

VEGF receptor signalling — in control of vascular function

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Abstract | Vascular endothelial growth-factor receptors (VEGFRs) regulate the cardiovascular system. VEGFR1 is required for the recruitment of haematopoietic precursors and migration of monocytes and macrophages, whereas VEGFR2 and VEGFR3 are essential for the functions of vascular endothelial and lymphendothelial cells, respectively. Recent insights have shed light onto VEGFR signal transduction and the interplay between different VEGFRs and VEGF co-receptors in development, adult physiology and disease.

Vasculogenesis

Establishment of the embryonic vascular system.

Angiogenesis

Formation of new blood vessels from already established vasculature.

Receptor tyrosine kinase

Single transmembrane growth-factor receptor with an intracellular enzymatic (tyrosine kinase) domain that is activated by growth-factor binding, resulting in the transfer of phosphate groups onto tyrosine residues.

Heparan sulphate proteoglycans

(HSPGs). Transmembrane, lipid-anchored or secreted proteins that interact, through covalently linked heparan sulphate chains, with many proteins, including VEGF.

Vascular endothelial growth factors (VEGFs) are crucial regulators of vascular development during embryogenesis (vasculogenesis) as well as blood-vessel formation (angiogenesis) in the adult. In mammals, five VEGF ligands, which occur in several different splice variants and processed forms, have been identified so far. These ligands bind in an overlapping pattern to three receptor tyrosine kinases (RTKs), known as VEGF receptor-1, -2 and -3 (VEGFR1–3), as well as to co-receptors (here defined as VEGF-binding molecules that lack established VEGF-induced catalytic function), such as heparan sulphate proteoglycans (HSPGs) and neuropilins.

In certain respects, VEGFs share regulatory mechanisms with other well-characterized RTKs, such as the platelet-derived growth-factor receptors (PDGFRs) and the epidermal growth-factor receptors (EGFRs). These mechanisms include receptor dimerization and activation of the tyrosine kinase, as well as creation of docking sites for signal transducers. Moreover, the VEGFRs induce cellular processes that are common to many growth-factor receptors, including cell migration, survival and proliferation.

However, the VEGFRs also seem to be unique, for example, in their ability to transduce signals that form the three-dimensional vascular tube, and in regulating vascular permeability that leads to oedema and swelling of tissues. **VEGFR1** is a positive regulator of monocyte and macrophage migration, and has been described as a positive and negative regulator of **VEGFR2** signalling capacity. Negative regulation is exerted, at least in part, by an alternatively spliced soluble VEGFR1 variant that binds to VEGF and thereby prevents VEGF from binding to VEGFR2. VEGFR2 is implicated in all aspects of normal and pathological vascular-endothelial-cell

biology, whereas **VEGFR3** is important for lymphatic-endothelial-cell development and function.

Recently, tumour therapies that are based on neutralizing anti-VEGF antibodies and small-molecular-weight tyrosine-kinase inhibitors that target the VEGFRs have been developed. These new strategies for tumour treatment show the clinical relevance of inhibiting VEGF signal-transduction pathways that are exaggerated in pathological angiogenesis. When using such therapies in the long-term treatment of cancer or other diseases that are associated with pathological angiogenesis, it will be important to preserve pathways that are important for the survival of blood vessels in healthy tissues. This review describes our current understanding of VEGFR-signal-transduction properties that regulate biological responses to VEGF, such as vessel survival, the need for balanced and convergent VEGFR signalling during development and the emerging picture of the role of co-receptors in directing the magnitude and quality of the signal output.

Characteristics of VEGFs and VEGFRs

The VEGF family members are secreted, dimeric glycoproteins of approximately 40 kDa. In mammals, the VEGF family consists of five members, **VEGFA**, **B**, **C**, **D** and placenta growth factor (**PLGF**). In addition, proteins that are structurally related to the VEGFs exist in parapoxvirus¹ (VEGFE) and snake venom² (a group of proteins known as VEGFFs). VEGFA, B and PLGF bind to VEGFR1, VEGFA and E bind to VEGFR2, and VEGFC and D bind to VEGFR3. Proteolytic processing of the human VEGFC and D allows for binding to VEGFR2, however, these factors bind to VEGF2 with lower affinity than to VEGFR3 (see FIG. 1a for mammalian ligand–receptor interactions). The VEGFFs interact with both VEGFR1 and 2 (REF. 1).

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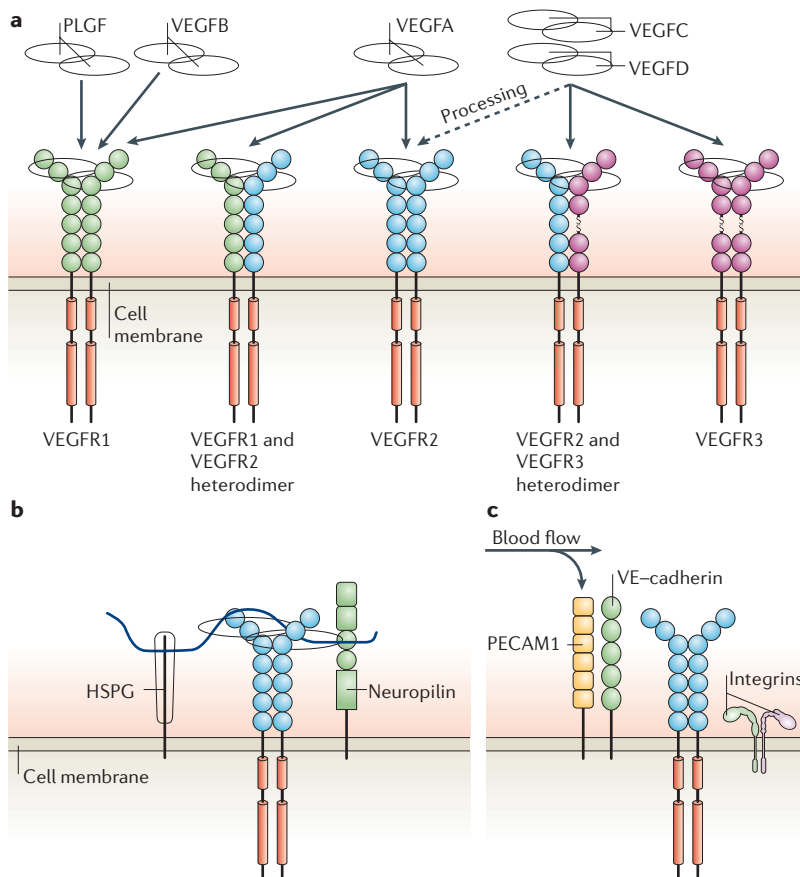


