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Membrane Trafficking and Endothelial-Cell Dynamics During Angiogenesis

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1. Introduction

The formation of new blood vessels, or neovascularization, involves multiple processes, including cell proliferation and migration, cell-cell and cell-matrix adhesion, and tube morphogenesis. Neovascularization can occur through one of two events: vasculogenesis, the *de novo* formation of blood vessels from angioblasts; or angiogenesis, the extension of new vessels from a pre-existing vasculature. Among these, angiogenesis in particular is relevant throughout life; its dysregulation has been causally related to several disorders that involve malignancy, inflammation, and ischemia. Angiogenesis is thought to depend on a set of signaling proteins – including certain kinases, integrins and vascular endothelial growth factor receptor-2 (VEGFR2) – that are enriched in specific plasma membrane domains. Both physiological and pathological angiogenesis rely on intracellular trafficking, a process that governs signaling by such proteins, as well as cell motility.

In this chapter, we discuss our current understanding of angiogenesis from the perspective of trafficking of the membrane components that are responsible for endothelial-cell (EC) dynamics.

2. Angiogenesis: Mechanism and importance

The vascular system carries out a variety of functions vital to vertebrates. It delivers oxygen and nutrients to tissues and organs. It is required for waste disposal, including the detoxification of toxic metabolites in the liver and their excretion through kidney. It is needed for the onset of immune responses against pathogens, since it transports immune cells to the site of infection and/or inflammation. Finally, its constituent vessels produce instructive signals for organogenesis.

Blood vessels are the main component of the vascular system and comprise: an EC monolayer that lines the vessel lumen, vascular smooth muscle cells that surround the EC monolayer, and a basement membrane that covers the vascular tube (**Figure 1**). The larger vessels, arteries and veins are stabilized by a thick layer of vascular smooth muscle cells, whereas the medium-sized vessels are supported by mural cells, for example pericytes (Gerhardt and Betsholtz, 2003).

The vascular system is among the earliest organ systems to develop in embryos; it first emerges when haemangioblast progenitors proliferate, migrate and differentiate into

ECs and form a primitive vascular plexus. The formation of new blood vessels, or neovascularization, then occurs either through vasculogenesis or angiogenesis, as mentioned above.

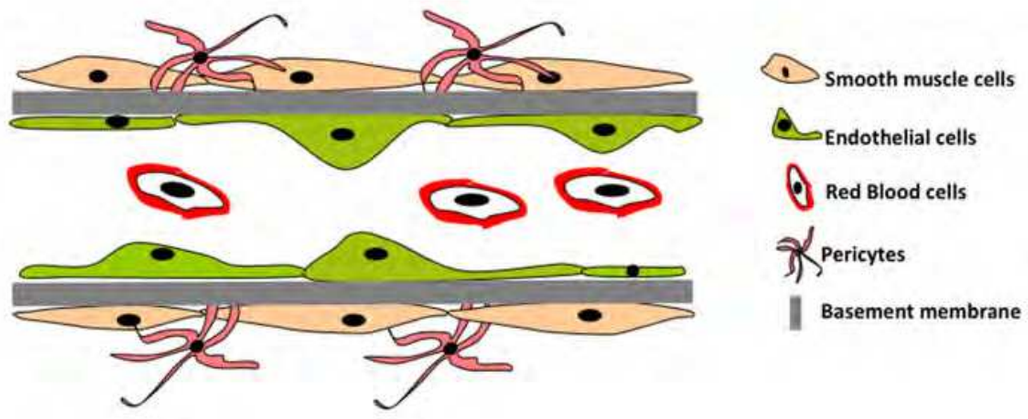


Fig. 1. Schematic representation of a mature blood vessel. Endothelial cells at the luminal side line tubular blood vessel. The smooth muscle cells and the pericytes that remain in contact with the endothelial cell lining through the basement membrane strengthen this tubular structure.

2.1 Vasculogenesis

Vasculogenesis, defined as *de novo* formation of blood vessels from precursor cells, starts with differentiation of precursor cells (angioblasts) into ECs and a primitive vascular network. In the first phase of vasculogenesis (during gastrulation), the splanchnic mesoderm gives rise to hemangioblasts, which are presumed to be the common progenitors of ECs and blood cells (Baron, 2001; Choi, 2002), and subsequently the outer cells of the blood island differentiate into angioblasts. In the second phase, angioblasts differentiate into ECs. In the third and final phase, the ECs participate in tube formation, giving rise to the primitive vascular plexus (**Figure 2**). Growth factor, fibroblast growth factor 2 (FGF2) generates hemangioblasts from the mesoderm, whereas vascular endothelial growth factor (VEGF) and its receptor, VEGFR, cooperate in hemangioblast generation and tube formation, and Angiopoietin-1 (Ang-1) regulates the connection between ECs and pericytes (Hiratsuka, 2011).

2.2 Angiogenesis

Angiogenesis, the establishment of new vessels from a pre-existing vasculature, starts with stimulation of ECs that line the luminal surface of blood vessels. The process of angiogenesis can be classified into two types: "sprouting" and "nonsprouting" (Risau, 1997). Sprouting angiogenesis refers to a process that entails proteolytic degradation of the extracellular matrix, the migration and proliferation of cells, the formation of a lumen, and maturation of ECs into functional capillaries. Specifically, ECs in pre-existing vessels respond to activation by a stimulatory molecule supplied by neighboring cells (e.g. VEGF), by producing a protease that degrades the surrounding basement membrane, to facilitate EC migration towards stimulatory signal. The sprout is comprised of a growth-arrested leading tip cell

and proliferating stalk cells (Gerhardt et al., 2003). The tips cells are migratory and extend long filopodia that guide the sprouts towards the stimulus. Subsequently, the sprout anastomoses with other newly formed vessels to allow circulation. The basement membrane reassembles pericytes and gets recruited to newly formed vessels (**Figure 2**). Sprouting angiogenesis occurs during yolk sac and embryo development, and later during brain development (Scappaticci, 2002). Non-sprouting angiogenesis is a process that occurs by invagination involving EC proliferation within a vessel, and results in an enlarged lumen that can be split by transcapillary or by the fusion and splitting of capillaries. Non-sprouting angiogenesis occurs mainly during development of the lung and heart (Scappaticci, 2002). Both sprouting and non-sprouting angiogenesis contribute to vessel formation in the adult, via a process known as "adult vasculogenesis". This process is thought to rely on endothelial progenitor cells (EPCs) that originate from the bone marrow. While circulating in the blood, EPCs are incorporated into vessels at the site of angiogenesis (Caplice and Doyle, 2005).

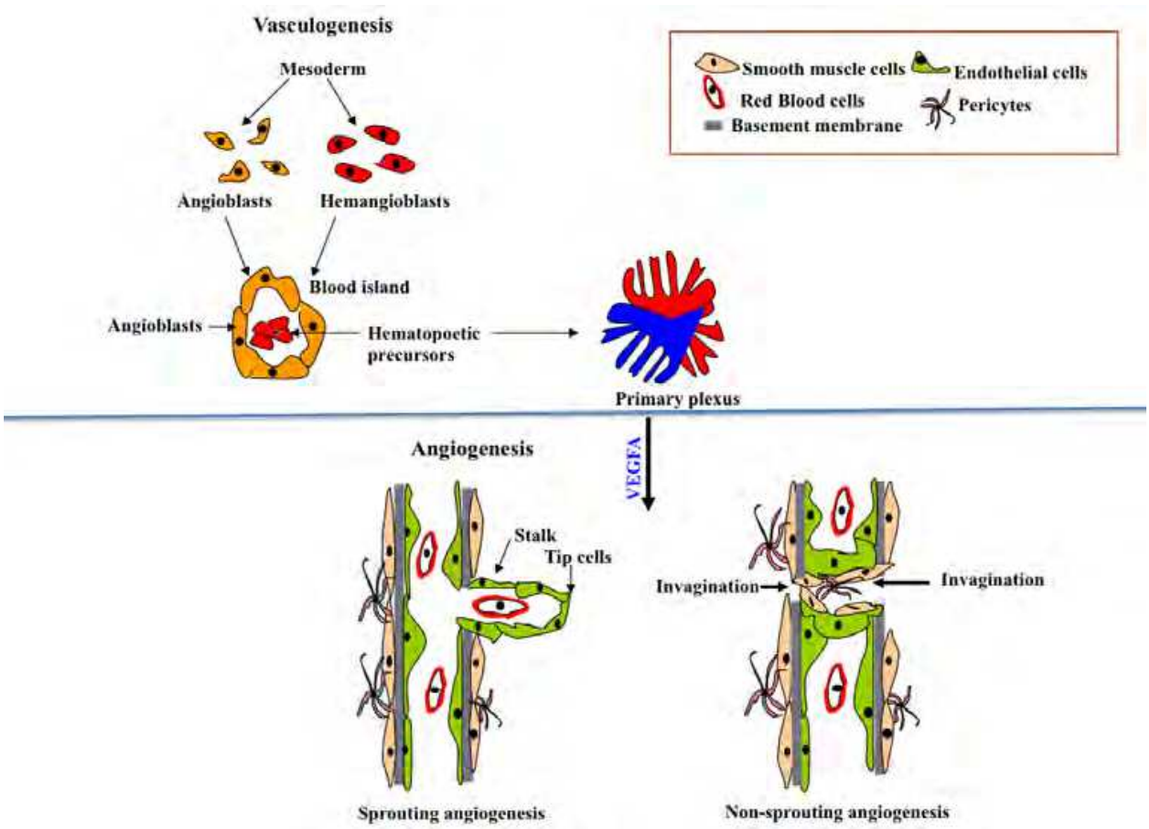


Fig. 2. Schematic outline of vasculogenesis (above) and angiogenesis (below). Both sprouting and non-sprouting mechanism of angiogenesis are shown.

