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An Inside View: VEGF Receptor Trafficking and Signaling

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Abstract

Vascular endothelial growth factors (VEGF) and their receptors play a central role in the development of cardiovascular system and in vasculature-related processes in the adult organism. Given the critical role of this signaling cascade, intricate control systems have evolved to regulate its function. A new layer of added complexity has been the demonstration of the importance of endocytosis and intracellular trafficking of VEGF receptors in the regulation of VEGF signaling. In this review, we consider an evolving link between VEGF receptor endocytosis, trafficking, and signaling and their biological function.

Vascular endothelial growth factor (VEGF) superfamily consists of VEGF ligands, receptors, and associated interacting molecules (Table 1) that orchestrate a number of critical events involving vascular development, angiogenesis, and arteriogenesis (32). VEGF-A is the single most important molecule regulating vascular development and angiogenesis in adult settings, and it exerts most of its actions via VEGF receptor 2 (VEGFR2; also known as KDR and FLK1) (7). In addition to VEGF-A, in eukaryotic organisms, VEGF family also includes VEGFs B, C, and D and a closely related peptide placental growth factor (PlGF). All VEGFs occur in a number of splice isoforms and possess an extensive spectrum of activities. VEGF-A binds to three receptors that regulate its activity: tyrosine kinases VEGF receptors 1 (VEGFR1; also known as FLT1) and VEGFR2, and a nontyrosine kinase neuropilin-1 (also known as Npn1 or Nrp1). Its binding to these receptors is further facilitated by transmembrane proteoglycans carrying heparan sulfate chains (32). Among VEGF-A receptors, VEGFR1 has the highest affinity and is thought to largely act as a negative regulator by effectively sequestering it from binding to VEGF-R2 or Nrp1. VEGFR2 is the key receptor mediating most cellular effects of VEGF-A in the endothelium, whereas the role of Npn1 in VEGF signaling remains rather mysterious at present.

The entire VEGF signaling system is very tightly controlled on multiple levels, including regulation of gene expression, message stability, protein stability and proteolytic processing, ligand-receptor interactions, and, finally, endocytosis and trafficking. The degree of fine tuning of VEGF signaling is demonstrated by its exquisite sensitivity to VEGF-A levels and

VEGFR1 and VEGFR2 expression, with even a 50% reduction in any of the molecules leading to early embryonic lethality (7). A similar fate results from deletion of other genes in VEGF-A/VEGFR2 signaling pathway, including *Plcg* (36, 37) and *Dll4* (17).

VEGFR2 is a classic tyrosine kinase receptor consisting of an extracellular ligand-binding domain, a transmembrane domain involved in receptor dimerization, and an intracellular kinase domain that is activated on ligand binding and dimerization. This in turn leads to phosphorylation of critical tyrosine residues that engage various adaptor molecules and initiate intracellular signaling. These include activation of MAPK signaling, PI3K/AKT, Src, and Rac (32).

Like a typical receptor tyrosine kinase (RTK), VEGFR2 was thought to signal from the plasma cell membrane after ligand-induced dimerization and activation (32). However, a number of recent studies have challenged this view of VEGFR2 signaling, demonstrating the importance of its endocytosis and trafficking in regulating its signaling. This newly emerging view of VEGFR2 signaling will be the subject of this review.

Endocytosis and Signaling

Endocytosis for a long time has been considered primarily as a means of extinguishing receptor signaling. Ligand binding to its particular receptor was thought to trigger internalization of the receptor-ligand complex into endosomes, with subsequent degradation in lysosomes. However, an avalanche of data over the last few years has amply demonstrated that signaling continues in various endosomal compartments and that trafficking of endosomes containing activated receptors (RTKs and GPCRs) serves as a major means of regulating receptor signaling (12, 46, 55, 56). Indeed, endosomes provide an attractive environment for signaling: their small volumes facilitate protein-protein interactions and assembly and maintenance of signaling complexes, whereas links to microtubular networks provide for rapid trafficking to various intracellular addresses. Furthermore, relatively slow sorting and trafficking processes prolong time available for signaling (55). Thus the knowledge of events involved in a receptor uptake and trafficking has become essential to the understanding of regulation of its activity. Since the detailed description of endocytosis and trafficking are beyond the scope of this review, the reader is referred to a number of outstanding recent reviews dealing with these subjects (12, 31, 43, 46, 55). Only highlights necessary for understanding VEGF-R2 endocytosis will be considered below.