Figure 1 | VEGF receptor-binding properties and signalling complexes.
a | Mammalian vascular endothelial growth factors (VEGFs) bind to the three VEGF receptor (VEGFR) tyrosine kinases, leading to the formation of VEGFR homodimers and heterodimers. Proteolytic processing of VEGFC and D allows for binding to VEGFR2.
b | VEGFR signalling is modulated by different co-receptors. VEGFs as well as VEGFRs bind to co-receptors such as heparan sulphate proteoglycans (HSPGs) and neuropilins. These interactions can influence VEGFR-mediated responses, for example, affecting the half-life of the receptor complex.
c | Mechanosensory complex formation. Blood flow might activate VEGFRs in a ligand-independent manner, by the formation of mechanosensory complexes that consist of platelet-endothelial-cell adhesion molecule-1 (PECAM1), vascular endothelial (VE)-cadherin, VEGFRs and integrins. PLGF, placenta growth factor.

Structure and expression of VEGFs. Structurally, the VEGFs are related to the PDGF family of growth factors, with intrachain and interchain disulfide bonds between eight cysteine residues in conserved positions. The crystal structure of VEGFA revealed two monomers that are organized in an anti-parallel fashion to form a dimer, with the receptor-binding sites located at each pole of the dimer³. The VEGFs preferentially form homodimers, although VEGFA and PLGF heterodimers have been identified⁴ (FIG. 1a).

Alternative splicing of several of the VEGF family members gives rise to isoforms with different biological activities. The human isoforms are denoted VEGFA121, VEGFA145, VEGFA165, VEGFA189 and VEGFA206 (see **Supplementary information S1** (figure)). The mouse isoforms are one amino-acid residue shorter than the corresponding human isoform, and they are

denoted VEGFA120 and so forth. The activities of the VEGFA isoforms are dictated by their different abilities to interact with VEGFR co-receptors, such as neuropilins and HSPGs (FIG. 1b,c). Another splice variant of VEGFA, known as VEGF165b, has been proposed to negatively regulate VEGFR activity⁵.

The bioactivity of VEGF family members is also regulated by proteolytic processing. This mechanism might enable specific interactions with different types of receptor. For example, in humans, processed VEGFC and D bind to VEGFR2, as well as to VEGFR3. Furthermore, proteolytic processing of VEGFA splice variants affects their ability to interact with the VEGF co-receptors HSPGs and neuropilins⁶.

Structure of VEGFRs. The VEGFRs are members of the RTK superfamily and they belong to the same subclass as receptors for PDGFs and fibroblast growth factors (FGFs). The VEGFRs are equipped with an approximately 750-amino-acid-residue extracellular domain, which is organized into seven immunoglobulin (Ig)-like folds. In VEGFR3, the fifth Ig domain is replaced by a disulfide bridge. The extracellular domain is followed by a single transmembrane region, a juxta-membrane domain, a split tyrosine-kinase domain that is interrupted by a 70-amino-acid kinase insert, and a C-terminal tail (FIG. 2). Structural and functional studies have yielded insights into how the distinct domains contribute to VEGFR activity. The crystal structure of part of the extracellular domain of VEGFR1, alone and in complex with ligand, shows that the Ig domain-2 constitutes the ligand-binding site on the receptor⁷. In addition, biochemical analyses showed that the Ig domain-3 in VEGFR2 is important for the determination of ligand-binding specificity⁸. Alternative splicing or proteolytic processing of VEGFRs give rise to secreted variants of VEGFR1 (REF. 9) and VEGFR2 (REF. 10), and in humans, to a C-terminal truncated VEGFR3 (REF. 11).

Although the VEGFRs are primarily expressed in the vascular system, more sensitive methodologies combined with improved reagent quality have allowed the detection of VEGFR expression in non-endothelial cells (see **Supplementary information S2** (table)). However, genetic models (TABLE 1) imply that the most important function of VEGF/VEGFRs is in the vascular system.

Regulation of VEGFR activity

The activity of RTKs is regulated by the availability of ligands. A particular feature of the VEGFA ligand is the dramatic upregulation of its expression levels under hypoxic conditions. Hypoxia allows the stabilization of hypoxia-inducible factors (HIFs) that bind to specific promoter elements that are present in the promoter region of VEGFA¹². Similarly, expression of VEGFR1 is directly regulated by HIFs¹³. VEGFR2 is also upregulated during hypoxia, but the role of different HIFs in this regulation remains to be clarified. VEGFR3 expression is upregulated in differentiating embryonic stem cells that are cultured in a hypoxic atmosphere¹⁴. However, the contribution of hypoxia to regulation of VEGFR3 expression *in vivo* is still unclear.

Neuropilins
 Transmembrane glycoproteins that have been characterized as receptors for semaphorins in neuronal guidance and as co-receptors for VEGFs in angiogenesis.

Oedema
 Abnormal and excessive accumulation of fluid in the tissue, which might be localized or generalised.

