Molecular Controls of Lymphatic VEGFR3 Signaling

Yong Deng, Xi Zhang, Michael Simons

Objectives—Vascular endothelial growth factor receptor 3 (VEGFR3) plays important roles both in lymphangiogenesis and angiogenesis. On stimulation by its ligand VEGF-C, VEGFR3 is able to form both homodimers as well as heterodimers with VEGFR2 and activates several downstream signal pathways, including extracellular signal-regulated kinases (ERK)1/2 and protein kinase B (AKT). Despite certain similarities with VEGFR2, molecular features of VEGFR3 signaling are still largely unknown.

Approach and Results—Human dermal lymphatic endothelial cells were used to examine VEGF-C–driven activation of signaling. Compared with VEGF-A activation of VEGFR2, VEGF-C–induced VEGFR3 activation led to a more extensive AKT activation, whereas activation of ERK1/2 displayed a distinctly different kinetics. Furthermore, VEGF-C, but not VEGF-A, induced formation of VEGFR3/VEGFR2 complexes. Silencing VEGFR2 or its partner neuropilin 1 specifically abolished VEGF-C–induced AKT but not ERK activation, whereas silencing of neuropilin 2 had little effect on either signaling pathway. Finally, suppression of vascular endothelial phosphotyrosine phosphatase but not other phosphotyrosine phosphatases enhanced VEGF-C–induced activation of both ERK and AKT pathways. Functionally, both ERK and AKT pathways are important for lymphatic endothelial cells migration.

Conclusions—VEGF-C activates AKT signaling via formation of VEGFR3/VEGFR2 complex, whereas ERK is activated by VEGFR3 homodimer. Neuropilin 1 and vascular endothelial phosphotyrosine phosphatase are involved in regulation of VEGFR3 signaling. (Arterioscler Thromb Vasc Biol. 2015;35:421-429. DOI: 10.1161/ATVBAHA.114.304881.)

Key Words: NRP1 ■ NRP2 ■ VE-PTP ■ VEGF-C ■ VEGFR2 ■ VEGFR3

The vascular endothelial growth factor (VEGF) family of vascular growth factors in mammals is composed of 5 proteins (VEGFs A–D) and of a closely related placenta growth factor. VEGFs act by binding to 3 closely related receptor tyrosine kinases (VEGFR1-3) and 2 nonkinase receptors, neuropilin (NRP) 1 and 2.1 Specificity of the biological activity of various VEGFs is determined, in part, by preferential binding of certain VEGFs to specific VEGFRs. Thus, VEGF-A and -B and placenta growth factor bind to VEGFR1, VEGF-A, and -C to VEGFR2, and VEGF-C and -D predominantly bind to VEGFR3.2 Specificity is further determined by differential expression of VEGF receptors, with VEGFR1 and VEGFR2 being the predominant isoforms in blood endothelial cells (EC). VEGFR3 is initially expressed during early embryonic development by both blood and lymphatic endothelial cells (LEC) later on becoming largely restricted to LECs.3 However, its expression can be induced in blood ECs during angiogenesis,4 and it may play an important role in retinal blood vasculature formation.5 VEGFR3 is also found in non-ECs, including neuronal progenitors, macrophages, and osteoblasts.6

VEGFR2 signaling has been extensively studied as a prototypical VEGF receptor. Recent advances include the appreciation of its interaction with vascular endothelial (VE)-cadherin,6 neuropilin 1,7 various phosphotyrosine phosphatases (PTPs),8 including density-enhanced phosphatase-1,9 vascular endothelial phosphotyrosine phosphatase (VE-PTP),10 and PTP1B,11 as well as the role of its endocytosis in the extracellular signal-regulated kinases (ERK) cascade activation.6,11 In contrast, relatively little is known about molecular controls of VEGFR3 signaling, although some details of VEGFR2 phosphorylation in blood ECs have been reported.12

Previous studies demonstrated that when bound by their ligands, both VEGFR2 and VEGFR3 form homodimers and undergo autophosphorylation of cytoplasmic tyrosine residues, leading to activation of their kinase activity. In addition to forming homodimers, on VEGF-C and VEGF-A stimulation, VEGFR3 can also form complexes with VEGFR2.13,14 Interestingly, VEGF-C–induced complexes concentrate on the leading edge of LECs, suggesting their involvement in cell migration. Yet the relative contribution of VEGFR3 homodimers versus VEGFR3/R2 complexes to activation of various intracellular signaling pathways and LECs biology has not been established.