Receptor endocytosis can proceed in a clathrin-dependent or -independent fashion. The latter includes caveolae-dependent endocytosis and macropinocytosis (4). Under normal conditions, clathrin-dependent endocytosis is by far the predominant route for uptake of most receptors, including VEGFR2 (34), although some receptors including TGF β (9) and FGF (14) receptors can be endocytosed in a clathrin-independent fashion. The “choice” of the endocytic pathway is affected by a number of factors including the identity and quantity of the ligand, in addition to the ability of a particular receptor to interact with various clathrin adaptor molecules. Furthermore, inhibition of one endocytosis pathway can shift receptor trafficking to another pathway.

Regardless of the mode of entry, the endocytosed receptor and its ligand will find its way to early endosomal compartment defined by the presence of Rab5. Membranes of the early endosomal compartment have a distinct lipid composition that includes high concentration of phosphatidylinositol-3-phosphate (PtdIns3P) that enables interactions with PX or FYVE protein domains and low concentration of PtdIns(4,5)P (46). In addition to this distinct membrane lipid content, early endosomes contain either EEA1 (early endosomal antigen 1) or APPL (adaptor protein, phosphotyrosine interaction, PH domain, and leucine zipper-containing) adaptor proteins. The presence of EEA1 or APPLs (APPL1 and APPL2) appears mutually exclusive since all of these adaptors bind to Rab5, competing for the same binding site (53, 66).

The presence of an early endosome-associated kinase PIK3C3 (VPS34) that mediates conversion of PtdIns to PtdIns3P results in accumulation of EEA1 and loss of APPLs, suggesting that as endosomes mature APPLs are replaced with EEA1. The precise role of all three adaptors is not clear. They may interact, directly or indirectly, with endosomal RTKs and their signaling complexes. In particular, depletion of AAPL1 but not AAPL2 expression during zebrafish development leads to widespread apoptosis (53) that has been attributed to decreased Akt signaling. At the same time, APPLs appear to be required for ERK activation in HeLa (66) and PC12 cells (38, 62). In the latter case, the involvement of APPLs in ERK activation requires another adaptor protein, synectin (GIPC1), that also plays an important role in VEGFR2 signaling and trafficking (see below). The switch from EEA1 to APPL may play an important role in the regulation of early endosome trafficking.

Following a stint in the early endosomal compartment, the receptor moves either into recycling Rab11 or Rab4 endosomes or into multivesicular bodies (MVB), organelles that form by invagination of small intraluminal vesicles that then pinch off in a process controlled by ESCRT complexes (64). Once in recycling endosomes, the receptor is returned to the plasma membrane either via sequence-dependent recycling, which in the case of β 2-adrenergic receptor involves its PDZ-binding domain, or via bulk recycling, as in the case of transferrin (48). In contrast, once in MVB, the receptor is moved on to lysosomes for degradation.

Receptor signaling may and does continue both in late endosomal and in MVB compartments. The transition to late endosomes and then to MVB is characterized by replacement of Rab5 with Rab7, a process termed “Rab conversion” (46), which is mediated by PtdIns3P binding of CCz1/Mon1 complex (47). Although in most cases transition of a signaling complex into MVB leads to termination of signaling, under some circumstances sequestration of a molecule or a molecular complex inside MVB can promote signaling. One example of this is Wnt signaling. In this cascade, Wnt receptors activation initiates a complex series of events that results in β -catenin-dependent transcription of Wnt target genes. Under normal circumstances β -catenin in the cytoplasm is rapidly degraded by a cytosolic “destruction” complex that includes kinase GSK3. Wnt binding to its receptors Frizzled and LRP6 leads to LRP6 endocytosis and subsequent phosphorylation of its cytoplasmic domain by GSK3 that promotes GSK3-LRP6 binding. Subsequent sequestration of LRP6-GSK3 complex into MVB results in prolonged downregulation of GSK3 activity

allowing for stabilization of β -catenin and transcriptional activation of Wnt-target genes (12).