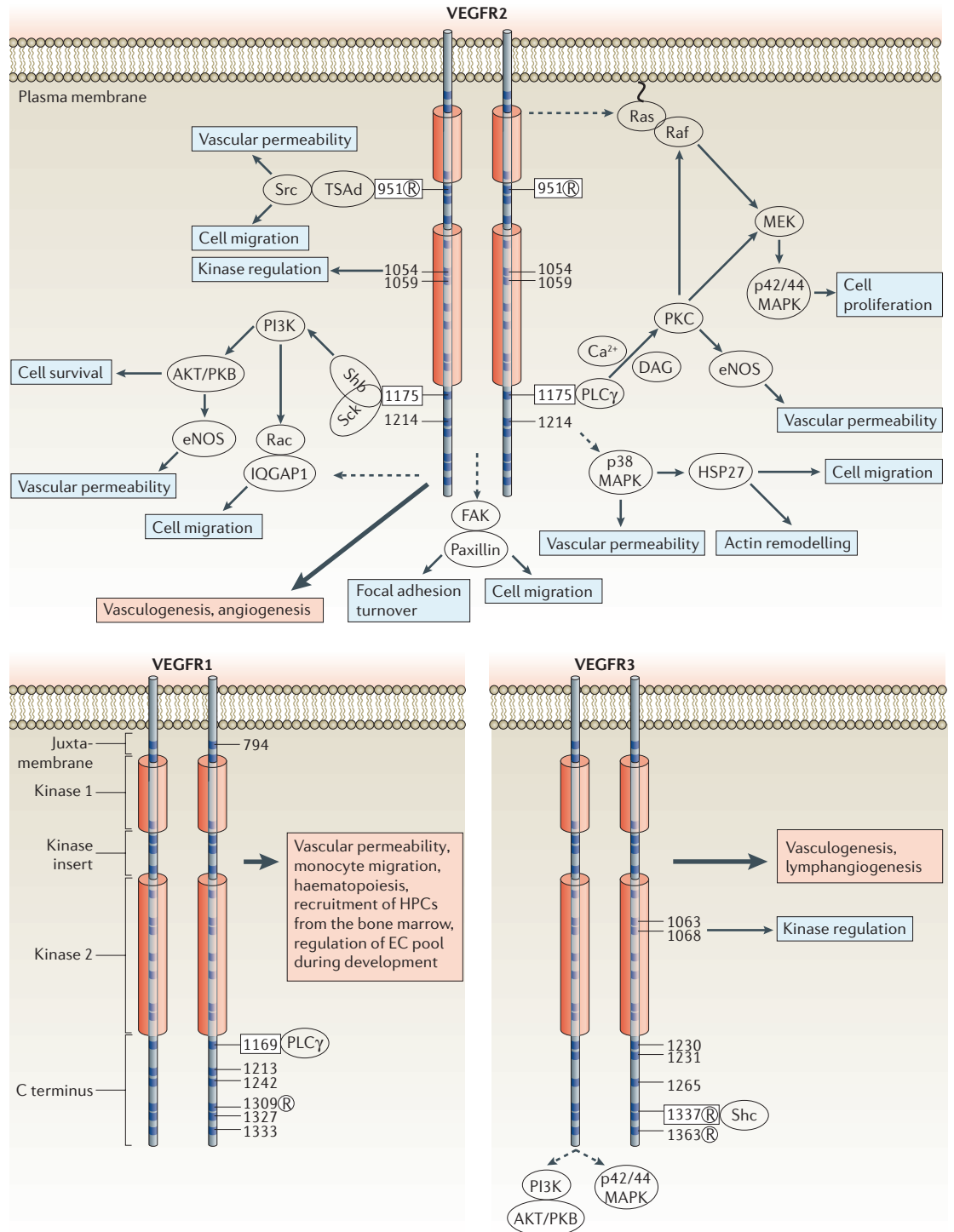


Figure 2 | **VEGFR phosphorylation sites and signal transduction.** Intracellular domains of dimerized and activated vascular endothelial growth-factor receptors (VEGFR1, 2 and 3) are shown with tyrosine-phosphorylation sites that are indicated by numbers. Circled R indicates that use of the phosphorylation site is regulated dependent on the angiogenic state of the endothelial cell (for VEGFR2) or is regulated by a particular ligand (for VEGFR1) or by heterodimerization (for VEGFR3). Dark blue squares in the receptor molecules indicate positions of tyrosine residues. Binding of signalling molecules (dark blue ovals) to certain phosphorylation sites (boxed numbers), initiates signalling cascades (light blue ovals), which leads to the establishment of specific biological responses (pale blue boxes). The mode of initiation of certain signalling chains is unclear (dashed arrows). Final biological outcomes that are coupled to the respective receptors are indicated in pink boxes. DAG, diacylglycerol; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; HPC, haematopoietic progenitor cell; HSP27, heat-shock protein-27; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase; PI3K, phosphatidylinositol 3' kinase; PKC, protein kinase C; PLCγ, phospholipase C-γ; Shb, SH2 and β-cells; TSAd, T-cell-specific adaptor.

Table 1 | VEGF/VEGFR and co-receptor function as assessed by gene targeting

Genotype	Phenotype	Refs
<i>Vegfa</i> ^{+/-}	Embryonic lethal at embryonic day (E)11–12, defective vascular development.	20,21
<i>Vegfa</i> ^{-/-}	Generated by aggregation of embryonic stem cells with tetraploid embryos, more severe defects in vascular development than heterozygote, embryonic lethal E9.5–10.5.	20
<i>Vegfa</i> ^{120/120}	50% die shortly after birth owing to bleeding in multiple organs, and the remaining mice die before postnatal day 14 owing to cardiac failure. Impaired myocardial angiogenesis, ischemic cardiomyopathy, skeletal defects, defects in vascular outgrowth and patterning in the retina.	88,123–125
<i>Vegfa</i> ^{164/164}	Viable, healthy.	88
<i>Vegfa</i> ^{188/188}	Impaired retinal arterial development, dwarfism, defective epiphysal vascularization, impaired development of growth plates and secondary ossification centres, knee-joint dysplasia.	88,126
<i>Vegfb</i> ^{-/-}	Reduced heart size, dysfunctional coronary vasculature, impaired recovery from cardiac ischemia.	127,128
VEGFC overexpression from the human keratin-14 (K14) promoter	Hyperplasia of lymphatic vessels.	129
<i>Vegfc</i> ^{-/-}	Prenatal death owing to oedema, lack of lymphatic vessels.	38
<i>Vegfc</i> ^{+/-}	Cutaneous lymphatic hypoplasia, lymphoedema.	38
<i>Vegfd</i> ^{-/-}	Normal development, slight reduction of lymphatic vessels adjacent to lung bronchiole.	130
<i>Plgf</i> ^{-/-}	Impaired angiogenesis during ischemia, inflammation, wound healing and cancer.	55
<i>Vegfr1</i> ^{-/-}	Embryonic lethal E8.5–9.0, increased hemangioblast commitment, vascular disorganization owing to endothelial-cell overgrowth.	23,131
<i>Vegfr1</i> (TK) ^{-/-}	Normal development, VEGF-induced macrophage migration suppressed, decreased tumour angiogenesis.	49,132
<i>Vegfr1</i> (TM-TK) ^{-/-}	50% of mice die during embryonic development, owing to vascular defects.	133
<i>Vegfr2</i> ^{-/-}	Embryonic lethal E8.5–9.5, defective blood-island formation and vasculogenesis.	22
<i>Vegfr3</i> ^{-/-}	Embryonic lethal before formation of lymphatics owing to cardiovascular failure. Embryos show vascular remodelling defects and pericardial fluid accumulation.	39
VEGFR3-Ig overexpression from K14 promoter	Inhibition of foetal lymphangiogenesis, regression of lymphatic vessels, lymphoedema.	134
<i>Neuropilin-1</i> ^{-/-}	Embryonic lethal, defective neural patterning, vascular regression.	135,136
Neuropilin-1 overexpression	Cardiovascular defects, heart malformation, excess blood-vessel formation, dilated blood vessels, haemorrhage, anomalies in nervous system and limbs.	137
<i>Neuropilin-2</i> ^{-/-}	40% show perinatal death close to birth. Survivors are smaller than littermates. Defects in neuronal patterning, severe reduction of small lymphatic vessels and capillaries.	86,138
<i>Neuropilin-1</i> ^{-/-} , <i>Neuropilin-2</i> ^{-/-}	Embryonic lethal E8.5, defective vascular development.	139