VEGFR signaling also involves coreceptor proteins neuropilin 1 and neuropilin 2.15 These transmembrane proteins are...
VEGFR2, these involve PTP1B,10,11 VE-PTP,9,19 and CD148/PTPs in regulating VEGF receptor signaling. In the case of neuropilin 1 and neuropilin 2 to VEGF-C/VEGFR3 signaling have not been defined.

Despite these phenotypic observations, specific contributions of neuropilin 1 and neuropilin 2 to VEGF-C–driven lymphatic vessel growth.18 Nevertheless, recent studies have highlighted the importance of PTPs in regulating VEGF receptor signaling. In the case of VEGFR2, these involve PTP1B,10,11 VE-PTP,9,19 and CD148/density-enhanced phosphatase-1.8 In contrast, PTPs involved in VEGF-C–driven lymphatic vessel growth.18

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In the present study, we set out to study VEGFR3 signaling in human dermal lymphatic endothelial cells (HDLECs). To this end, we examined VEGF-C versus VEGF-A–dependent activation of ERK1/2 and protein kinase B (AKT) signaling and examined the contribution of key signaling proteins, including VEGFR2 and R3, neuropilin 1 and neuropilin 2, and various PTPs. We find that VEGFR2 and neuropilin 1 are required for VEGF-C–induced AKT activation. Moreover, we found that VE-PTP, but not other phosphatases, is able to modulate VEGFR3 activation. Finally, we determined that VEGF-C stimulation of LEC migration requires activation of both ERK and AKT pathways.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**Molecular Differences in VEGFR3 Versus VEGFR2 Signaling in the Lymphatic Endothelium**

To examine the differences in VEGFR3- versus VEGFR2-induced activation of key endothelial signaling pathways, such as ERK and AKT, we compared the effect of VEGF-A versus VEGF-C stimulation of HDLECs that express both VEGFR2 and VEGFR3. Stimulation with VEGF-A resulted in a strong and rapid (within 5 minutes) activation of ERK1/2 pathway and a relatively mild activation of phosphoinositide 3-kinase 3-kinase signaling as determined by AKT phosphorylation (Figure 1A and 1B). In contrast, when stimulated with VEGF-C, both ERK and AKT were activated with a much slower kinetics, but the extent of AKT activation was far greater than with VEGF-A (Figure 1A and 1B). These findings are in agreement with a previous in vitro study of VEGF-C signaling.21

We next asked whether VEGFR3 signaling shares some of the key molecular elements involved in regulation of VEGFR2 signaling, such as synectin (an adaptor protein which is critical to VEGFR2 trafficking and VEGF-A–induced ERK activation) and PTP1B (an intracellular protein tyrosine phosphatase that has been shown to colocalize to VEGFR2 early endosomes and regulate its activation of ERK1/2).22 A knockdown of either synectin or PTP1B had no effect on VEGF-C–induced ERK or AKT activation in HDLEC (Figure IA and IB in the online-only Data Supplement). Moreover, VEGF-C failed to induce PTP1B colocalization with internalized VEGFR3 (Figure IC in the online-only Data Supplement).