Since most RTK are rapidly internalized upon ligand binding, endosomal signaling is important to achieving sufficient signal duration. EGF receptor (EGFR), the most extensively studied RTK in this regard, remains ligand bound, phosphorylated, and activated until late stages of endosomal trafficking (56). In the case of MAPK cascade, one of the canonical signaling pathways activated by virtually all RTKs, activation and assembly of the signaling complex takes place with the receptor still on the plasma membrane. However, the complex remains active in early and even late endosomes. This endosomal targeting of MEK-ERK complex requires Raf kinase activity and clathrin-dependent endocytosis (18) with the entire MAPK activation complex including GRB2, SOS, RAS, RAF, ERK1, and ERK2 found in early endosomes (55) leading to continued signaling. Following the transition to late endosomes, Raf1-bound K-Ras activates the protein complex that include adaptors p14 and MP1 (40) that in turn recruits and binds MEK1, thereby facilitating ERK1/2 entry into the nucleus (59). Similarly, in the case of neuronal growth factor (NGF) signaling, TrkA receptor in late endosomes is found in complex with Rap1 and PDZ-GEF1 that maintains its signaling and facilitates nuclear entry (27).

VEGFR2 Endocytosis

With this general background of receptor endocytosis and signaling in mind, we will now focus on events involved in endocytosis of VEGF receptors. By necessity, the discussion will concentrate almost exclusively on VEGFR2, since essentially nothing is known about VEGFR1 or VEGFR3 uptake and trafficking.

Cellular uptake of almost all RTK is initiated by their ligand binding, with a few exceptions such as T-cell receptor (21) and EGF receptor (23) that demonstrate constitutive recycling. VEGFR2, in addition to ligand-activated endocytosis, is also subject to constitutive recycling of inactive receptor (19). Both constitutive and ligand-induced VEGFR2 endocytosis utilize clathrin- and dynamin-dependent pathways and direct the receptor to EEA1-positive endosomes (34). A significant portion of the total VEGFR2 pool undergoes constitutive endocytosis in unstimulated HUVEC and is rapidly recycled back to the plasma membrane via Rab4-containing endosomes (19). Interestingly, the recycling pathway of ligand-induced VEGFR2 has not been determined.

VEGFR2 on the plasma membrane can exist in a complex or complexes with a large number of other transmembrane proteins including other VEGF RTKs (VEGFR1 and VEGFR3) (32), non-RTK VEGF receptor Nrp1 (54), adhesion molecules such as VE-cadherin (60), certain integrins (32), and other receptors including thrombospondin receptor CD47 (30) and ephrin-B2 (52, 63). Similarly, on the intracellular side, VEGFR2 can be found in association with a number of protein tyrosine phosphatases (PTPs) including VE-PTP (44), PTP1b (35), DEP1-/CD148 (34), and TCPTP (42). All of these proteins can and do play an important role in regulation of VEGFR2 signaling during various endocytic and trafficking events (FIGURE 1 and Table 1).

VEGF-A binding initiates active uptake of VEGFR2. The exact series of events and whether endocytosis will even take place at all are very much dependent on the state of endothelial cell and the surrounding extracellular matrix (ECM). When plated on fibronectin, endothelial cells respond to matrix-bound VEGF-A by prolonged phosphorylation of VEGFR2 at Y¹²¹⁴ site and activation of p38-MAPK signaling. This response is not seen following stimulation with non-ECM binding VEGF-A isoform or following inhibition of β 1-integrin activity (8). This association of VEGFR2 signaling with α 5 β 1-integrin activity is particularly interesting given the dependence of α 5 β 1 uptake on endocytosis and trafficking of another VEGF-R2 interacting protein, neuropilin-1. Specifically, neuropilin-1 regulates endocytosis of active α 5 β 1 by controlling its intracellular trafficking via Rab5-endosomes and synectin/myosin VI complex (61), which will be discussed in detail below.