Ig, immunoglobulin; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

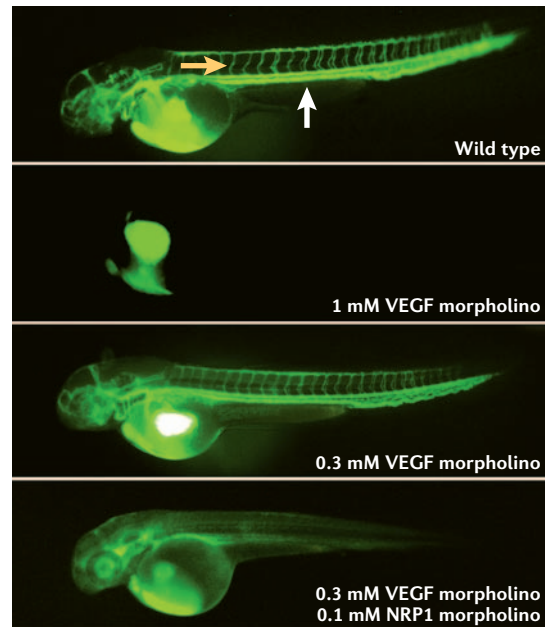
Guided by the binding properties of the ligands, the VEGFRs are able to form both homodimers and heterodimers¹⁵ (FIG. 1a). The signal-transduction properties of the VEGFR heterodimers compared with homodimers remain to be elucidated. Dimerization of receptors is accompanied by activation of the receptor-kinase activity that leads to the autophosphorylation of the receptors. Phosphorylated receptors recruit interacting proteins and induce the activation of signalling pathways that involve an array of second messengers, as described below.

Although positive regulation of VEGFR2 activity is achieved through interaction with the trimeric G proteins G α q/G α 11 (REF. 16), the molecular details of this regulation have not yet been determined. Negative regulation of RTKs is important for limiting the response of the target cells; for example, induction of the kinase activity is known to be counteracted by rapid dephosphorylation of the receptors by tyrosine-specific phosphatases. It has been shown that the activation of VEGFR2 is negatively regulated by the phosphotyrosine phosphatases Src-homology phosphatase-1 (SHP1) and

Box 1 | VEGFR studies in model organisms

Genetic models such as zebrafish and *Drosophila melanogaster* have been useful in delineating the pathways that regulate angiogenesis and tubular morphogenesis, respectively. Moreover, *Xenopus laevis* tadpoles have recently emerged as an interesting new model for developmental angiogenesis and lymphangiogenesis¹⁰². *D. melanogaster* lacks a proper vascular system, but the molecular mechanisms that regulate the formation of the tracheal tube seem to be similar to those that are involved in shaping the vascular tube in mammals. One common receptor for platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), known as PVR (PDGF/VEGF receptor), has been identified in *D. melanogaster*. The PVR cooperates with the *D. melanogaster* epidermal growth-factor receptor (EGFR) in guiding cell migration, in a manner that is dependent on receptor endocytosis¹⁰³. Analysis of *Pvr* mutants showed a role for PVR in the survival of blood cells in the *D. melanogaster* embryo, which implies similarities to the haematopoietic system of the vertebrates¹⁰⁴.

In zebrafish, formation of intersegmental vessels (yellow arrow in the figure) is considered to represent capillary sprouting during mammalian development, whereas the axial vessels (white arrow in the figure) correspond to arteries and veins. Disruption of VEGFR2 signalling impairs intersegmental sprouting angiogenesis¹⁰⁵. Furthermore, gene targeting has shown convergence between the Hedgehog, VEGF and Notch signalling pathways in blood-vessel formation¹⁰⁶ and the involvement of phospholipase C- γ (PLC γ) downstream of VEGF in arterial development¹⁰⁷. Inactivation of neuropilin-1 (zNRP1) results in vascular defects, similar to those that are induced by an inhibitor of VEGFR2-kinase activity¹⁰⁸. Co-injection of zNRP1 and VEGF morpholinos, at concentrations that individually allow blood-vessel development, results in defects in both intersegmental and axial vessels¹⁰⁸ (see figure, vessels in green were visualized by fluorescein isothiocyanate–dextran). *Etsrp*, an ETS-domain transcription factor, is specifically expressed in zebrafish vascular-endothelial-cell precursors¹⁰⁹. Inactivation of *etsrp* results in the loss of functional blood vessels, whereas overexpression induces ectopic expression of vascular endothelial markers in many cell types. *etsrp* gene function is also required for VEGF and stem-cell leukemia (*scl/tal1*) function, which establishes a fundamental role of *Etsrp* in vasculogenesis¹⁰⁹. The figure is reproduced with permission from REF. 108 © (2002) The National Academy of Sciences.