2.3 Pathological angiogenesis

The process of angiogenesis is highly regulated in healthy adults and occurs only rarely, for example during ovulation and the endometrial growth that is central to the menstrual cycle. Angiogenesis is also required for wound healing, and ceases once this process is completed.

Since vessels nourish nearly every organ of the body the strict limitation of angiogenesis to such contexts is essential; deviation from normal vessel growth – in either direction – leads to fatal disease. Excess angiogenesis is characteristic of conditions such as retinopathy, rheumatoid arthritis, psoriasis and tumor growth (Folkman, 1995), and insufficient angiogenesis is a feature of ischemic heart and limb disease, stroke and gastrointestinal ulcers (Carmeliet and Jain, 2000).

The process of angiogenesis plays a crucial role in cancer metastasis – the major cause of mortality in cancer patients. In tumor diseases, angiogenesis is stimulated by the secretion of signaling molecules, e.g. VEGFA, by either the tumor cells themselves or tumor-infiltrating macrophages. In most cancers, tumor angiogenesis is crucial for disease progression, as it supplies the tumor cells with nutrients and oxygen, enabling them to survive and spread (Folkman, 2002). Notably, tumor-supporting vessels tend to be disorganized and leaky; thus vessel function is suboptimal and angiogenesis is further stimulated by hypoxia-driven expression of VEGFA in the tumor tissue. These findings have stimulated great interest in targeting tumor vessels as a means of developing novel cancer therapies. Indeed, intensive research efforts toward this end have revealed that, in patients with metastatic colorectal cancer, supplementing chemotherapy with a neutralizing antibody against VEGFA (Avastin/Bevacizumab) results in significantly improved survival (McCarthy, 2003).

Angiogenesis can also be regulated by microRNA (miRNA) (Wang and Olson, 2009). MicroRNA is a class of highly conserved, single stranded, non-coding small RNAs influencing gene expression by inhibiting translation of protein from mRNA or by promoting the degradation of mRNA (Bartel, 2004; Kim, 2005). The direct evidence of miRNA importance in angiogenesis was provided by observation of defective vascular remodeling during developmental angiogenesis in hypomorphic mouse line with EC-specific deletion of Dicer, one of key enzymes involved in miRNA generation (Otsuka et al., 2008; Yang et al., 2005). Endothelial cell primarily express miRNA-221/222, miRNA-21, the Let-7 family, the miR-17-92 cluster, the miRNA-23-24 cluster, and miRNA-126 (Harris et al., 2008; Kuehbacher et al., 2007; Suarez et al., 2007). miRNA-210 which gets activated upon hypoxia is an important regulator of EC survival, migration and differentiation during angiogenesis (Fasanaro et al., 2008). miRNA-210 overexpression under normoxic conditions stimulates angiogenesis and VEGF-induced cell migration whereas its blockade by anti-mRNA inhibits tube formation stimulated by hypoxia (Fasanaro et al., 2008). miRNA-210 may play an important role in pathological angiogenesis since hypoxia is associated with tumor development and organ ischemia. miRNA-21 and miRNA-31 are the other pro-angiogenic miRNAs which are upregulated in various various cancers to stimulate invasion and metastasis in cancer (Tsai et al., 2009).

3. Ligands and receptors in angiogenesis

At the molecular level, the initiation of vascular development is dependent on a number of cell-surface receptors and their respective ligands. Upon binding to a wide range of peptide growth factors, members of the receptor tyrosine kinase (RTK) family of transmembrane proteins transduce proliferative and morphogenic signals, thereby fine-tuning the orchestration of vascular remodeling and angiogenic processes. Although angiogenesis is controlled by a wide range of extracellular signals, the most potent pro-angiogenic signaling is initiated by binding of VEGF (vascular endothelial growth factor) to EC-resident VEGF

receptors (VEGFRs) like VEGFR1 [Flt (Fms-like tyrosine kinase)-1], VEGFR2 [Flk-1 (fetal liver kinase 1)/KDR (kinase insert domain receptor)] and VEGFR3 (Flt-4) (Zachary, 2003).

Remodeling from existing vessels during angiogenesis is governed by VEGF-mediated EC proliferation, and their sprouting from points of loose contact between capillaries and the extracellular matrix. The nascent capillary network then matures through the actions of TGF-β and platelet-derived growth factor (PDGF). The arterial and venous ECs within the final primary capillary express Ephrin B2 and its receptor EphB4, respectively, in their cell membranes; this enables correct fusion between arterial and venous vessels.

3.1 The VEGF-VEGFR system coordinates the process of angiogenesis

3.1.1 VEGF

The VEGF family is a branch of the PDGF/VEGF supergene family and its members have a homodimeric structure (Ferrara and Davis-Smyth, 1997). The VEGF family consists of five related growth factors: VEGFA, VEGFB, VEGFC, VEGFD and PlGF (placental growth factor). Although VEGFs are homodimeric polypeptides, naturally occurring heterodimers have also been reported (DiSalvo et al., 1995). VEGFA, which was also isolated as vascular permeability factor (VPF), is alternatively spliced to generate VEGFA121, VEGFA145, VEGFA165 and VEGFA189 (the numbers indicate final residue in each polypeptide in humans). Alternative splicing and processing regulates ligand binding to VEGF receptors, heparan sulfate and neuropilins (NRPs) (Grunewald et al., 2010). VEGFA165 and VEGFA189 bind to both heparan sulfate and NRP1. VEGFA plays an important role in the proliferation and migration of ECs, and also acts on monocytes and macrophages, neurons, cancer cells, and kidney epithelial cells. VEGFA is known to be regulated by hypoxia-inducible factor (HIF) (Germain et al., 2010), an event that leads to increased expression during embryonic development and wound healing, and also in the context of cancer. VEGFA produced by most parenchymal cells, act in paracrine manner on adjacent ECs to regulate signaling by the VEGF receptors. Autocrine VEGFA is essential for EC survival (Lee et al., 2007), and is consistent with their requirement for complete development of the vasculature in mice; both homozygosity and heterozygosity for knockout of the VEGFA gene result in embryonic lethality characterized by incomplete development of the vasculature (Carmeliet et al., 1996; Ferrara et al., 1996). The biological activity of VEGF is manifested after it binds to VEGF receptors, which are of 3 different types in human, and bind VEGF with distinct affinities.

3.2 VEGF receptors

Orthologs of each of the VEGF receptor tyrosine kinases (VEGFRs) – namely VEGFR1, VEGFR2 and VEGFR3 – have been identified in humans, mice and other mammals. Structurally, the VEGFRs have a common organization consisting of: an extracellular, ligand-binding domain that features 7 immunoglobulin (Ig)–like loops, a transmembrane domain; a juxtamembrane domain; a split kinase domain and a C-terminal tail **(Figure 3)**. Structurally, the VEGFRs are distantly related to the PDGFRs, which have five extracellular Ig-like domains (Matthews et al., 1991; Terman et al., 1991). All three VEGFRs undergo alternative splicing to generate more than one receptor form. The truncated form of VEGFR1, known as soluble VEGFR1 (sVEGR1, sFlt-1) (Kendall and Thomas, 1993), is implicated as causative agent in preeclampsia, a major disorder that can occur during

pregnancy (Levine et al., 2004). A naturally occurring soluble form of VEGFR2 has also been described; it could potentially arise as a result of alternative splicing or through proteolytic processing (Ebos et al., 2004). In humans, alternative splicing of VEGFR3 generates two isoforms with distinct C-terminal tails (Hughes, 2001).