On the other hand, confluent HUVEC plated on collagen-1 demonstrated only minimal VEGFR2 endocytosis in response to VEGF. This is thought to be due to phosphotyrosine phosphatase (PTP) TCPTP that is found in association with α 2 β 1-integrin (42). TCPTP binds to VEGFR2 and specifically dephosphorylates tyrosines Y¹⁰⁵⁴ and Y¹⁰⁵⁹ in the TK activation site and Y¹²¹⁴ in Rac activation site. TCPTP activation by intracellular domain of α 2 β 1 diminishes the kinase activity of VEGFR2 and blocks its internalization from the cell surface. Another ubiquitous PTP, SHP2 has also been reported to enhance internalization of VEGFR2 (3, 28).

Another adhesion molecule that regulates VEGFR2 endocytosis is VE-cadherin. Association between the two proteins in cell-cell junctions plays an important role in density-dependent growth inhibition (33). VEGFR2 endocytosis in this setting is prevented by VEGFR2 co-localization with VE-cadherin in adherence junction, which brings VEGFR2 into close association with VE-cadherin-bound DEP1/CD148. The latter dephosphorylates the receptor and prevents its internalization, thereby decreasing the magnitude and duration of its signaling. In non-confluent endothelial cells or in the absence of VE-cadherin, VEGFR2 uptake, phosphorylation and duration of signaling are all increased, whereas inhibition of VEGFR2 endocytosis reduces its signaling to the level seen in confluent endothelial cells (34).

Cerebral cavernous malformation protein CCM3 can also regulate VEGFR2 internalization (25). CCM3 binds VEGFR2 via its COOH-terminal domain and stabilizes the receptor at the cell surface. In patients with cerebral vascular malformations caused by *CCM3* mutations that affect its COOH-terminal end, the stability of VEGFR2 at the membrane and its endocytosis are reduced. Indeed, the early embryonic lethality (~E.8.5) and abnormalities in vascular development following *Ccm3* deletion in mice are similar to defects observed with VEGFR2 knockout (25).

Finally, another RTK, ephrin-B2, also regulates VEGFR2 uptake and signaling in a yet poorly understood manner. In the absence of ephrinB2, virtually no constitutive or VEGF-A-induced uptake of VEGFR2 occurs, and its signaling is profoundly impaired (52). Given that virtually nothing is known about how ephrin-B2 plays such a major permissive role in the initiation of VEGFR2 uptake, it is difficult to speculate about the mechanisms involved. It should be noted, however, that ephrin-B2 is a PDZ binding protein and the presence of its

PDZ domain is required for the effect on endothelial cell morphology, proliferation, and other biological functions. One may speculate that an ephrin-B2-PDZ-dependent interaction may somehow facilitate VEGFR2 movement into clathrin pits. Another interesting aspect of ephrin-B2 biology is its ability to stimulate $\alpha 5\beta 1$ -integrin clustering at sites of cell-cell contacts (29). Since $\beta 1$ -integrins, as already discussed, have been indirectly implicated in VEGFR2 endocytosis, it is possible to envision a VEGFR2-ephrinB2- $\beta 1$ -integrin complex that regulates VEGFR2 uptake. It should also be noted that ephrin-B2 plays a similar role in endocytosis of VEGFR3 (63), suggesting analogous molecular processes in lymphatic and blood vessel endothelial cells.

VEGFR2 Trafficking

Upon entry into cell, VEGFR2, like many other RTK receptors, continues to signal. The effectiveness of signaling, however, is dependent on its location in a specific endosomal compartment and subsequent trafficking (51). VEGFR2 upon endocytosis is initially found in APPL-positive Rab5 early endosomes and then transitions to EEA1⁺Rab5⁺ endosomes (FIGURE 2). In general, cargo movement from APPL to EEA1⁺Rab5⁺ endosomes is thought to occur due to EEA1 displacing APPL 1/2 from the common Rab5 binding site.