SHP2 (REFS 17,18). RTK activity is also downregulated by rapid degradation through the proteasome pathway, as well as through internalization of the receptor and degradation in the lysosomes. Rapid and controlled endocytosis and inactivation of VEGFR2 is probably important for accurate and localized responses to VEGF gradients, as indicated from studies in *Drosophila melanogaster* (BOX 1). Internalization and degradation of VEGFR2 requires protein kinase C (PKC)-dependent phosphorylation of the receptor C-terminal tail¹⁹. However, detailed analyses of the fate of activated VEGFRs have not yet been performed, and the role of proteasome-mediated degradation remains to be determined.

Embryogenesis and stem-cell recruitment

VEGFA was originally discovered as an efficient and rapid inducer of vascular permeability (therefore, known as a vascular permeability factor (VPF) (BOX 2). Knockout studies subsequently showed the importance of several of the VEGF family members, as well as the VEGFRs, in cardiovascular, haematopoietic and lymphatic development.

Cardiovascular development. Inactivation of a single *Vegfa* allele in mice results in early lethality at embryonic day (E)11–12 owing to deficient endothelial-cell development and lack of vessels^{20,21} (TABLE 1). *Vegfr2*^{-/-} animals show a similar phenotype, but they die at E8.5 (REF. 22). By contrast, depletion of VEGFR1 expression allows endothelial-cell development, but embryos die at E8.5–9.0 because of excessive endothelial proliferation and obstruction of the vessel lumen^{23,24}. The expansion of the endothelial-cell pool in *Vegfr1*^{-/-} animals is probably a consequence of increased accessibility of VEGFA for binding and activation of VEGFR2. On the other hand, elevated production of the soluble VEGFR1 variant (sFlt1) in the placenta and consequent immobilization of VEGFA and decreased VEGFR2 signalling is implicated in preeclampsia²⁵.

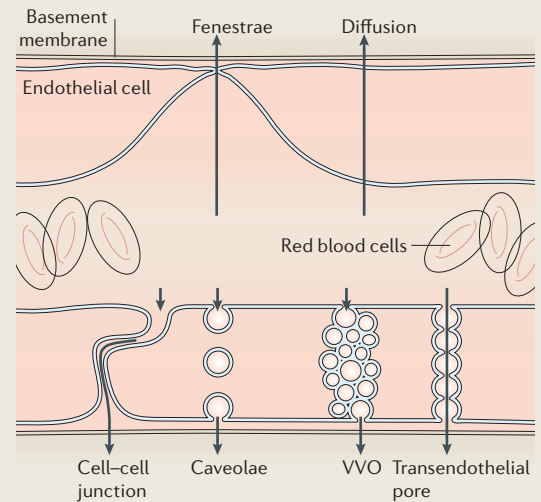
Moderate overexpression of VEGFA from its endogenous locus results in aberrant heart development and lethality at E12.5–14, which shows the essential role of regulated VEGFA signalling during development²⁶. Balanced VEGF function is also important in post-natal development. Inducible deletion of VEGFA in neonatal mice results in stunted growth and increased apoptosis

Preeclampsia

A pregnancy disorder that is characterized by hypertension and proteinuria occurring after the 20th week of gestation.

Box 2 | Regulation of vascular permeability

Vascular endothelial growth factor-A (VEGFA) was originally discovered as a vascular-permeability factor (VPF). It induces permeability with unusually rapid kinetics and 50,000 times more potency than histamine¹¹⁰ (reviewed in REF. 111). Vascular permeability is a prerequisite for physiological processes such as wound healing, but might also aggravate pathologies such as cancer by promoting the formation of oedema and ascites, and facilitating the distant spread of metastases. VEGFA-induced vessel fenestrations¹¹² allow leakage of small solutes, but larger molecules are still retained. Passage of small proteins has instead been attributed to VEGF-induced formation of caveolae, the assembly of caveolae into vesiculovacuolar organelles (VVOs), and/or the induction of trans-endothelial pores^{113,114} (see figure). Leakage of larger proteins and extravasation of red blood cells depend on vascular endothelial (VE)-cadherin-mediated loosening of adherens junctions, which allow passage between endothelial cells¹¹⁵. Several signal transducers that are downstream of the VEGFRs (FIG. 2)



have been implicated in the regulation of the permeability process (reviewed in REF. 115). VEGF-induced permeability depends on nitric oxide (NO) production, which requires activation of endothelial NO synthase (eNOS), either as a consequence of phospholipase C- γ activation and calcium influx, or through phosphorylation of eNOS by AKT/protein kinase B (PKB)^{111,116}. In support of this finding, targeted deletion of eNOS abrogates VEGF-induced permeability¹¹⁷. Furthermore, VEGFR2-induced activation of Src and Yes, but not the related kinase Fyn, is important for vascular permeability in different pathologies, notably tumours^{115,118}. Interestingly, VEGFE, which activates VEGFR2 but not VEGFR1, induces angiogenesis without signs of oedema and haemorrhaging¹¹⁹. This finding shows that signalling through VEGFR2 alone does not induce permeability. Rather, cross-communication between VEGFR1 and 2 (for example, through heterodimerization) might be required.

of endothelial cells²⁷. Even a short-term, pharmacological block of the interaction between VEGFA and VEGFR2 in adult mice leads to vessel regression²⁸.

Haematopoietic cell development. It has been proposed that the development of haematopoietic and endothelial cells are closely linked, possibly through a common early progenitor that expresses VEGFR2. In agreement with this notion, there is ample evidence that VEGFs are important for the regulation of haematopoietic cell development, survival, differentiation and migration^{29,30}. Indeed, it has been shown that survival of haematopoietic stem cells is promoted by VEGFA³¹. Moreover, the migration of haematopoietic progenitor cells (HPCs), as well as monocytes, towards a gradient of VEGF is mediated through VEGFR1 (REF. 1). The physiological relevance of this finding has been shown by treatment of mice with VEGFR1 neutralizing antibodies, which leads to suppression of inflammatory reactions owing to reduced migration of HPCs and VEGFR1-expressing leukocytes³². The important role of VEGFR1 in haematopoietic-cell function is furthermore underscored by the fact that PLGF-stimulated VEGFR1-positive HPCs can rescue haematopoiesis after bone-marrow destruction³³. The VEGFR1-dependent regulated migration of HPCs has also been implicated in the establishment of tumour metastases, as HPCs home to tumour-specific pre-metastatic sites³⁴. On the other hand, recruitment of circulating endothelial precursors (CEPs) to sites of active angiogenesis is mediated through VEGFR2 (reviewed in REF. 35).