VEGFR3 has previously been reported to form complexes with VEGFR2 on VEGF-C stimulation in cultured LEC and tip cells in vivo.13,14 However, whether such complex formation affects VEGFR3 endocytosis and signaling has not been defined. To determine the role of VEGFR2 in these processes, we examined formation of VEGFR2/R3 complexes after VEGF-C and VEGF-A stimulation. In agreement with previous studies, VEGFR2 communoprecipitated with VEGFR3 in HDLECs that were stimulated by VEGF-C, whereas only marginal VEGFR2/R3 communoprecipitation was observed after VEGF-A treatment (Figure 1C). This was confirmed using confocal microscopy analysis of endocytosed receptors after VEGF-A or VEGF-C stimulation. Treatment of HDLEC with VEGF-C induced both VEGFR3 and VEGFR2 internalization and a significant degree of colocalization between the 2 receptors (Figure 1D and 1F). In contrast, VEGF-A induced strong VEGFR2, but only marginal VEGFR3 internalization and a much lesser extent of colocalization of the 2 receptors was observed (Figure 1E and 1F).

**VEGFR2 Is Involved in VEGFR3 Endocytosis and AKT Activation**

To examine the contribution of VEGFR2 to VEGFR3 signaling, we used VEGFR2 siRNA to knockdown its expression in HDLEC. Reduction in VEGFR2 expression significantly impaired VEGF-C–induced AKT activation, but surprisingly had no effect on ERK activation (Figure 2A and 2B). It also did not affect VEGF-C–induced VEGFR3 internalization (Figure 2C).

Phosphorylation of VEGFR2 at Y1175 is crucial for activation of both ERK1/2 and AKT by the receptor. Similar to VEGF-A, VEGF-C was also able to induce VEGFR2 Y1175 phosphorylation in HDLEC (Figure 2D). However, the extent of VEGF-C–induced phosphorylation was much weaker and the kinetics slower than that by VEGF-A (Figure 2E). To determine whether VEGFR2 kinase activity is required for VEGFR3 signaling, we expressed either a control (wild-type) or a dominant-negative kinase-dead VEGFR2 mutant (K868SR) construct23 in HDLECs (Figure II in the online-only
Data Supplement). The expression of the mutant receptor had no effect on VEGF-C–induced ERK or AKT activation (Figure 2F) or formation of VEGFR2/R3 complexes (Figure 2G), indicating that VEGFR2 kinase activity is dispensable for VEGFR3 signaling.

Neuropilin 1 but not Neuropilin 2 Is Involved in VEGFR3 Signaling in Lymphatic Endothelial Cells

Besides VEGFR2, neuropilin 2, highly expressed in lymphatic and venous ECs, is thought to play a role in VEGFR3 signaling. In addition, neuropilin 1, although predominantly found in arterial ECs, is also expressed in a portion of lymphatic ECs. Therefore, we next set out to examine the roles of neuropilin 1 and neuropilin 2 in VEGF-C signaling in HDLECs.

We first tested whether the 2 neuropilins interact with VEGFR3 on VEGF-C stimulation. Neuropilin 2 but not neuropilin 1 could be immunoprecipitated with VEGFR3 after VEGF-C stimulation (Figure 3A and 3B). However, although a knockdown of neuropilin 2 expression had no effect on
VEGF-C–induced ERK or AKT activation (Figure 3C and 3D), neuropilin 1 knockdown severely impaired VEGF-C–induced AKT but not ERK activation (Figure 3C and 3E), thus resembling the effect of VEGFR2 knockdown. Knocking down both neuropilin 1 and neuropilin 2 had a similar effect on VEGFR3 signaling to that observed with a single neuropilin.
1 knockdown, indicating that neuropilin 1 but not neuropilin 2 plays a predominant role in regulating VEGF-C signaling (Figure 3C).

To determine whether either neuropilin is involved in VEGF-C–induced VEGFR3 internalization, we followed the fate of surface biotinylated VEGFR3 after VEGF-C stimulation.
stimulation. A knockdown of neither neuropilin 2 (Figure 3F) nor neuropilin 1 (Figure 3G) had any significant effect on VEGF-C–induced VEGFR3 internalization. Both neuropilins also had no significant role in formation of VEGF-C–induced VEGFR2/R3 complex (Figure IIIA and IIIB in the online-only Data Supplement). A knockdown of VEGFR2 did not have any effect on VEGF-C–induced VEGFR3/neuropilin 2 interaction (Figure IIC in the online-only Data Supplement).