EEA1 endosomes containing VEGFR2 undergo intracellular trafficking that utilizes synectin/myosin VI complex. Synectin (*Gipc1*) is a single PDZ domain protein that can bind a number of transmembrane proteins including VEGFR2 interacting proteins neuropilin-1 (6) and ephrin-B2, adhesion molecules $\alpha 5/\alpha 6$ -integrins (58) and proteoglycan syndecan-4 (20), RTKs including TrkA/B (39) and IGF-1 (5, 65), and a number of cytoplasmic proteins such as RGS-GAIP (11) and RhoA GEF Syx1 (22, 38a). In addition to its PDZ domain, synectin also has a myosin VI binding site, and the two proteins have been shown to bind and orchestrate inward vesicle trafficking in a number of cell types (1, 45). Although ubiquitously expressed, synectin knockout in mice or knockdown in zebrafish results in a unique vascular phenotype characterized by decreased arterial vasculature size and branching complexity and reduced arteriogenesis in adult tissues (10). A similar arterial phenotype is seen in myosin VI knockout mice and zebrafish (35). Interestingly, impaired lymphangiogenesis was observed after synectin knockdown in zebrafish but not in mice (26).

VEGF signaling is significantly impaired in synectin- and myosin VI-null EC as are in vivo responses to VEGF stimulation such as angiogenesis in implantable Matrigel pellets (10, 35). The molecular basis of this defect turns out to be rooted in impaired VEGFR2 trafficking that in turn reduces its ability to activate ERK signaling. In the absence of synectin or myosin VI, following endocytosis, VEGFR2 remains for a prolonged period of time in EEA1-positive endosomal compartment before moving on. This delay is associated with a decrease in phosphorylation of Y¹¹⁷⁵ site in VEGFR2 that is critical for activation of PLC γ and ERK signaling. At the same time, phosphorylation of other critical VEGFR2 tyrosine residues (including Y¹⁰⁵⁴ and Y¹⁰⁵⁹) required for activation of tyrosine kinase activity, and Y⁹⁵¹, thought to be involved in Src and PI3K activation, remains unaffected. As a result, ERK phosphorylation is reduced and shortened in duration, whereas Src and PI3K signaling is normal (35).

The reduced phosphorylation of Y¹¹⁷⁵ site is restored by inhibition of expression of PTP1b, an endoplasmic reticulum-resident PTP that has been shown to affect activation and trafficking of multiple RTKs but not other PTP that are known to interact with VEGFR2, including VE-PTP, CD148, and Shp2. This suggests that VEGFR2 in early endosomes is in close contact with PTP1b and that prolonged residence in this endosomal compartment leads to a selective reduction in Y¹¹⁷⁵ phosphorylation and VEGF-induced ERK activation (FIGURE 2). The evidence for the functional significance of this VEGFR2 trafficking delay comes from studies demonstrating that a knockdown of PTP1b expression in synectin or myosin VI-null endothelial cells fully restores VEGFR2-dependent ERK activation. Likewise, suppression of PTP1b activity in vivo restores arteriogenesis in synectin-null mice (35).

Exactly how VEGFR2 in EEA1-positive endosomes interacts with a synectin/myosin VI complex is unclear at the present time. One possibility is that this is orchestrated by neuropilin-1 since it can both interact with VEGFR2 and bind synectin. This hypothesis is supported by two observations. First, VEGF-A_{165a}, an isoform capable of binding both VEGFR2 and Nrp1, directs VEGFR2 to the Rab5/Rab4/Rab11 recycling pathway (presumably via EEA1), whereas non-Nrp1-binding isoform VEGF-A_{165b} directs it toward degradation via the Rab7 pathway (2). Second, Nrp1 targeting of endocytosed VEGFR2 to the Rab5/Rab11 pathway requires the presence of its PDZ binding domain (50), suggesting a synectin-dependent process.

The balance between endocytosis and exocytosis is another means of regulating VEGFR2 signaling. Binding of VEGF to VEGFR2 on the plasma membrane stimulates trafficking of intracellular VEGFR2 sequestered in recycling endosomes back to the plasma membrane (19). In addition, the same binding event also initiates trafficking of newly synthesized, Golgi-resident VEGFR2 to the membrane (41). The process of VEGFR2 trafficking through the Golgi network and, subsequently, to the membrane is controlled by a Golgi-localized target membrane-soluble *N*-ethylmaleimide attachment protein receptor (t-SNARE) syntaxin 6. A reduction in syntaxin-6 levels results in decreased VEGF-induced endothelial cell proliferation, migration, and tube formation, whereas in vivo an inhibitory form of syntaxin 6 reduces VEGF-induced angiogenesis and permeability (41).