Lymphatic development. Lymphatic development requires the activation of the homeobox transcription factor PROX1 (REF. 36), which in turn regulates the expression of VEGFR3 (REF. 37), a receptor for VEGFC and D (FIG. 1a). Lymphatic endothelial cells (LECs) that express VEGFR3 bud off and migrate away from the embryonic cardinal vein at E10.5 in response to a gradient of VEGFC, which is produced by nearby mesenchymal cells³⁸. The migrating LECs subsequently assemble into lymph sacs, which extend through sprouting, to lay down the framework of the lymphatic system. *Vegfc*^{-/-} embryos lack lymphatic vessels and die prenatally because of severe tissue oedema³⁸, whereas *Vegfr3*^{-/-} mice die from defective vascular remodelling before the establishment of the lymphatics³⁹. These findings indicate that, during early embryogenesis, VEGFR3 is essential for blood vascular development. Similarly, VEGFR2 has been implicated in lymphangiogenesis. Whether this effect is mediated through binding of the proteolytically processed forms of VEGFC and D to VEGFR2 remains to be determined. Also, VEGFA has been shown to induce lymphangiogenesis, presumably through VEGFR2, as indicated by the effect of VEGFA-encoding adenovirus delivery into the mouse ear⁴⁰.

The detailed roles of VEGF/VEGFRs in vascular developmental processes and in context-guided formation of tubular structures have been analysed extensively in different model organisms, including zebrafish, *D. melanogaster* and *Xenopus laevis* tadpoles (BOX 1).

Ascites
Accumulation of serous fluid in the peritoneal cavity.

Caveolae
Specialized micro-investigations of the plasma membrane.

Vesiculovacuolar organelle (VVO). Transendothelial channel created by fusion of vesicles, for example, in response to VEGF.

Cardinal vein
An important drainage vessel for deoxygenated blood in the embryo.

Mesenchymal cell
Embryonic connective (supporting)-tissue cell.

VEGFR1 — a complex regulator

The VEGFR1 tyrosine kinase exhibits all the conserved motifs that are required for kinase activity. However, the level of phosphorylation of VEGFR1 in response to VEGFA is low^{41,42} and can be readily detected only in overexpression models (for example mammalian or insect cell lines that are transfected to stably or transiently overexpress VEGFR1). Analyses of chimeric VEGFR1/VEGFR2 receptors has implicated the juxta-membrane domain of VEGFR1 in repression of its kinase activity⁴³. Repression might be exerted through folding of the intracellular domain of VEGFR1 in a way that prevents the exposure of regulatory sequences in the kinase domain. Supporting this idea, the tyrosine residue in the kinase domain that has a position that corresponds to the classic positive regulatory tyrosine residue Tyr416 in the Src kinase is not phosphorylated in the activated VEGFR1 (REF. 44). Similarly positioned tyrosine residues in VEGFR2 and 3 have been identified as phosphorylation sites and have been implicated in the activation of the kinase activity (see below) (FIG. 2).

Interacting partners of VEGFR1. Several VEGFR1 tyrosine-phosphorylation sites and their potential interacting partners have been described in different overexpression models. Such proteins include the p85/phosphatidylinositol-3 kinase (PI3K), phospholipase C- γ (PLC γ), SHP2, growth-factor-receptor-bound-2 (Grb2) protein and Nck⁴⁵. However, despite the many potential interactions of VEGFR1 that have been reported, the downstream signalling events remain to be delineated. For example, it is unclear how signal transduction is induced in primary cells, in which VEGFR1 is known to exert a significant biological response, such as in monocytes/macrophages that migrate in response to VEGFA through VEGFR1 (REF. 46).

A potential caveat in the analysis of signalling responses that are downstream of VEGFR1 is that mutation of VEGFR1 to replace potential phosphotyrosine with phenylalanine residues leads to misfolding of the intracellular domain of the receptor and nonspecific kinase inactivation⁴⁷. This reinforces the notion that the folding of the intracellular domain of VEGFR1 is particular (see above) and different from that of other VEGFRs, for which single-point mutations to replace phosphotyrosine residues is compatible with intact kinase activity.

Another interesting but unclear feature of VEGFR1 is that its different ligands (VEGFA, VEGFB and PLGF; FIG. 1a) transduce distinct biological responses. In an attempt to understand the potential mechanisms that underlie these different responses, Autiero *et al.* showed that VEGF and PLGF might induce different phosphorylation-site patterns in VEGFR1 (REF. 48). Tyr1309 is phosphorylated only in response to PLGF, and it results in the activation of AKT/protein kinase B (PKB)⁴⁸.

Dissecting the functions of VEGFR1. The vascular phenotype of the *Vegfr1*^{-/-} embryos (TABLE 1) strongly implicates VEGFR1 in the negative regulation of the

endothelial-cell pool, but it is apparently not required for endothelial-cell development *per se*. Importantly, deletion of the VEGFR1 tyrosine-kinase domain is compatible with normal vascular development, as demonstrated by the phenotype of *Vegfr1*(TK)^{-/-} mice⁴⁹. Therefore, VEGFR1 seems to function as a 'trap' for VEGFA during embryogenesis by regulating its accessibility for VEGFR2 on developing blood vessels. Anchoring of the extracellular domain of VEGFR1 to the cell membrane is important, as 50% of the mice that lack both the tyrosine-kinase domain and the transmembrane domain die at E8.5–9, owing to vascular malformations. Remarkably, the remaining 50% develop normally⁵⁰. Although the tyrosine-kinase activity of VEGFR1 is dispensable for vascular development, it is a prerequisite for recruitment of HPCs in different pathologies (see above)^{32,51}.