**Figure 4.** Vascular endothelial phosphotyrosine phosphatase (VE-PTP) negatively regulates vascular endothelial growth factor receptor 3 (VEGFR3) signaling and endocytosis. A, Human dermal lymphatic endothelial cells (HDLECs) transfected with VE-PTP or control siRNAs were serum-starved and stimulated with 100 ng/mL VEGF-C. Activation of VEGFR3 signaling was examined by Western blotting as indicated. B, Quantitative analyses of extracellular signal-regulated kinases (ERK), protein kinase B (AKT), and VEGFR2 phosphorylation shown in panel A. Data represents mean±SEM of 3 independent experiments. Statistical analysis was performed using 2-way analysis of variance. C, HDLECs transfected with VE-PTP or control siRNAs were serum-starved and stimulated with 100 ng/mL VEGF-C. VEGFR3 tyrosine phosphorylation was examined by blotting of VEGFR3 antibody immunoprecipitate with antityrosine antibody. D, Quantification of panel C. Data represents mean±SEM of 3 independent experiments. E, Serum-starved HDLECs were stimulated with 100 ng/mL VEGF-C, and then VEGFR3/VE-PTP interaction was examined by Western blotting with anti-VE-PTP antibody of VEGFR3 immunoprecipitate. Ctrl IgG, negative control. F and G, Surface biotinylation assay analysis of VEGF-C–induced VEGFR3 internalization after HDLEC treatment with VE-PTP or control siRNAs. Upper panel, Western blot analysis of internalized VEGFR3. Lower panel, Western blots of total cell lysates corresponding to the upper panel. Quantification was performed as described in Figure 3F and 3G. Note that 20% of lane S sample was loaded. Data in G is a summary of the quantification and represented mean±SEM of ≥3 independent experiments. Statistical analysis was performed using 2-way analysis of variance. H, Serum-starved HDLECs treated with anti-VE-PTP or control siRNAs were stimulated with 100 ng/mL VEGF-C followed by Western blotting with anti-VEGFR2 antibody of VEGFR3 immunoprecipitates.
VE-PTP Regulates VEGF-C/VEGFR3 Signaling

To determine whether a protein tyrosine phosphatase is involved in regulation of VEGF3 signaling, we knocked down PTPs expressed in LEC and observed the effect of these knockdowns on VEGF-C–dependent activation of ERK and AKT activation. Although knockdowns of PTP1B, PTPs, Src homology region 2 domain-containing phosphatase-1, and density-enhanced phosphatase-1 did not show any significant effect on VEGF-C–induced ERK and AKT activation, a VE-PTP knockdown significantly activated these pathways (Figure 4A and 4B). In agreement with a previously established role of VE-PTP in regulation of VEGFR2 phosphorylation,9,19 the activation of this receptor, as judged by Y1175 phosphorylation, was also increased (Figure 4A and 4B).

To find out whether VE-PTP regulates VEGFR3 phosphorylation, we performed phosphotyrosine blotting of immunoprecipitated VEGFR3 after VEGF-C stimulation from HDLEC treated with VE-PTP or control siRNA sequences. VE-PTP knockdown resulted in a nearly 6-fold increase in VEGFR3 tyrosine phosphorylation compared with control knockdown (Figure 4C and 4D). To further confirm the interaction between these 2 molecules, we next examined the presence of VE-PTP in VEGFR3 immunoprecipitate. Although essentially no VE-PTP was present before VEGF-C treatment, there was a marked increase in its presence after VEGF-C exposure (Figure 4E).

The presence of VE-PTP in fact also has a significant effect on VEGFR3 internalization. Using a cell surface biotinylation assay, we observed a significant increase in VEGF-C–induced VEGFR3 internalization after siRNA VE-PTP knockdown (Figure 4F and 4G). However, VE-PTP is not involved in VEGFR2/VEGFR3 interaction because knocking down VE-PTP had no effect on the kinetics or extent of complex formation (Figure 4H).