Finally, another level of VEGFR2 regulation is proteolytic cleavage that releases nearly complete cytoplasmic domain of VEGFR2. The proteolytically cleaved fragment is able to activate PLC γ and ERK signaling on its own, further demonstrating an intracellular site of ERK activation by VEGFR2 (5a).

Biological Effects of VEGFR2 Trafficking

We now have a rapidly increasing knowledge of functional consequences of abnormal VEGFR2 endocytosis and trafficking. Remarkably, every known alteration in these processes results in vascular defects with a high degree of arterial specificity in addition to angiogenic defects (Table 2).

When VEGFR2 uptake is impaired, as happens in endothelial cells expressing ephrin-B2 with a mutated PDZ binding site or in ephrin-B2-null cells, there is a pronounced reduction

in the number of tip cells in the developing retina, whereas in pathological settings tumor angiogenesis and growth are reduced. At the cellular level, there is a decrease in the number of filopodia extensions, and phosphorylation of the VEGFR2 Y¹²¹² site, thought to be involved in Rac1 activation, is profoundly reduced (52). Akt-1 activation is also reduced, as was the phosphorylation of the Y⁹⁵¹ site (52) that may be responsible for activation of this pathway (49). Similar to VEGFR2, endothelial ephrin-B2 deletion also results in marked impairment of VEGFR3 endocytosis and a compromise of Rac1, Akt, and Erk signaling (63). It should be noted that, although there is a tendency to conflate neuropilin-1- and VEGFR2-dependent effects of VEGF signaling, it is not clear that Nrp1 does not, indeed, have VEGFR2-independent VEGF-induced signaling effects. It also is not clear whether defective neuropilin-1 and VEGFR2 endocytosis are involved in various vascular phenotypes (10, 15, 35).

Indirect evidence implicates neuropilin-1 in shuttling VEGFR2 trafficking to the clathrin pathway (50), and it may direct interactions with Rab11 endosomes during the intracellular sorting step (2). Endothelial-specific disruption of neuropilin-1 expression results in marked reduction in arterial branching and increased vessel diameter. Furthermore, the origin of coronary arteries is frequently abnormal as is the septation of the ventricular outflow tract resulting in persistent truncus arteriosus (41a). Another defect associated with neuropilin-1 deletion is the persistent association of endothelial tip cell filopodia with the radial glia in the subventricular zone of the developing hindbrain. This likely results from the failure of the vascular sprouts to turn and extend across this region, leaving them blind-ended and forming vascular tufts (22). Although the molecular details of neuropilin-1-VEGFR2 interaction remain unclear, the cytoplasmic domain of neuropilin-1 appears critical for spatial separation of arterial and venous circulation in the retina (16).

The next pair of proteins controlling VEGFR2 intracellular trafficking is synectin or myosin VI. As already discussed, both are involved in trafficking of early VEGFR2 endosomes away from the PTP-1b-rich cytoplasmic domains. Unlike knockouts of ephrin-B2 and neuropilin-1, knockouts of these genes are not lethal, but both result in arterial-specific phenotypes characterized by decreased vascular branching and reduced lumen size (10, 35).

Summary

These recent developments have a number of interesting implications regarding VEGFR2 signaling. First, it appears that ERK activation occurs predominantly in endosomal compartments (35) and not on a plasma membrane. This is in agreement with studies showing the existence of an ERK scaffold complex in endosomes (46, 55). Second, different VEGFR2 phosphorylation sites may be dephosphorylated by specific PTPs (32, 35). If true, this would allow considerable fine-tuning of VEGFR2 signaling output with dephosphorylation events channeling RTK activation to specific pathways. Third, regulation of receptor trafficking after its endocytosis is another important means of regulating its activity (13). This additional control layer may be particularly important in temporal control of certain signaling outputs.