The biological outcome of stimulating or inhibiting the activity of VEGFR1 might be influenced through 'crosstalk' with VEGFR2. Several groups have reported that VEGFR1 negatively regulates VEGFR2 signals. VEGFR2-mediated proliferation of endothelial cells can be suppressed by VEGFR1 (REFS 52,53), and this effect is dependent on PI3K⁵². Moreover, embryonic stem cells that lack VEGFR1 show increased levels of VEGFR2 phosphorylation⁵⁴. However, there are also reports, which indicate that VEGFR1 might instead promote VEGFR2 activity, for example, during pathological conditions⁵⁵. Activation of VEGFR1 by PLGF results in increased phosphorylation of VEGFR2 (REF. 56), possibly through displacement of VEGFA that is bound to VEGFR1, thereby making it available for VEGFR2. Alternatively, signals that are induced by VEGFR1 might positively modulate the output of VEGFR2, for example, through attenuation of intracellular negative regulatory pathways that possibly involve phosphotyrosine phosphatases. Whether these apparently opposing effects of VEGFR1 on VEGFR2 activity operate under different conditions or in different types of endothelial cell remains to be investigated.

VEGFR2 signalling and vascular function

The important role of VEGFR2 signalling during development and in neovascularization in physiological or pathological conditions *in vivo* (TABLE 1) has allowed the design of clinically beneficial therapies (BOX 3). The possibility that VEGFR2-blocking therapies could be tailored to attenuate only specific signal-transduction pathways (see Future directions section) to reduce side effects of the treatment has promoted a general interest in dissecting VEGFR2 signalling. In contrast to VEGFR1, autophosphorylation of VEGFR2 after stimulation with VEGFA is readily detected in intact cells, and the positions of several phosphorylated tyrosine residues in the receptor have been mapped (FIG. 2). Of these, phosphorylation of Tyr1054 and Tyr1059 is required for maximal kinase activity⁵⁷.

VEGFR2 activates PLC γ and PI3K. Only a few SH2-domain-containing molecules have been shown to interact directly with VEGFR2. PLC γ binds to phosphorylated Tyr1175 (Tyr1173 in the mouse), and mediates

Neovascularization
Formation of new blood
vessels, often in conjunction
with disease processes.

Box 3 | **Angiogenic therapy**

Several different strategies have been designed to target vascular-endothelial-growth-factor-receptor (VEGFR) signal transduction. Clinically, the most successful so far is the VEGF-neutralizing antibody Bevacizumab/Avastin, which was approved by the United States Food and Drug Administration (FDA) in early 2004. When given in combination with chemotherapy, Bevacizumab prolongs the survival of patients with lung, colon and breast cancer (for an extensive review on the current status of anti-VEGF therapy in clinical trials for cancer see REF. 120). It has been suggested that anti-VEGF treatment transiently 'normalizes' the tumour blood vessels, which results in more efficient delivery of the chemotherapeutic drug¹²¹. Other strategies to target the VEGF/VEGFR pathway include soluble VEGFRs (Traps), receptor tyrosine-kinase inhibitors (RTKI) that target VEGFR2, as well as neutralizing aptamers. The RTKIs generally target several kinases in addition to VEGFR2, possibly directly inhibiting tumour-cell proliferation or survival. The kinase inhibitors Sorafenib (BAY43-9006) and Sunitinib (SU11248) have been tested in large phase III clinical trials for treatment of metastatic kidney cancer and gastrointestinal stromal tumours, respectively, and are showing promising results as monotherapies. The second anti-angiogenic drug that has been approved by the FDA is the anti-VEGF aptamer Pegaptanib/Macugen, which is used in the treatment of age-related macular degeneration (AMD)¹²². Ranibizumab/Lucentis, a small antibody fragment that was designed to bind to all VEGF isoforms, is currently in phase III clinical trials for treatment of AMD. Owing to a significantly better outcome in the treated group compared with the control group, Lucentis has been granted a priority review by the FDA (see table showing anti-VEGF/VEGFR therapies that have been approved or are in phase III clinical trials, Q1, 2006). There is also a clinical interest in promoting angiogenesis in conditions such as stroke and ischemia. Therapeutic angiogenesis through gene transfer of various pro-angiogenic molecules, including VEGFs, is currently under evaluation in clinical trials.

Drug	Mechanism	Application/phase
Bevacizumab* (Avastin)*	Monoclonal anti-VEGF antibody	Increases survival from several different types of cancer when combined with chemotherapy Approved by the FDA
Vatalanib* (PTK787/ZK222584)	VEGFR1, VEGFR2, VEGFR3, PDGFRβ, c-Kit kinase inhibitor	Solid tumours Phase III
AE-941* (Neovastat)	Inhibits binding of VEGF to VEGFR, inhibitor of matrix metalloproteinase-2 (MMP2) and MMP9	Broad spectrum of malignancies Phase III
Sorafenib* (BAY 43-9006)	VEGFR2, PDGFRβ, FLT3, c-Kit multitargeted kinase inhibitor	Renal-cell cancer Phase III
Sunitinib* (SU11248)	VEGFR2, PDGFRβ, FLT3, c-Kit multitargeted kinase inhibitor	Gastro-intestinal stromal tumour Approved by the FDA
Pegaptanib† (Macugen)	VEGF-neutralizing aptamer	Age-related macular degeneration Approved by the FDA
Ranibizumab‡ (Lucentis)	VEGF-neutralizing antibody fragment	AMD Application filed for approval by the FDA

*For further information, see REF. 120. †For information, see Macugen website (see Online links box). ‡For information, see Genentech website (see Online links box). PDGFRβ, platelet-derived growth-factor receptor-β.

the activation of the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase-1/2 (ERK1/2) cascade and proliferation of endothelial cells⁵⁸. PLCγ activates PKC by the generation of diacylglycerol and increased concentrations of intracellular calcium. Mice that express a mutated Tyr1173Phe VEGFR2 die at E8.5–9.5 because of vascular defects that resemble the defects of *Vegfr2*^{-/-} mice⁵⁹. These data show an essential function of the Tyr1173 residue during vascular development.

Besides PLCγ, the adaptor molecule Shb binds to phosphorylated Tyr1175 (REF. 60). VEGF-induced migration and activation of PI3K is inhibited by small interfering RNA (siRNA)-mediated knockdown of Shb in endothelial cells⁶⁰. Indeed, Tyr1173/1175 has been coupled to VEGF-induced PI3K activation previously⁶¹. The serine/threonine kinase AKT/PKB is activated downstream of PI3K and mediates survival of the endothelial cells⁶².