VEGFR2 and Neuropilin 1 Are Required for VEGF-C–Induced Cell Migration

Having established roles played by NRPs, VE-PTP, and VEGFR2 in VEGFR3 signaling, we next examined the effect of these signaling changes on LEC migration using a wound healing assay. Knockdown of VEGF2 and neuropilin 1 abolished cell migration of HDLEC in response to VEGF-C, whereas knocking down VE-PTP strikingly showed no effect (Figure 5A). In agreement with the minor role played by neuropilin 2 in VEGFR3-dependent ERK and AKT activation, its knockdown had only a marginal effect on VEGF-C–induced migration (Figure 5A).

To compare the role of the same proteins in VEGF-A signaling, we examined the effect of their knockdown on VEGF-A–induced HDLEC migration. As expected, knockdown of VEGF2 completely abolished VEGF-A–induced cell migration (Figure 5B). However, we did not observe any significant effect of neuropilin 1 knockdown on VEGF-A–induced cell migration, indicating that neuropilin 1 plays a distinct role in VEGF-C versus VEGF-A–induced cell migration. Similar to our observation in VEGF–C–induced cell migration, knockdown of neuropilin 2 and VE-PTP did not show any effect on the ability of HDLEC to migrate in response to VEGF-A, indicating that both of the 2 molecules are likely dispensable for these processes. Finally, we investigated the importance of ERK and AKT activation in VEGF-C–versus VEGF-A–induced cell migration. Inhibition of either ERK or AKT suppressed VEGF-C–induced cell migration, whereas only ERK but not AKT inhibition suppressed VEGF-A–induced cell migration (Figure 5C and 5D).

Discussion

The results of this study define relative contributions of various VEGFR3 partners to VEGF-C–induced signaling in LECs. In particular, we find that although VEGFR3/VEGFR2 complex formation is critical to VEGF-C–induced AKT activation, ERK activation by this growth factor is primarily driven by VEGF3 homodimer. Furthermore, although neuropilin 1 plays an essential role in AKT activation, it is dispensable for ERK activation. At the same time, a plasma membrane protein VE-PTP acts as a VEGFR3 tyrosine phosphatase and modulates both ERK and AKT activation by VEGF-C. Functionally, both ERK and AKT pathways play an equally important role in regulating LEC migration (Figure 5E).

One unexpected finding is the role of VEGFR2/R3 complex in VEGF-C–driven AKT activation. The role of VEGF2 in this complex is apparently to bring in its partner neuropilin 1. This is suggested by the fact that VEGFR2 kinase activity is not required for either the complex formation or AKT activation, as well as by the observed decrease in AKT activation after neuropilin 1 knockdown. The fact that NRP1 was not detected on co-IP with VEGFR2 is likely attributable to low levels of this protein in LECs. The function of neuropilin 1 in this setting is distinctly different from the role it plays in VEGF2-driven ERK activation, where it is involved in intracellular trafficking of VEGFR2.7 How neuropilin 1 facilitates AKT activation in the case of VEGFR3/R2 complex is not known.

Taken together with the observation that knockdown of VEGFR2 and neuropilin 1 did not affect VEGFR3 endocytosis and ERK activation but specifically abolished AKT activation, our data argues that VEGFR3-driven ERK but not AKT activation is dependent on receptor endocytosis. Although this endocytosis dependence of VEGFR3/ERK signaling is similar to that of VEGFR2 activation of ERK,22 in the distinction to the latter, VEGFR3 intracellular signaling does not seem affected by its subsequent intracellular trafficking because neither an intracellular phosphatase PTP1b nor synectin, regulators of VEGFR2 trafficking, affected this pathway. These findings are further in agreement with recent in vivo demonstration of the role of VEGFR2 in lymphatic vessel morphogenesis.24

The other key finding is the role of VE-PTP in modulating VEGFR3 signaling. VE-PTP knockdown enhanced both ERK and AKT activation. The effect of VE-PTP is likely attributable to a direct dephosphorylation of VEGF-C–activated VEGFR3 on the plasma cell membrane, thus diminishing ERK and AKT activation. Although VEGFR3/VE-PTP complexes have not been previously reported, we observed coinmunoprecipitation of the 2 proteins after, but not before, VEGF-C treatment of HDLECs. An unexpected finding is that VE-PTP knockdown does not increase HDLEC migration above baseline. This is
likely because migration is already fully activated by the normal VEGF signaling.