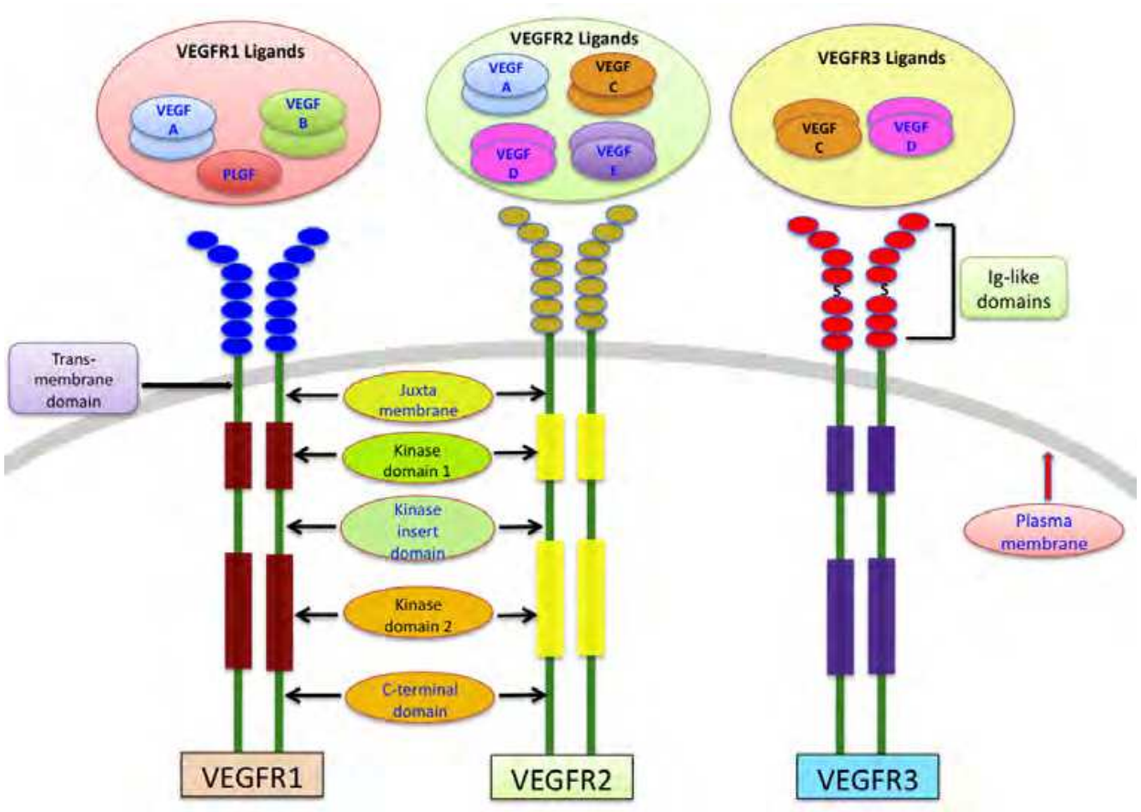


Fig. 3. VEGF receptors and their ligands. Schematic presentation of VEGF receptors (VEGFR1, VEGFR2 and VEGFR3). The signaling domain of all three receptors is present within cytosol. The Ig-like domains of VEGFRs are involved in VEGF binding, shown in elliptical structures that are present extracellularly. More than one kind of VEGF can bind to one receptor with different affinities.

3.2.1 VEGFR1

VEGFR1 (also known as Flt1 in mouse) is a glycoprotein of 150-184 kDa (de Vries et al., 1992; Shibuya et al., 1990) that gets activated upon binding of VEGFA, VEGFB and PLGF. VEGFR1 is expressed in vascular ECs at relatively high levels, throughout development and in the adult (Peters et al., 1993). It is also expressed in various other cell types such as monocytes, macrophages, human trophoblasts, renal mesangial cells, vascular smooth muscle cells, dendritic cells and a variety of human tumor-cell types (Barleon et al., 1996; Dikov et al., 2005; Sawano et al., 2001).

Alternative splicing of VEGFR1 generates soluble VEGFR1 (sVEGFR1), which is abundantly expressed in the placenta. Although the affinity of VEGFR1 for VEGFA is greater than that of VEGFR2, VEGFR1 transduces only weak signals for EC and pericyte growth and survival,

as well as for macrophage migration (Barleon et al., 1996; Nomura et al., 1995). In response to ligand binding, VEGFR1 undergoes autophosphorylation at various tyrosines within the intracellular domain (TYRs1169, 1213, 1242, 1327, 1333) (Ito et al., 1998; Sawano et al., 1997). Phosphorylation of TYR1169 allows binding and activation of phospholipase C (PLC)γ1, which regulates EC proliferation via the mitogen-activated protein kinase (MAPK) pathway (Sawano et al., 1997). Tyr1213 binds a variety of SH2-containing proteins, including PLCγ, growth-factor-receptor bound protein (GRB) 2, non-catalytic region of tyrosine kinase adaptor protein (Nck) and SH2-domain-containing protein tyrosine phosphatase 2 (SHP-2) (Igarashi et al., 1998; Ito et al., 1998). Tyr1309 is however phosphorylated in response to PLGF but not VEGF (Autiero et al., 2003) (**Figure 4)**. Although the exact role of VEGFR1

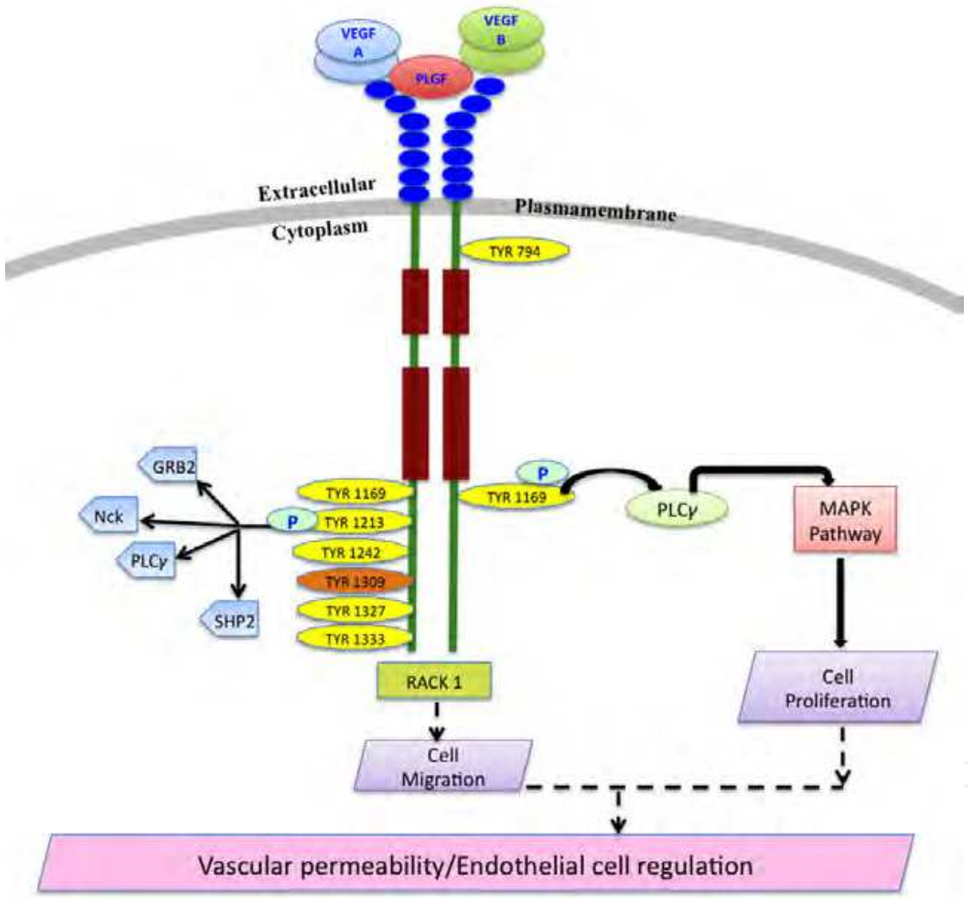


Fig. 4. VEGFR1 tyrosine phosphorylation and signaling. Schematic presentation of intracellular dimerized VEGFR1. VEGFA/VEGFB binding induces tyrosine phosphorylation of VEGFR1 at different tyrosine (TYR) positions shown in yellow ellipses. PLGF binding stimulates phosphorylation at TYR1309 shown in orange ellipse. Signaling molecules (shown within boxes) binds to certain phosphorylated tyrosine residues (circled P) and activates downstream signaling events leading to specific physiological outcome (shown in purple boxes) required for vascular permeability and endothelial cell regulation. PLCγ, phospholipase C-γ; MAPK, mitogen-activated protein; RACK1, receptor for activated C-kinase 1; SHP2, SH2-domain-containing protein tyrosine phosphatase 2.