VEGFR2 membrane localization, internalization, and trafficking activation regulate its functions in a specific biological context. In a quiescent EC monolayer characterized by tight cell-cell junctions, the major role of VEGF signaling is maintenance of the monolayer (prevention of EC apoptosis) and production of nitric oxide. Both of these activities are the result of activation of PI3K/Akt pathway. VEGFR2 endocytosis in this setting is prevented by several mechanisms, including close proximity of VE-cadherin-bound DEP1/CD148 (33, 34) and β 1-integrin-bound TCPTP (42). Both of these phosphatases dephosphorylate VEGFR2 and reduce its endocytosis. This leads to VEGFR2 remaining on the plasma membrane from where it is able to activate PI3K/Akt but not ERK signaling.

In contrast, in settings when endothelial proliferation and migration are required, as occurs, for example, following injury when the monolayer integrity is broken and endothelial cells are no longer contact-inhibited, VEGF stimulation leads to VEGFR2 internalization into EEA1-containing endosomes. That by itself is not enough to activate ERK signaling since VEGFR2 in EEA1 endosomes finds itself in close association with PTP1b that selectively dephosphorylates the PLC γ /ERK pathway activation site. Trafficking of EEA1⁺ VEGFR2 endosomes, a process that requires synectin/myosin VI interaction, finally allows full activation of ERK signaling (35).

Once the vascular repair process is complete, the endothelial cells are once again arranged in a tight monolayer and a new basement membrane composed of collagen I and IV has formed, engagement of α 1 β 1 integrin activates TCPTP, whereas co-localization with VE-cadherin brings DEP1/CD148 once again into the picture. As the result, VEGFR2 internalization and ERK activation are inhibited, and membrane localization and PI3K/Akt activation are once again favored.

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FIGURE 1. VEGF-R2 interacting proteins

Inactive VEGFR2. In resting endothelial cells, VEGFR2 is found in complexes with VE-cadherin and β 1-integrins. This brings VEGFR2 close to DEP1 and TCPTP tyrosine phosphatases, thereby maintaining its inactive state. CD47-bound Tsp2 also prevents VEGFR2 activation. *Active VEGFR2.* VEGFR2 monomers dimerize after VEGF-A binding, leading to phosphorylation of tyrosine kinase domain tyrosines 1054 and 1059 and internalization into clathrin pathway in association with neuropilin-1 and its cytoplasmic partner synectin. Uncertainty in this function of neuropilin is indicated by Nrp1?. Dimerized VEGF-A-bound VEGFR2 in endosomes signals via three key phosphotyrosine sites: Y951, 1175, and 1214.

FIGURE 2. VEGF-R2 trafficking and signaling

VEGFR2 in early Rab5/AAPL endosomes in a complex with synectin and myosin VI. Neuropilin-1 probably serves as a bridge between VEGFR2-containing endosomes and synectin/myosin VI complex. VEGFR2 is partially deactivated on Y1175 site due to proximity to PTP1b leading to decreased ERK signaling. VEGFR2 endosomes undergo APPL to EEA1 conversion. VEGFR2 activity is still partially impaired. Synectin-/myosin VI-dependent trafficking of VEGFR2 away from subplasma membrane regions and close proximity to PTP1b result in increased phosphorylation of Y1175 site and full activation of ERK signaling.