Another unexpected finding is the lack of significant contribution of neuropilin 2 to VEGFR3 signaling, despite its role in lymphatic biology. The 2 proteins do form a complex as suggested by immunoprecipitation experiments, but there seems to be little effect from neuropilin 2 knockdown on either VEGF-C–driven ERK and AKT activation or LEC migration. This may reflect intrinsic limitations of an in vitro study, although it should be noted that there is no clear data supporting or refuting neuropilin 2 contributions to lymphatics biology in vivo. In addition, AKT activation in this study was dispensable for VEGF-A–induced LEC migration, though a previous report suggested its involvement in this process. The reason for this difference is not clear.
In summary, these studies have uncovered novel feature of VEGFR3 lymphatic endothelium signaling involving regulation of AKT activation via VEGFR3/VEGFR2/neuropilin 1 complex, ERK via VEGFR3/R3 homodimer, as well as regulatory roles of VE-PTP.

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Disclosure

None.

References


Significance

Lymphatic endothelial cells play a key biological role in many disease processes. Yet our knowledge of how signal transduction takes place in this cell type is incomplete. The principle signaling receptors in the lymphatic endothelium are vascular endothelial growth factor (VEGF) receptor 2 and 3. In this study, we investigated how these receptors transmit signal from VEGF-C, the growth factor responsible for much of the lymphatic biology. We find that VEGFR3 homodimer activates extracellular signal-regulated kinases-1/2 pathway, whereas VEGFR2/R3 complex activates protein kinase B signaling. Furthermore, VEGF3-driven extracellular signal-regulated kinases but not protein kinase B activation is dependent on the receptor endocytosis, whereas activation of both pathways is modulated by a transmembrane phosphatase vascular endothelial phosphotyrosine phosphatase.
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MATERIALS AND METHODS

Cell culture, Transfection and Infection.

HMVEC-dLyAd, adult Human Dermal Lymphatic Microvascular Endothelial Cells (HDLEC) were purchased from Lonza and cultured in EBM-2MV medium (Lonza). For siRNA transfection, HDLECs were growth to 40-60% confluence and transfected with siRNA using RNAiMAX (Invitrogen) according to manufacturer’s manual. For adenovirus infection, HDLECs were growth to 40-60% confluence and infected with adenovirus at an MOI of 100 in culture medium. Lentiviruses production and infection were performed as previously described 1.

Antibodies and Reagents.

Antibodies used for Western blotting were: anti-pERK1/2, ERK1/2, pAKT S473, panAKT, pVEGFR2 Y1175, VEGFR2, NRP1, PTP1B, PTPµ, SHP1 (Cell Signaling), anti-human NRP2, human VEGFR3, DEP-1 (R&D), anti-α-tubulin (12G10, Developmental Studies Hybridoma Bank) and anti-VE-PTP (a gift from Dr. Dietmar Vestweber, Max Planck Institute for Molecular Biomedicine, Munster, Germany). Antibodies used for endocytosis and for immunofluorescence stainings were: anti-human VEGF-R3 (R&D), anti-EEA1, anti-PTP1B (BD Biosciences), anti-human VEGFR2 (ReliaTech). DAPI was obtained from Sigma. Secondary antibodies for immunoblotting were from Vector Labs and for immunofluorescence staining from Invitrogen. Reduced glutathione and iodoacetamide were from Sigma. The negative control (SI03650318), human VEGFR2 (SI00605528) and human NRP1 (SI02663213, SI02663220, SI00057113) siRNAs were from Qiagen. The siRNAs from Sigma are: human PTP1B (SASI_Hs01_00230699), PTPµ (SASI_Hs02_00324044), DEP1 (SASI_Hs01_00241067), VEGFR (#1:SASI_Hs02_00324723, #2:SASI_Hs02_00324725, #3:SASI_Hs02_00324728), VEGFR2 (#1:SASI_Hs01_00073462, #2:SASI_Hs01_00073461), NRP2 (#2, SASI_Hs01_00086002, #3: SASI_Hs01_00086001, SASI_Hs01_00085996) Human VEGF-C and VEGF-A164 were purchased from R&D. EZLink Sulfo-NHS-SS-Biotin and NeutrAvidin beads were from Thermo Scientific. U0126 was from Cell Signaling. LY294002 was from Sigma.  Ad-NRP1 adenovirus production was previously described 7.