remains a subject of debate, studies using VEGFR1-neutralizing antibodies have implicated this receptor in actin reorganization within, and the migration of ECs (Kanno et al., 2000), and suggested that receptor for activated C-kinase 1 (RACK1) is its downstream effector in this context (Wang et al., 2011). VEGFR1-dependent activation of PI3K/Akt may play a role in EC differentiation and organization (Huang et al., 2001) **(Figure 4)**. Under *in vitro* conditions, VEGFR1 and VEGFR2 are known to form heterodimers on cells co-expressing these receptors (Huang et al., 2001), so it is believed that VEGFR1 can regulate EC functions via cross-talk with VEGFR2, through dimerization as a result of VEGFA binding to both. Binding of PLGF can also lead to crosstalk between VEGFR2 and VEGFR1 through transphosphorylation, leading to sensitization of VEGFR2 subsequent to activation by VEGFA (Autiero et al., 2003). Soluble VEGFR1 (sVEGFR1, sFlt1) can negatively influence vascular development, either by sequestering VEGFA from signaling receptors or by forming non-functional heterodimers with VEGFR2 (Kendall et al., 1994). Although, VEGFR1 and sVEGFR1 are considered VEGF decoys that control signaling by VEGFR1 and the formation of angiogenic sprouts (Kappas et al., 2008), the importance of VEGFR1 in angiogenesis is demonstrated by the fact that the VEGR1 null (*vegfr1*$^{-/-}$) mouse dies at embryonic day 9, due to increased proliferation of ECs, disorganization and dysfunction of the vascular system (Fong et al., 1995).

3.2.2 VEGFR2

VEGFR2 (also known as KDR or Flk-1) is generally accepted to be the main receptor tyrosine kinase responsible for transducing the angiogenic activities of VEGFA, a factor that stimulates vascular-cell survival/growth and promotes angiogenesis. VEGFR2 knockout results in embryonic lethality due to deficiencies in vasculogenesis and hematopoiesis (Shalaby et al., 1995). VEGFR2 is highly expressed in vascular endothelial progenitors during early embryogenesis and generates a variety of angiogenic signals, not only for the proliferation of ECs but also their migration and morphogenesis, and as such has an important role in vascular tube formation. VEGFR2 expression is upregulated under conditions that trigger pathological angiogenesis, such as in tumors (reviewed in (Matsumoto and Claesson-Welsh, 2001). The binding of VEGF to VEGFR2 leads to receptor dimerization and promotes EC differentiation, proliferation, migration and vascular-tube formation. Both homo- and heterodimerization occurs following the trans-phosphorylation of tyrosine in the receptor intracellular domain. The major phosphorylation sites on VEGFR2 are TYR951 (in the kinase-insert domain), TYR1054 and TYR1059 (within the kinase domain), and TYR1175 and TYR1214 (in the C-terminal domain) (Matsumoto et al., 2005; (Takahashi et al., 2001). Additional phosphorylation sites on VEGFR2 have been identified at postions 1223, 1305, 1309 and 1319, but their function remains to be established (Matsumoto et al., 2005). Although TYR801 within the juxtamembrane can be phosphorylated when the intracellular portion of VEGFR2 is tested in isolation (Solowiej et al., 2009), its phosphorylation in the context of the intact protein has not yet been demonstrated. Phosphorylation at TYR951, within the kinase-insert domain, leads to binding and tyrosine phosphorylation of the SH2-domain–containing signaling molecule T-cell specific adapter (TSAd)(Matsumoto et al., 2005). TSAd, which is equipped with Src Homology 2 (SH2) and protein tyrosine binding (PTB) domains, in turn associates with the cytoplasmic tyrosine kinase Src, thereby regulating actin stress fiber organization and the migratory responses of ECs to VEGFA (**Figure 5)**. Phosphorylation at TYR1054 and TYR1059, both of which are located within the kinase domain activation loop (Kendall et al., 1999), is

preceded by autophosphorylation at TYR801 (Solowiej et al., 2009). Phosphorylation at TYR1059 induces Src binding, which in turn phosphorylates TYR1175 of VEGFR2, as well as residues within downstream signal transducers, such as the actin binding protein IQ-motif-contaning GTPase-activating protein 1 (IQGAP1), which is implicated in the regulation of cell-cell contacts, proliferation and migration (Meyer et al., 2008; Yamaoka-Tojo et al., 2006).

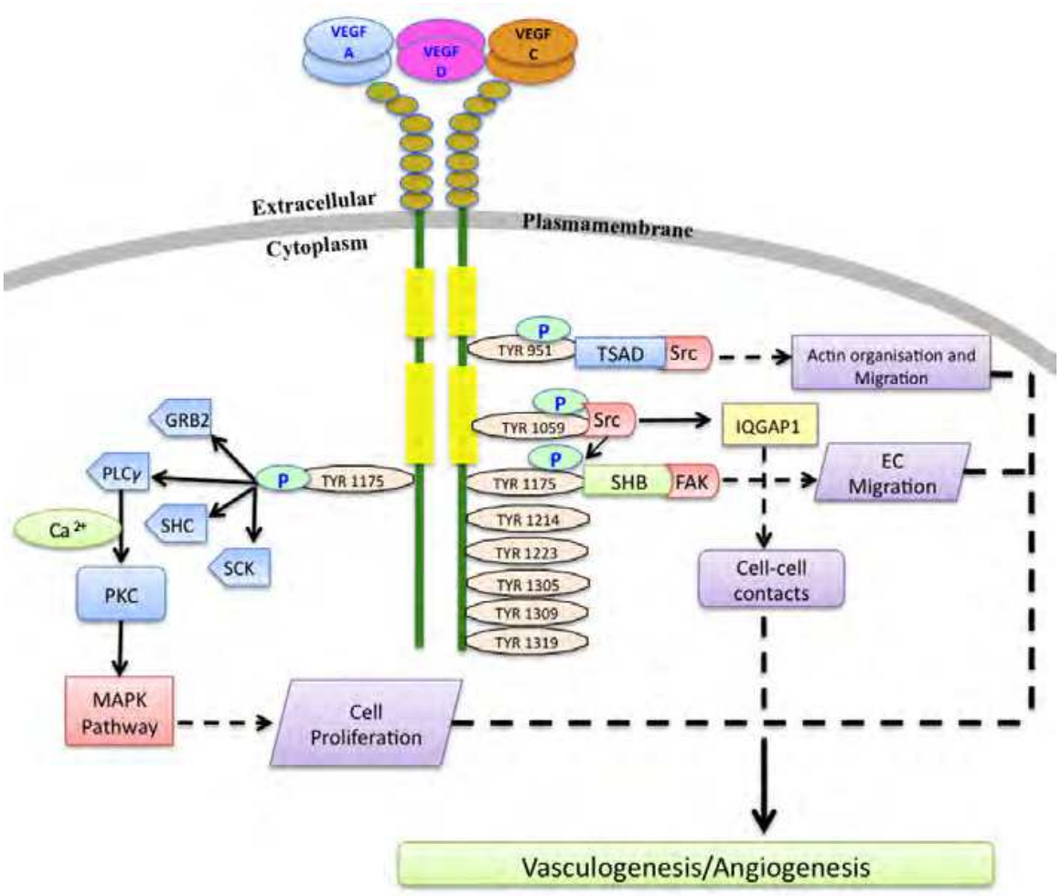


Fig. 5. VEGFR2 tyrosine phosphorylation and signaling. Schematic presentation of dimerized VEGFR2. VEGFA/VEGFB/VEGFC binding induces tyrosine phosphorylation of VEGFR2 at different tyrosine (TYR) positions shown in ellipses. Signaling molecules (shown within boxes) binds to certain phosphorylated TYR residues (circled P) and activates network of downstream signaling events required for variety of physiological outcomes (shown in purple boxes) essential during vasculogenesis and angiogenesis.

Phosphorylation at TYR1175 is required for the binding of PLCγ, which mediates activation of the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase-1/2 (ERK1/2) cascade and the proliferation of ECs (Takahashi et al., 2001). Binding of PLCγ stimulates the protein kinase C (PKC) pathway, leading to inositol triphosphate generation and calcium mobilization. In addition to interacting with PLCγ, phosphorylated TYR1175 binds to the SH2-domain–containing adaptor protein B (SHB) and Src homology and collagen homology (Sck/SHC) (Holmqvist et al., 2004; Warner et al., 2000). SHB binds to Tyrosine-phosphorylated focal adhesion kinase (FAK) in VEGF-treated cells (Abu-Ghazaleh

et al., 2001) and thereby contributes to EC attachment and migration (Holmqvist et al., 2003). The binding of SHC to TYR1175-phosphorylated VEGFR2 is believed to control Ras activation and mitogenicity in response to VEGF (Meadows et al., 2001).

