered ultrastructure. VEGF-A gene expression is also increased in β3-integrin null mice in the male heart, with abnormal endothelial ultrastructure as a consequence [108].

Blood flow and shear stress may induce VEGFR activation and signal transduction independently of VEGF-binding. Thus, a mechanosensory complex consisting of VEGFR2, platelet-endothelial cell adhesion molecule-1 (PECAM-1) and VE-cadherin has been described [109]. Formation of this complex induces integrin activation and signal transduction. It is quite well established that many aspects of endothelial cell biology involve convergence of integrin and VEGFR signaling pathways (for a review, see [110]). Other signaling systems also converge with that of the VEGFRs, such as the angiopoietin/Tie receptor complex, which plays an essential role in angiogenic remodeling. A characteristic feature of the angiopoietin ligands is their opposing effects on Tie2 receptor activation, where the eventual endothelial cell response is influenced by concurrent VEGFR signaling (for a review, see ref. [111]). Furthermore, signal transduction by TGF-β family of growth factors and receptors strongly modulates VEGF-induced angiogenesis [112]. Thus, VEGF-signaling is context-dependent and is influenced by cell–cell and cell–matrix interactions and by a wealth of soluble and cell-associated mediators of different kinds. One recent example is the Notch family of ligands and receptors which are critical in VEGF-dependent formation of tip cells in angiogenic sprouts [113].

Conclusions

The recent advances in development of VEGF-targeted therapy to arrest deregulated vascularization in conjunction with chronic inflammation and cancer have clearly demonstrated

the critical in vivo contribution of VEGFR signaling in angiogenesis. Moreover, such therapy may be further developed, as combined neuropilin-1 and VEGF-neutralization appears superior in arresting tumor vascularization compared to VEGF-neutralization alone [114]. From studies on the consequence of VEGF-neutralization on normal vasculature, however, it seems that VEGF has a prominent and perhaps unique role in mediating endothelial cell survival in quiescent vessels [66]. Therefore, current VEGF-targeted therapy needs to be refined to preserve VEGF-induced survival, while suppressing other VEGF-induced responses such as permeability and formation of the three-dimensional vascular tube. Clearly, future antiangiogenic therapy will benefit from the steady accumulation of data on signaling in endothelial cells.

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