Receptor Internalization Analysis by Cell-surface Biotinylation Assay.

Serum-starved confluent HDLECs were incubated with EZLink Sulfo-NHS-SS-Biotin (0.25mg/ml) in EBM-2 at 4°C for 30 min, quenched with cold 50 mM glycine in PBS and rinsed with cold EBM-2 with 1% BSA. To determine total cell surface receptors, cells were lysed prior to stimulation. To determine receptor internalization, cells were stimulated with VEGF-C (100 ng/ml) at 37°C and then rinsed with cold PBS. Cell surface-bound Sulfo-NHS-SS-Biotin was then stripped by incubating with reduced glutathione (45 mM) in 75mM NaCl, 75mM NaOH, 1mM EDTA, 1% BSA for 20 min twice on ice. Glutathione was quenched with cold PBS with iodoacetamide (5 mg/ml). Cells were then lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA and 1 mM EGTA) and biotin bound internalized receptors was immunoprecipitated with NeutrAvidin beads at 4°C overnight. Samples were analyzed by SDS-PAGE followed by Western blotting. To quantitatively analyze receptor internalization, densitometry of western blots was performed using Image J (NIH). The amount of total cell surface receptors prior to stimulation was designated as 100% and the percentage of internalized receptors in total cell surface receptors was calculated.

Endocytosis assay and immunofluorescent staining.

For endocytosis assays, HDLECs were grown to confluence on gelatin-coated 35-mm glass-bottom cell culture dishes (MatTek) and starved overnight with EBM-2 with 0.5% FBS. The cells were incubated on ice for 20 min to stop endocytosis, labeled with antibodies against human
VEGFR3 or VEGFR2 in EBM-2 containing 0.5% FBS for 15 min on ice and rinsed with cold PBS to remove unbound antibodies. Cells were then stimulated with VEGF-C (100 ng/ml) in EBM-2 containing 0.5% FBS at 37°C, followed by washing with ice-cold PBS pH 2.5 to remove remaining cell surface antibodies. Cells were fixed for 10 min at room temperature in 4% paraformaldehyde and permeabilized with 2.5% PFA/0.1% Triton X-100/0.1% NP-40 for 10 min. Cells were then labeled with antibodies against indicated endosome markers overnight, followed by incubation with secondary antibodies. Z-stack images with a 1µm step size were acquired under an UltraVIEW VoX spinning disc confocal microscope with a 60X oil immersion lens (Perkin Elmer) and equipped with a Ti-E microscope stand (Nikon) using a Volocity software (Perkin Elmer). The overlap coefficient index according to Pearson’s was calculated using the co-localization function of Volocity. To visualize cell surface protein only, cells were pre-cooled on ice for 30 minutes and then incubated with primary antibodies for 30 minutes on ice followed by ice cold PBS washing to remove unbound antibodies. The cells were then fixed with 4% PFA for 10 minutes on ice, incubated with secondary antibodies and visualized by confocal microscopy.

**Western blotting, Immunoprecipitation and Quantitative RT-PCR (qRT-PCR).**

For Western blotting, cells were lysed in RIPA buffer (Boston Bioproducts) supplemented with protease and phosphatase inhibitors. Cell lysates were then separated with 4-20% gradient gel (Bio-Rad) and subjected to immunoblotting. For immunoprecipitation, cells were lysed in NP-40 Lysis Buffer supplemented with protease and phosphatase inhibitors. A densitometry of Western blot was performed using Image J (NIH). For qRT-PCR, total RNA was purified using an RNaseasyPlus Mini kit (Qiagen). cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad). qPCR was performed using iQ SYBR Green Supermix (Bio-Rad) on a Bio-Rad CFX96 Real Time System. Primers used for qPCR are: human VE-PTP, for: 5' TGCTAAGTGGAAAATGGAGGCT 3', rev: 5' GCCCACGACCACCTTTCTCAT 3'; human FLT4, for: 5' GGTGTCGATGACGTGTGACT 3', rev: 5' CTCTGCTGGGACTCCTG 3'; human GAPDH, for: TGCACCACCAACTGCTTAGC, rev: GGCATGGACTGTGGTACATGAG.