3.2.3 VEGFR3

VEGFR3, also denoted Flt4, is a protein with a molecular weight of around 195 kDa, and becomes activated when bound to VEGFC or VEGFD. VEGFR3 is an essential protein; its inactivation results in embryonic death at E9.5, due to abnormal remodeling of the primary vascular plexus (Dumont et al., 1998). Although VEGFR3 plays a role in vascular development in the early embryo, its expression is later largely confined to ECs of the lymphatic system (Kaipainen et al., 1995). The exception is when its expression is induced in vascular ECs during active phases of angiogenesis, for example in the tumor vasculature or the endothelial tip cells of angiogenic sprouts in the retina (Tammela et al., 2008). VEGFR3 has five tyrosine phosphorylation sites – at positions 1230, 1231, 1265, 1337 and 1363 in the C-terminal tail (Dixelius et al., 2003) – that are activated and phosphorylated upon binding of VEGFC or VEGFD. TYR1337 when phosphorylated is known to bind to Shc and Grb2 to initiate signaling by the MAPK pathway. Both TYR1063 and TYR1068, which are located within the kinase activation domain, are crucial for kinase activity. Phosphorylation of TYR1230 and TYR1231 creates a docking site for SHC–GBR2, which promotes signaling by the ERK1/2 and PI3K/Akt pathways **(Figure 6)**. Phosphorylated TYR1063 has been shown to interact with adaptor protein C10 regulator of kinase (CRK I/II), which activates the c-Jun N-terminal kinase (JNK) pathway to promote cell survival (Salameh et al., 2005).

3.2.4 Neuropilins (NRP)

The cell-surface glycoprotein neuropilin is a transmembrane protein with a small cytoplasmic tail that lacks intrinsic catalytic function (Fujisawa et al., 1997). The neuropilin homolog NRP1 is expressed in arteries, whereas NRP2, is expressed in the venous and lymphatic vessels (Yuan et al., 2002). NRP1 was originally identified as a receptor for the collapsin/semaphorin family of neuronal guidance molecule (Chen et al., 1997). Later it was reported to also be expressed in ECs, where it acts as a coreceptor for VEGFA164 and VEGFA165 in mice and humans, respectively (Miao et al., 1999; Soker et al., 1998). NRP1 modulates VEGFR signaling, leading to enhanced survival and migration of ECs *in vitro* (Favier et al., 2006). NRP1 has also been implicated in VEGFR2-mediated vascular permeability (Becker et al., 2005) and VEGFA-induced vessel sprouting and branching (Kawamura et al., 2008). Although the exact influence of NRP1 on VEGFA mediated VEGFR2 signaling remains to be deciphered, the importance of NRP1 in angiogenesis was established by the fact that overexpression or deletion of NRP1 in mice leads to embryonic lethality and vascular abnormalities (Kawasaki et al., 1999). Neuropilin is also expressed in tumors and is believed to enhance tumor angiogenesis (Miao et al., 2000); it probably does so by stabilizing VEGF/VEGFR signaling on adjacent cell in trans.

3.3 Role of the extracellular matrix (ECM) in endothelial-cell interactions during angiogenesis

The angiogenic process is influenced not only by ligand/receptor systems, but also overall composition of extracellular matrix (ECM) surrounding the vasculature. Indeed, the ECM

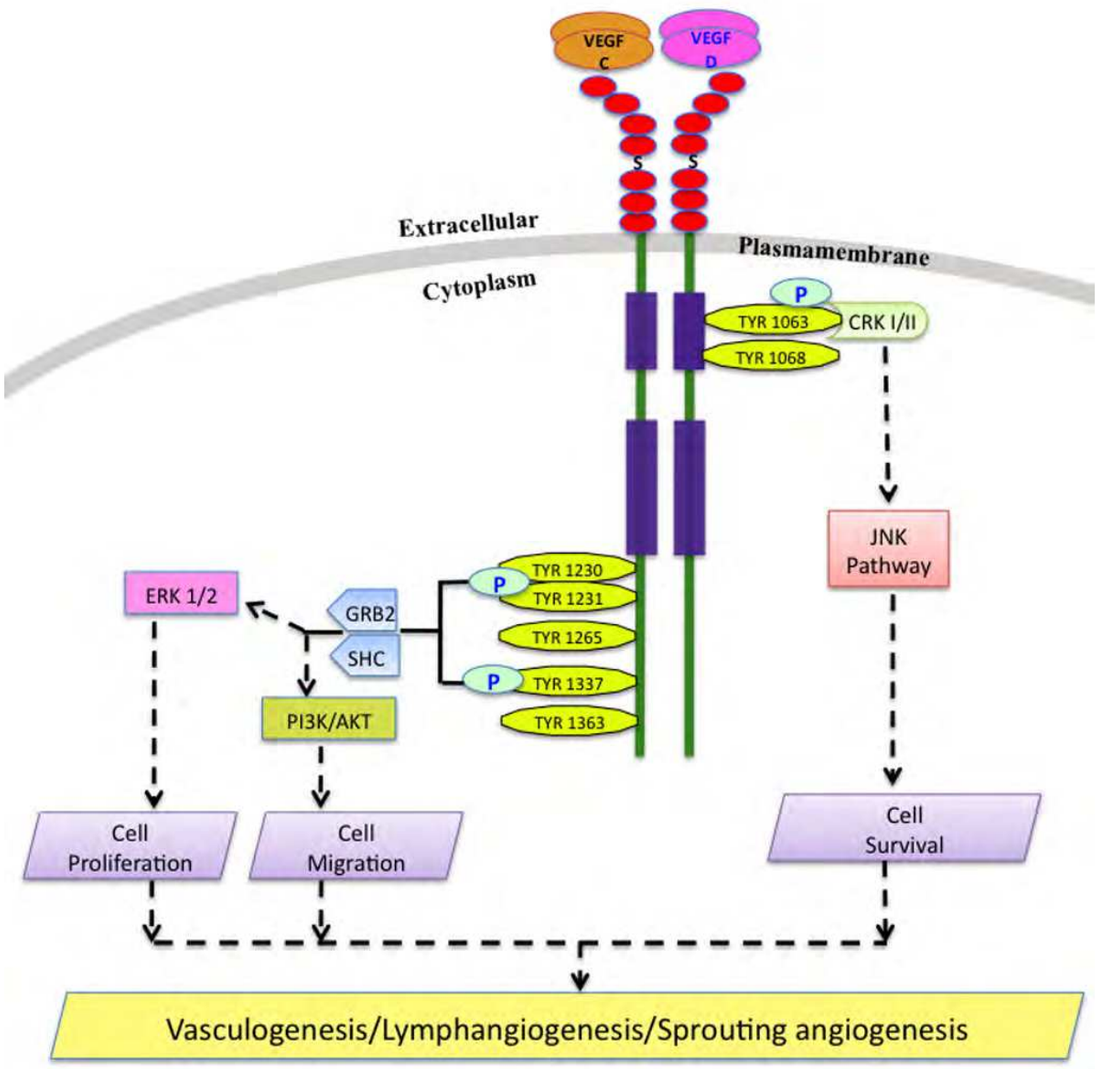


Fig. 6. VEGFR3 tyrosine phosphorylation and signaling. Schematic presentation of intracellular dimerized VEGFR3. VEGFC/VEGFD binding induces tyrosine phosphorylation of VEGFR3 at different tyrosine (TYR) positions shown in green ellipses. Signaling molecules (shown within boxes) binds to certain phosphorylated tyrosine residues (circled P) and activates network of downstream signaling events required for variety of physiological outcomes (shown in purple boxes) essential during vasculogenesis and angiogenesis

provides an essential connection between ECs and the surrounding tissues and affect angiogenesis either positively or negatively (Nyberg et al., 2005). ECs are attached to a basement membrane (BM), which forms a continuous coat around the vessels. The main components of the vascular BM are type IV collagen, laminins, fibronectin, heparan-sulphate proteoglycans and nidogens. The BM provides structural support to the ECs (Kalluri, 2003), and interaction of ECs with components in the BM is important for maintaining integrity of the vessel wall (Hallmann et al., 2005). When ECs are exposed to