Table 1
VEGF system and its components

Component	Interactions	Comments
<i>Ligands</i>		
VEGF-A		
VEGF-A121	VEGF-R1, VEGF-R2	Non-HS-binding isoform; free circulation in the blood
VEGF-A165	VEGF-R1, VEGF-R2, Nrp-1	Predominant isoform; some HS binding; circulates in the blood
VEGF-A189, 205	VEGF-R1 VEGF-R2, Nrp-1	Tight HS binding; ECM-bound
VEGF-B	VEGF-R1	Metabolic regulator
VEGF-C	VEGF-R2, VEGF-R3, Nrp-2	Requires proteolytic processing to VEGF-C N/ C (mature form)
VEGF-D	VEGF-R2, VEGF-R3, Nrp-2	Requires proteolytic processing to VEGF-D N/ C (mature form)
PIGF	VEGF-R1	Predominantly placental VEGF; regulates pathologic angiogenesis
<i>Receptors and transmembrane proteins</i>		
VEGF-R1	VEGF-A, VEGF-B, PIGF	Present in monocytes and other MNC
sVEGFR1	Same VEGF-R1	Major regulator of blood VEGF-A levels
VEGFR1 C	Same as VEGF-R1	50% <i>in utero</i> mortality (only sVEGFR1 form present)
VEGFR1-TK ⁻	Same as VEGF-R1	Normal development and angiogenesis; reduced inflammatory response
VEGF-R2	VEGF-A, VEGF-C, VEGF-D	Central VEGF-R in the adult blood endothelium; part of the shear stress sensor complex
VEGFR2Y1175F	Same as VEGFR2	embryonic lethal, same as VEGFR2 knockout. Lacks ERK activation
VEGF-R3	VEGF-C, VEGF-D	Key VEGF-R in lymphatics; also involved in developmental angiogenesis
Nrp-1	VEGF-A, Sema-3A, $\alpha 5\beta 1$, synectin	Predominantly arterial; VEGF-R-dependent and independent signaling
Nrp-2	VEGF-C, VEGF-D, synectin	Predominantly lymphatic; VEGF-R-dependent and independent signaling
VE-cadherin	VEGF-R2, DEP-1/CD148	Localizes VEGF-R2 to adherence junctions; part of shear stress sensor
Ephrin-B2	VEGF-R2, VEGF-R3	Regulation of cellular uptake
CD47	Thrombospondin-1, VEGF-R2	Thrombospondin-1 inhibits VEG-R2/CD47 association in the membrane
Integrins ($\alpha 5\beta 1$, $\alpha 2\beta 1$, $\alpha v\beta 3$)	Nrp-1, TCPTP	Stabilize VEGF-R2 at the cell surface near ECM adhesion sites
<i>Intracellular components</i>		
CCM3	VEGF-R2	Stabilizes VEGF-R2 at the cell surface
Synectin	Nrp-1, Nrp-2, Myosin-VI	Regulation of VEGFR2 trafficking
Myosin-VI	Synectin	Regulation of VEGFR2 trafficking
Syntaxin 6	VEGF-R2	Facilitates lysosomal degradation
TCPTP	$\alpha 2\beta 1$, VEGF-R2	Dephosphorylates Y ¹⁰⁵⁴ and Y ¹⁰⁵⁹ (TK site) and Y ¹²¹⁴ (Nck/p38-MAPK site)
VE-PTP	VEGF-R2	Dephosphorylation on an all sites
DEP-1/CD148	VEGF-R2, VE-cadherin	VEGF-R2 dephosphorylation
PTP1b	VEGF-R2	Dephosphorylates Y1175 (PLC γ /ERK activation site)

Table 2
Functional effects of disruption of VEGF-R2 endocytosis and trafficking

Gene	VEGFR2 E and T Role	Phenotype
VEGF-A	Initiation of active uptake	Vegfa ^{-/-} : complete failure of vascular development
VEGF-R2	Signaling receptor	Vegfr2 ^{-/-} , Vegfr2Y1175F: complete failure of vascular development
Ephrin-B2	Initiation of endocytosis	Ephrinb2 ^{ECKO} : reduced tip cell number, decreased angiogenesis
Neuropilin-1	Sorting to clathrin pathway	Npn1 ^{ECKO} : reduced arterial branching, absence of small/medium size arteries
CCM3	Endocytosis and stabilization at/near PM	Ccm3 ^{ECKO} : angiogenic and vascular remodeling defects
Synectin	Trafficking of early endosomes	Reduced arterial branching and lumen size
Myosin-VI	Trafficking of early endosomes	Reduced arterial branching and lumen size
Syntaxin 6	Trafficking to lysosomes	Reduced VEGF-induced EC migration, proliferation and angiogenesis

ECKO, endothelial-specific knockout; PM, plasma membrane.