**Endothelial Cell Migration Assay**

Cell migration was measured by “wounding” assays using a modified protocol as previously described. In brief, to stop cell proliferation, HDLECs were treated with 10 µg/ml mitomycin C in culture medium for 2 hours at 37 degree. Cells were serum starved in EBM-2 with 0.5% FBS for overnight. A wound was created using a 200 µl tip. Cell migration was then allowed in EBM-2 with 0.5% FBS in the absence or presence of VEGF-C (100 ng/ml) or VEGF-A (50 ng/ml) for indicated times. To inhibit MEK/ERK or PI3K/AKT activation, cells were pretreated with U0126 (10 ng/ml) or LY294002 (10 ng/ml) for 30 minutes prior to experiment. The distance of wound closure was analyzed for each experiment and six replicates for each condition were measured using NIH ImageJ. To calculate VEGF-induced cell migration, cell migration in the presence of VEGF was subtracted from that measured in the absence of VEGF stimulation.

**Data Analysis.**

Differences between two groups were tested for statistical significance with a two-tailed Student's t-test or two way’s ANOVA using GraphPad Prism 6.0. With two way’s ANOVA analysis, a p value of the interaction between column and row factors was calculated and shown. A p value less than 0.05 was considered significant. When p>0.05, “ns” was shown to represent the non-significance.
**Figure I.** Key protein components of VEGFR2 endocytosis and signaling are not involved in VEGFR3 endocytosis and signaling. (A) HDLECs transfected with synectin or control siRNAs were serum-starved and stimulated with 100 ng/ml VEGF-C as indicated. Activation of VEGFR3 signaling was examined by Western blotting. (B) HDLECs transfected with PTP1B or control siRNAs were serum-starved and stimulated with 100 ng/ml VEGF-C as indicated. Activation of ERK and AKT was examined by Western blotting. (C) Serum-starved HDLECs with surface VEGFR3 labeled with anti-VEGFR3 antibody were stimulated with 100 ng/ml VEGF-C for the indicated periods of time to induce VEGFR3 endocytosis. Co-localization of internalized VEGFR3 (red) with PTP1B (green) was visualized by confocal microscopy. Nuclei were labeled with DAPI (blue).
Figure II. Validation of VEGFR2 WT and K868R constructs. HDLECs were infected with ad-VEGFR2 WT or K868R. Response to VEGF-A stimulation was examined by determining ERK1/2 phosphorylation by western blotting.

Figure III. Interaction of NRP2 and VEGFR2 with VEGFR3. (A-C) HDLECs treated with control and NRP1 (A), NPR2 (B) or VEGFR2 (C) siRNAs were subjected to immunoprecipitation with anti-VEGFR2 (A,B) or anti-NRP2 (C) antibodies following VEGF-C stimulation. The immunoprecipitates were then probed with VEGFR2 (A,B) or NRP2 (C) antibodies.
**Figure IV. Screen of phosphatases involved in VEGFR3 signaling.** (A) HDLECs were treated with control siRNA or siRNA against phosphatases indicated on the blot. The cells were then serum-starved and stimulated with 100 ng/ml VEGF-C. Activation of ERK and AKT was determined by Western blotting at 0, 10 or 30 min after VEGF-C stimulation. Note that only VE-PTP knockdown increases both ERK and AKT activation increased. (B) qRT-PCR analysis showing the knockdown efficiency of VE-PTP in cell used in Fig. 4A. VEGFR3 mRNA was not significantly affected by the VE-PTP knockdown. Statistical analysis was performed using t-test.