angiogenic growth factors such as VEGFA, several kind of matrix-degrading enzymes, such as matrix metalloproteinases, dissolve the BM. Matrix-degrading enzymes are produced by ECs and stromal cells, as well as by tumor and inflammatory cells (Egeblad and Werb, 2002). Endothelial sprouting is activated not only by growth factors, but also signals from the matrix proteins. Degradation of the BM reveals so-called cryptic sites that can activate the angiogenic properties of ECs. As the BM is degraded and vascular permeability increases, the blood-clotting protein fibrinogen leaks out of the vessels and polymerizes into a fibrin gel (Iivanainen et al., 2003). Constituents of the degraded BM, fibrin, and EC-produced extracellular matrix (ECM) components then form a provisional matrix that ECs invade with guidance from both growth factors and matrix proteins. Once through the provisional matrix, the endothelial sprout further invades the interstitial matrix, which is composed of many proteins including fibrillar collagens (such as collagen I) and fibronectin. These proteins may also promote angiogenesis, collagen I for example, has pro-angoigenic effects (Davis and Senger, 2005; Senger et al., 2002). The signals from the ECM are transmitted by cell-surface expressed adhesion receptors called integrins, which bind to different ECM proteins in a specific manner (Stupack and Cheresh, 2004). In conjunction with the fusion of newly formed vessel sprouts to allow perfusion, the BM reassembles and pericytes are recruited to the vessel. ECs recruit pericytes by secreting PDGF, which binds to the PDGFβ-receptor expressed on pericytes; this process is crucial for vessel stabilization, and lack of pericyte engagement causes remodelling defects and leaky vessels (Uemura et al., 2002)

3.4 Role of Integrin in endothelial-cell dynamics during angiogenesis

Integrins are heterodimeric transmembrane glycoproteins consisting of non-covalently associated α and β subunits, and they promote cell-matrix adhesion and migration on the surrounding ECM. Integrins have no intrinsic enzymatic or kinase activity, but activate signaling pathways by co-clustering with kinases and adaptor proteins in a focal adhesion complex comprising: protein kinases such as FAK and Src; adaptor proteins such as Shc; signaling intermediates such as GTPases of the Rho family; and actin-binding cytoskeletal proteins such as talin, α-actinin, paxillin, tensin and vinculin (Mitra et al., 2005; Mitra and Schlaepfer, 2006). Integrin signaling promotes cell migration, proliferation and survival (Avraamides et al., 2008). Eighteen α and eight β subunits can associate to form 24 unique integrin heterodimers. EC integrins that regulate cell growth, survival and migration during angiogenesis include heterodimers α1β1, α2β1, α4β1, α5β1, α6β1, α6β4, α9β1, αvβ3 and αvβ5 (Avraamides et al., 2008). Integrin αvβ3 (a receptor for RGD-containing ECM proteins such as vitronectin, fibronectin, fibrinogen and osteopontin) was the first αv integrin shown to regulate EC survival and migration during angiogenesis. The expresssion of αvβ3 integrin on resting ECs is negligible, but is upregulated by the presence of angiogenic growth factors such as bFGF, TNFα and IL8 (Brooks et al., 1994). Integrin αvβ3 also regulate pathological angiogenesis during processes such as wound healing. The VEGFR2–αvβ3-integrin association is important for full VEGFR2 signaling activity, for activation of p38MAPK and FAK, and for the recruitment of actin-binding vinculin as needed to initiate EC migration (Mahabeleshwar et al., 2008). The ligation of endothelial αvβ3 integrin has also been shown to activate FAK, Src, and other kinases, resulting in cell proliferation, differentiation and migration (Eliceiri et al., 2002). Whereas αvβ3 dimers initiate angiogenesis in response to bFGF and TNFα, the related integrin αvβ5 is required for TNFα- and VEGF-mediated angiogenesis (Friedlander et al., 1995). β1

integrin, which pairs with variety of α integrin subunits, plays an important role in angiogenesis. Mice with EC-specific deletion of β1 integrin die during embryonic stages due to severe vascular defects (Tanjore et al., 2008). Matrix-bound VEGF induces the formation of a complex between VEGFR2 and β1 integrin, which leads to prolonged phosphorylation of VEGFR2 at TYR 1214 and association of β1 integrin with focal adhesions (Chen et al., 2010).

Fibronectin secreted by ECs is a key ECM component. It is deposited by ECs during normal and tumor angiogenesis (Clark et al., 1982; Kim et al., 2000). Fibronectin interacts with integrins such as α5β1, αvβ5 and αvβ3 (Plow et al., 2000). During embryonic vascular development, as well as during tumor angiogenesis, the ECM protein fibronectin serves as an adhesive support and signals through α5β1 integrin to regulate the spreading, migration and contractility of ECs (Francis et al., 2002). Although the expression of α5β1 in quiescent endothelium is low, it is upregulated by exposure to a subset of angiogenic stimuli including bFGF, IL8 and the ECM protein (Kim et al., 2000). Integrin α5β1 promotes EC migration and survival in *in vivo* and *in vitro* models of angiogenesis by suppressing the activity of protein kinase A (PKA) (Kim et al., 2000b(Kim et al., 2002). Further, our recent study has demonstrated that integrin α5β1 recycling is essential for EC adhesion and migration on fibronectin (Tiwari et al., 2011), a process required during angiogenesis. Integrin α5β1 promotes the formation of focal adhesions and signaling through FAK (Schlaepfer and Hunter, 1998), which is required for EC migration (Mitra et al., 2005). The reduction in EC surface associated adhesion through integrin α5β1 in the context of impaired recycling leads to further reduction in total and activated FAK (Tiwari et al., 2011). During embryogenesis, integrin α5 is required for the development of early blood vessels and other tissues, as revealed by the fact that α5 integrin-deficient mice exhibit a mesodermal defect and are embryonic lethal (Yang et al., 1993). Integrin α4β1, another fibronectin receptor, affects the adhesion and extravasation of lymphocytes by binding to VCAM1, a member of the immunoglobulin superfamily, that is expressed on inflamed ECs. Deletion of integrin α4 in a mouse model leads to defects in plancentation, heart development and coronary artery development, and thus to embryonic lethality (Yang et al., 1995). Integrin α4β1 promotes adhesion of the endothelium to VCAM1-expressing vascular smooth muscle cells during blood vessel formation (Garmy-Susini et al., 2005). Integrin α4β1–VCAM1 facilitates cell-cell attachment between ECs expressing the pericyte chemoattractant PDGF and pericytes expressing VEGF, in response to growth and survival signals that emanate from each cell type during angiogenesis (Garmy-Susini et al., 2005). Integrin α9β1 is another fibronectin-binding integrin known to have role in angiogenesis (Vlahakis et al., 2007). Although structurally similar to integrin α4β1, integrin α9β1 can bind to a number of ECM proteins and cell-surface receptors including tenascin C, thrombospondin, osteopontin, fibronectin, VCAM1 and other ligands (Liao et al., 2002; Marcinkiewicz et al., 2000; Staniszewska et al., 2007). α4β1 binds only to VEGF, and promotes VEGFA-induced angiogenesis. β1 integrin, which pairs with variety of alpha integrin subunits, plays a key role in angiogenesis. Mice with an EC-specific deletion of β1 integrin die at embryonic stages due to a severe vascular defect (Tanjore et al., 2008). Matrix-bound VEGF induces the formation of complex between VEGFR2 and β1 integrin, which leads to prolonged phosphorylation of VEGFR2 at TYR 1214 and to an association of β1 integrin with focal adhesions (Chen et al., 2010).

4. Membrane trafficking

Membrane trafficking is an active process that relocates proteins from one region of a cell to another, and contributes to the regulation of cell migration (Ulrich and Heisenberg, 2009). Signaling by the membrane-resident proteins/receptors is regulated by their availability at the cell surface or correct locations that are controlled by membrane trafficking events. The trafficking of membrane receptors and their signaling are intertwined: trafficking itself affects signal transduction, and signaling by RTKs regulates the trafficking machinery (Sorkin and von Zastrow, 2009). The secretory and endocytic pathways, which are made up of a network of membrane-bound compartments, modify newly synthesized proteins, deliver them to their appropriate locations, and regulate the uptake and turnover of those that are targeted to the cell surface. Trafficking accomplishes the specific and regulated transfer of molecules between distinct membrane-enclosed organelles. The transport process involves the budding of vesicular or tubular carriers from donor membranes, followed by their delivery to specific acceptor membranes. Budding requires the formation of cargo-laden vesicles or tubules at a donor compartment, and also the involvement of (i) specific coat proteins like COPI or COPII; (ii) adaptor proteins such as clathrin, AP-1, 2, 3; and (iii) membrane-deforming proteins like Bar-family proteins (Doherty and McMahon, 2009). The cargo-containing donor vesicle then docks to the acceptor compartment with the help of rab GTPases and a tether. Finally, fusion of the donor and acceptor membranes for the delivery of the cargo is accomplished with the help of N-ethylmaleimide-sensitive factor (NSF)-attachment protein receptor (SNARE) protein complexes (Jahn and Scheller, 2006; Sudhof and Rothman, 2009).

4.1 Biosynthetic/secretory pathway

After being synthesized in the cytoplasm, new proteins are translocated to the endoplasmic reticulum (ER) (Lee et al., 2004) and then move to Golgi via membranous vesicles. After moving through the cis and medial cisternae of the Golgi, the ER-derived cargoes move to trans-Golgi cisternae, where the proteins destined for secretion or presentation on the PM are packed into secretory vesicle that subsequently fuse with the PM (Emr et al., 2009). The Golgi apparatus is the major sorting compartment of the cell; its cargo is sorted not only to the PM for presentation or secretion, but also to endosomes and lysosomes, or back to the ER (Emr et al., 2009).

4.2 Endocytic and exocytic pathways

The endocytic pathway regulates internalization and recycling of proteins internalized from the PM, through a variety of mechanisms, to early and/or late endosomes. Depending on the receptor type and the particular requirements of a particular cell, protein from the endosomes are either sorted to the lysosome, a major degradation site for both internalized and cellular proteins, or recycled back to the PM (Doherty and McMahon, 2009).

Efficient protein trafficking within endomembrane system is further regulated by cross-talk between the two pathways. Endosomal and lysosomal proteins that maintain their integrity and functionality shuttle from the ER, via the Golgi, to endosomes and lysosomes (Ghosh et al., 2003). In polarized cells, movement of the proteins from one side of the cell to the other, via the transcytotic pathway, involves both the biosynthetic/secretory and the

endo/exocytic routes (Tuma and Hubbard, 2003). Thus, the membrane trafficking events control the cell communication network connected with signaling events, determining not only the intensity and duration, but also the final biological outcome.

The membrane trafficking process is also important for the signaling activities required for cell survival and migration during the normal physiological response, as well as for those that take place during angiogenesis. In ECs, modulation of receptor tyrosine activity through endocytosis and vesicle trafficking affects downstream targets such as endothelial nitric oxide synthase (eNOS) and VE-cadherin. Further, activation of RTKs results in the dissolution of EC–specific adhesion through endocytosis of VE-cadherin, thereby promoting cell migration and vascular permeability (Mukherjee et al., 2006). Directional cell migration requires the trafficking of adhesion and growth factor receptors including VEGFR2, which is involved with angiogenesis (Lanahan et al., 2010). The angiogenic signals generated in response to VEGFR2 receptor activation are highly regulated by sorting pathways during intracellular trafficking (Manickam et al., 2011). VEGFR2 signaling is regulated by a broad range of angiogenic regulators that in turn regulates receptor trafficking through the endosomal system (Manickam et al., 2011; Scott and Mellor, 2009). Rab GTPases regulate key events in VEGFR2 trafficking between the PM, early endosomes and late endosomes (Bruns et al., 2009). In addition, the Golgi (which is the central hub for membrane trafficking across the mammalian cell) coordinates the cell-surface expression of VEGFR2 by regulating the secretory transport of newly synthesized VEGFR2 (Manickam et al., 2011).

4.3 Endocytic trafficking of VEGFR2

Resting ECs have two pools of VEGFR2: a stable cell-surface pool that can form a complex with VE-cadherin at cell-cell junctions and does not undergo rapid internalization (Lampugnani et al., 2006), and a pool that continuously cycles between the surface and sorting endosomes and is independent of VEGF binding (Gampel et al., 2006). The VEGFR2 is associated with the caveolin-containing and cholesterol-enriched membrane microdomain (Labrecque et al., 2003). Binding of VEGF to extracellular domain of VEGFR2 triggers internalization of receptor subsequent to its dimerization and phosphorylation at TYR1054 and TYR1059. Upon activation, VEGFR2 dissociates from caveolin and transported to endosomes (Salikhova et al., 2008). Multiple modes of VEGFR2 internalization exist, since VEGF-stimulated endocytosis of VEGFR2 is clathrin-dependent (Lampugnani et al., 2006), and VEGFR2 is known to be translocated to perinuclear caveosomes through caveolar endocytosis (Bauer et al., 2005; Labrecque et al., 2003). Thus, the intracellular distribution of VEGFR2 depends on VEGF stimulation. Endosomal trafficking and the signaling of VEGFR2 is dependent on the Rab5a protein, a Ras-related small GTPase associated with early endosomes (Jopling et al., 2009). Depletion of Rab5a enhances VEGFR2 tyrosine phosphorylation and MAPK signaling, whereas overexpression of a Rab5a GTPase-deficient (constitutively active mutant, Q79L) causes VEGFR2 accumulation within endosomes (Jopling et al., 2009). Rab7a GTPase regulates VEGFR2 trafficking from early to late endosomes. Rab7a depletion is inhibitory whereas Rab5a depletion is stimulatory for EC migration (Jopling et al., 2009). VEGF stimulation also can increase the rate of VEGFR2 recycling from sorting endosomes. The vesicles containing VEGFR2 /Src complex traffic to a late endosomal compartment after a longer duration (30 mins) of VEGF stimulation (Gampel et al., 2006). Since Src is an important downstream target of VEGFR2 in an angiogenesis and

vascular permeability pathway (Zachary, 2003), it is believed that recycling of VEGFR2/Src to the PM would sensitize pro-angiogenic signals. VEGF stimulation direct VEGFR2 from sorting endosomes to late endosomes finally directs it toward lysosomes for degradation (Gampel et al., 2006).

Regulation of VEGFR2 degradation is important for angiogenesis since VEGFR2 down-regulation controls the sensitivity of ECs to VEGF stimulation. Reports of VEGFR2 degradation upon VEGF stimulation are varied. One study suggests that VEGFR2 degradation is complete upon VEGF A stimulation (Ewan et al., 2006), whereas another study revealed degradation of only 30-40% of the total receptor population (Gampel et al., 2006). Degradation of activated VEGFR2 is promoted by its ubiquitination by c-Cbl, a protein that forms a complex with phospholipase Cγ1 (PLCγ1), a mediator of VEGFR2 signaling (Singh et al., 2007). VEGFR2 trafficking can also be regulated by the co-receptors such as VEGFR1 and NRP1, which can interact with VEGFR2. VEGFR1 is ubiquitylated upon VEGF stimulation and recruits Cbl (Kobayashi et al., 2004). As discussed above, VEGFR1 forms a heterodimer with VEGFR2 upon VEGF binding, so that the decision for sorting of VEGFR2 along a degradative pathway could be influenced by VEGFR1. NRP1, one of the other VEGFR2 co-receptors, which forms a complex with VEGFR2 upon VEGF binding, could stabilize VEGFR2 on the EC surface since loss of NRP1 increases the degradation of VEGFR2 upon VEGF stimulation (Holmes and Zachary, 2008).

4.4 Secretory transport of VEGFR2

Although the endocytic transport of VEGFR2 has been characterized in detail, the reports on secretory transport, i.e transport of newly synthesized VEGFR2, are scant. The Golgi apparatus regulates the trafficking of newly synthesized VEGFR2, since the Golgi receives newly synthesized proteins and lipids from the endoplasmic reticulum (ER), the central organelle in which trafficking-route decisions are made. Recently, studies from our group demonstrated that a significant amount of VEGFR2 is present in the Golgi apparatus, and that VEGF mobilizes this pool from the Golgi compartment (Manickam et al., 2011). The post-Golgi trafficking of cargo occurs in vesicular fashion, where the cargo-loaded vesicles bud from Golgi, delivering the cargoes to their target destinations by membrane fusion events. Membrane fusion steps in eukaryotic cells require SNAREs (Chen and Scheller, 2001; Hong, 2005). The SNAREs are classified into 2 major groups based on the presence of a glutamine (Q SNAREs or t-SNAREs) or an arginine (R SNAREs or v-SNAREs) in the center of the SNARE motif. The trans-Golgi network- and endosome-localized t-SNARE syntaxin 6 (STX6), but not syntaxin 10 and syntaxin 16, regulates secretory transport of VEGFR2 as well as VEGF-induced angiogenic processes (Manickam et al., 2011). Earlier studies demonstrated that STX6 participates in post-Golgi transport of components of membrane microdomains to the PM (Choudhury et al., 2006). Inhibition of STX6 either by loss-of-function approaches, applying an siRNAs against *STX6* or by expressing the inhibitory, cytosolic domains of STX6 interferes with trafficking of Golgi-resident pool of VEGFR2 and targets it to lysosomes for degradation in human ECs, as described in model **(Figure 7).** Further, inhibition of STX6 in cell culture reduced VEGF-induced cell proliferation, cell migration, and vascular tube formation (Manickam et al., 2011). Thus, the t-SNARE STX6 plays a crucial role in maintaining cellular VEGFR2 levels and, subsequently, in physiological processes associated with VEGF-mediated angiogenesis.

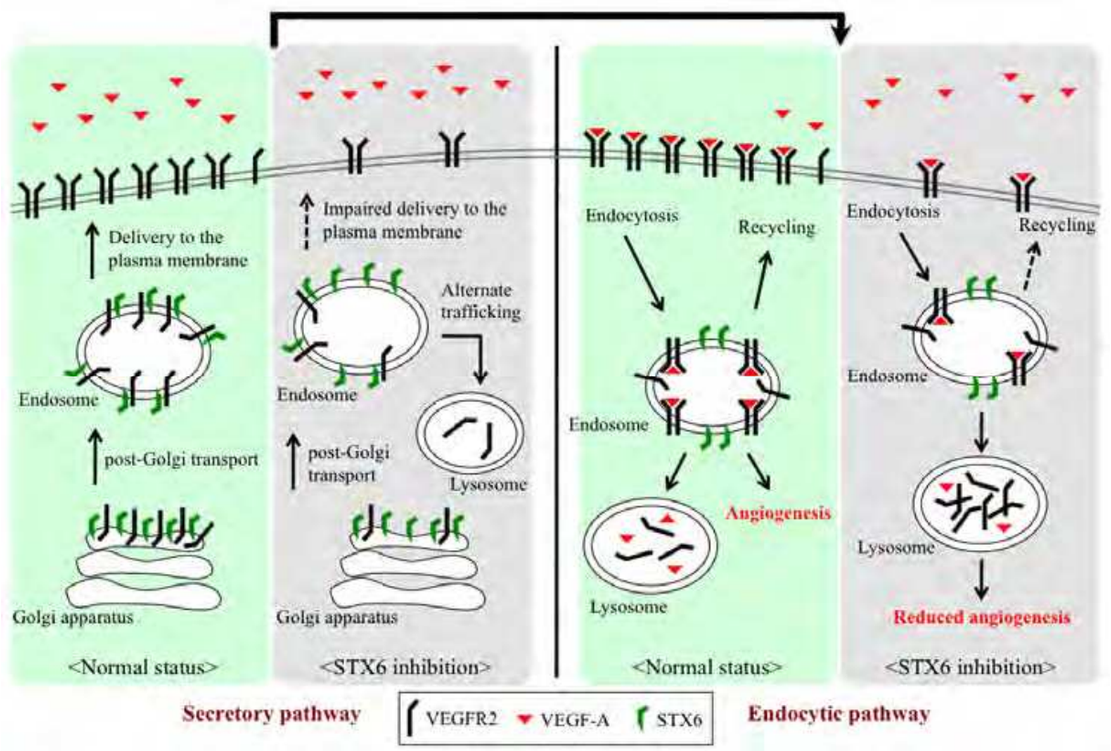


Fig. 7. Schematic summary of VEGFR2 trafficking. In endothelial cells, VEGFR2 is enriched in the plasma membrane, endosomes and Golgi apparatus. These VEGFR2 pools are maintained by endocytic and secretory transport pathways. The subcellular localization of VEGFR2 is essential for VEGF mediated signaling and angiogenesis, and is regulated by syntaxin 6, which colocalizes with VEGFR2 at the Golgi apparatus and endosomes. When syntaxin 6 function is inhibited, the cellular pool of VEGFR2 is depleted as a consequence of enhanced degradation in lysosomes. Syntaxin 6 contributes to trafficking of VEGFR2 from the Golgi and/or endosomes and the maintenance of proper levels of this receptor in different subcellular compartments required for efficient receptor signaling and angiogenesis. (Modified from Manickam et al., 2011. Blood *117*, 1425-1435. © the American Society of Hematology.)

4.5 VEGFR2 trafficking and angiogenesis

The process of angiogenesis is regulated by the response of VEGFR2 to VEGF binding. The availability of VEGFR2 at the PM may directly control the signaling response of ECs to VEGF for the onset of pro-angiogenic events. Thus, the trafficking mechanisms, such as secretory transport, recycling or degradation that affects the surface level VEGFR2, would determine the response of ECs to VEGF during angiogenesis. The EC-surface proteins that stabilize VEGFR2 at the plasma membrane contribute to the VEGF-driven cellular response during angiogenesis. A reduction in the engagement of VE-cadherin with ECs present in the stable vasculature leads to a reduction in surface levels of VEGFR2, thus reducing the sensitivity of ECs to VEGF (Lampugnani et al., 2006). The trafficking of VEGF receptors becomes more important under pathological conditions such as wound healing or ischemic heart disease, which may require rapid recycling and/or secretory transport of VEGF

receptors to the surface, ensuring continuous activation of the VEGF/VEGFR pathway to guide angiogenic events.

As discussed above, a study by our group (Manickam et al., 2011) demonstrated that enhanced VEGFR2 degradation due to reduced secretory transport of VEGFR2 from the Golgi to PM (due to lack of functional STX6) leads to reductions in VEGF-induced proliferation, migration, and tube formation. Such *in vitro* effects may be responsible for reduced VEGFA-induced angiogenesis in the context of interference with STX6 function via adenoviral gene transfer of cytosolic domain of STX6 (STX6-cyto, inhibitory form) in ear angiogenesis assay in nude mice **(Figure 8).** This ear angiogenesis model demonstrated that trafficking of VEGFR2 is essential for angiogenesis. Also, in our most recent study, we demonstrated that STX6 is essential for maintenance of EC surface-localized integrin α5β1, which plays a crucial role in angiogenesis (Tiwari et al., 2011). The finding that expressing a cytosolic form of STX6 significantly blocks VEGF-induced angiogenesis raises the prospect that pharmacologic manipulation of STX6 function in the setting of vascular disorders may be an effective therapeutic tool.



Fig. 8. Mice Ear Angiogenesis Assay: Representative images showing gross appearance of angiogenesis in mock, syntaxin 6-cyto– or syntaxin 16-cyto–injected mouse ears, 5 days before adenovirus expressing VEGF164 (Ad-VEGF164) administration. Nude mice (Nu/Nu strain) were given 2 injections under anesthesia. The first set of injections (1st) was PBS, syntaxin 6-cyto, or syntaxin 16-cyto. The second (2nd) was PBS or Ad-VEGF164, and given 2 days later at the site of first injection. At 7 days after the first set of injections, animals were euthanized and the ears were photographed. (from Manickam et al., 2011. Blood *117*, 1425-1435. © the American Society of Hematology).

5. Conclusions

Angiogenesis is regulated by VEGF-stimulated sensitization of the VEGF receptor and subsequent signaling events that are required during this process. Intracellular trafficking of VEGFR2 controls the VEGF signals that govern angiogenesis. Deciphering the mechanism underlying this trafficking and the roles of mediators in the transport of other VEGF receptors, as well as identifying other angiogenic components that play a role in the formation of new vessels, may provide better insight into angiogenesis. Syntaxin 6-regulated membrane trafficking events control outside-in signaling via the haptotactic and chemotactic mechanisms that regulate integrin α5β1-mediated EC movement on fibronectin and VEGF-mediated VEGFR2 signaling– important components of the angiogenic process (Manickam et al., 2011; Tiwari et al., 2011). A great deal has been deciphered about angiogenesis-related signaling pathways, but a detailed investigation of the intracellular and membrane trafficking of molecules associated directly or indirectly with angiogenesis would add to our knowledge of the angiogenic process, as well as help us to design therapeutic strategies for pathological angiogenesis therapeutic strategies for pathological angiogenesis.

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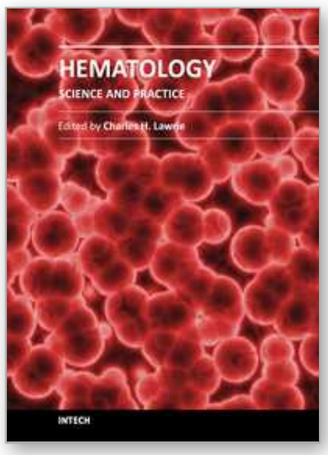
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Hematology encompasses the physiology and pathology of blood and of the blood-forming organs. In common with other areas of medicine, the pace of change in hematology has been breathtaking over recent years. There are now many treatment options available to the modern hematologist and, happily, a greatly improved outlook for the vast majority of patients with blood disorders and malignancies. Improvements in the clinic reflect, and in many respects are driven by, advances in our scientific understanding of hematological processes under both normal and disease conditions. Hematology - Science and Practice consists of a selection of essays which aim to inform both specialist and non-specialist readers about some of the latest advances in hematology, in both laboratory and clinic.

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