AKT/PKB also regulates nitric oxide (NO) production by direct phosphorylation and activation of endothelial NO synthase (eNOS) (BOX 2). In addition to PLCγ and Shb, phosphorylated Tyr1175 has also been shown to interact with Sck/ShcB⁶³, an adaptor molecule that is dispensable for normal development⁶⁴.

VEGF does not seem to be a powerful mitogen for endothelial cells, and the significance of the classic Ras–Raf–MEK–MAPK pathway downstream of VEGFR2 is unclear. However, VEGF stimulates activation of Ras in human umbilical-vein endothelial cells (HUVECs)^{65,66} and Ras activation has been coupled to an angiogenic phenotype of endothelial cells⁶⁶. Ras-independent induction of the Raf–MEK–MAPK pathway after VEGF stimulation, through PLCγ-activated PKC, has been shown in primary liver sinusoidal endothelial cells⁶⁷. Conflicting results exist in the literature with respect to the interaction of VEGFR2 with ShcA or Grb2, which

Age-related macular degeneration

(AMD). Degeneration of the cells of the macula (part of the retina) in the eye and neovascularization in the choroids, which results in blurred vision and can cause blindness. Leading cause of blindness among the elderly in the western world.

recruit the Ras-activating nucleotide-exchange factor son of sevenless to the receptor^{63,68}.

Factors involved in cell migration. Another important phosphorylation site in VEGFR2 is Tyr951 (Tyr949 in the mouse), which is a binding site for the signalling adaptor TSAd (T-cell-specific adaptor; also known as VEGF receptor-associated protein (VRAP))⁶⁹. The phosphorylated Tyr951–TSAd pathway has been shown to regulate endothelial-cell migration^{69,70}. Reduced vascularization and growth of tumours in *Tsad*^{-/-} mice showed that this is an important pathway for endothelial cells that are engaged in active angiogenesis⁶⁹. VEGFA induces the formation of a complex between TSAd and Src⁶⁹, which indicates that TSAd might regulate Src activation and vascular permeability downstream of VEGFR2 (BOX 2).

Mice that express mutant Tyr1212Phe (corresponding to the human Tyr1214) VEGFR2 are viable and fertile⁵⁹. Phosphorylation of Tyr1212/1214 has been implicated in VEGF-induced actin remodelling through the sequential activation of CDC42 and p38 MAPK⁷¹. Inhibition of the p38 MAPK augments VEGF-induced angiogenesis in the chicken chorioallantoic membrane (CAM)^{72,73}, without an accompanying increase in vascular permeability⁷². Moreover, p38 MAPK induces phosphorylation of the heat-shock protein-27 (HSP27), a molecular chaperone that positively regulates VEGF-induced actin reorganization and migration^{74,75}.

Other signalling molecules that have been implicated in VEGF-induced migration through VEGFR2 include the focal-adhesion kinase (FAK) and its substrate paxillin^{76,77}, which are involved in focal-adhesion turnover during cellular migration. Endothelial-cell motility seems also to be regulated through a newly identified binding partner of phosphorylated VEGFR2, IQGAP1, which binds to and activates Rac1 by inhibiting its intrinsic GTPase-activity⁷⁸. IQGAP1 co-localizes with phosphorylated VEGFR2 at the leading edge of migrating endothelial cells and IQGAP1 knockdown by siRNA prevents VEGF-induced migration⁷⁹.

VEGFR3 signalling and lymphatic function

Analyses of a spectrum of genetic models have strongly implicated VEGFR3 in the establishment and maintenance of the lymphatics (TABLE 1). Interestingly, VEGFR3 is the only VEGFR for which naturally occurring mutations have been described. Therefore, dysfunction of lymphatic vessels might be caused by congenital inactivating mutations in VEGFR3 (REF. 80).

The kinase activity of VEGFR3 is probably regulated by phosphorylation of conserved residues in the kinase domain (Tyr1063 and Tyr1068). Phosphorylation of the Tyr1337 is required for association of the Shc–Grb2 complex to VEGFR3 (REF. 81), whereas the role of other phosphorylation sites (Tyr1230, Tyr1231, Tyr1265, Tyr1337 and Tyr1363) (FIG. 2) in signal transduction downstream of VEGFR3 remains to be identified. VEGFR3 forms homodimers or heterodimers with VEGFR2 in response to processed VEGFC¹⁵. These heterodimeric receptors might form *in vivo* both in lymphatic endothelial cells and in certain endothelial cells, such as in fenestrated capillaries,

which express both receptor types⁸². Importantly, the dimerization partner directs the use of potential phosphorylation sites, which is a reflection of the different substrate specificities of kinases. Therefore, in the heterodimer, VEGFR3 is not phosphorylated at the two C-terminal tyrosine residues Tyr1337, which is the Shc-binding site, and Tyr1363 (REF. 15) (FIG. 2).

Mäkinen and co-workers reported that VEGFR3 mediates activation of the ERK1/2 in a PKC-dependent manner, as well as activation of the PI3K–AKT/PKB pathway⁸³. These pathways might be important during embryonic development, when VEGFC guides migration and sprouting of lymphendothelial precursor cells from restricted regions of the cardinal vein³⁸. Other signal transducers that are potentially used by VEGFR3 include PLC γ , SHP2 (REF. 84) and the transcription factors STAT3 (signal transducer and activator of transcription-3) and STAT5 (REF. 85).

Signal transduction by VEGFR3 is also modulated by co-receptors such as neuropilin-2. The crucial role of this interaction has been shown by the phenotype of *Neuropilin-2*^{-/-} mice, which fail to form normal lymphatic vessels and capillaries⁸⁶ (TABLE 1).

Modulation of VEGFR signalling

Some co-receptors for the VEGFs modulate VEGFR signal transduction by affecting the distribution of the growth factor in the tissue, as well as the composition and stability of the signalling complex on the target cell. The human *VEGFA* gene consists of nine alternatively spliced exons⁵ that regulate interactions with HSPGs and neuropilins. The shortest isoform, VEGFA121 (VEGFA120 in the mouse), lacks the HSPG- and neuropilin-binding domains (see **Supplementary information S1** (figure)). The consequent reduced retention of VEGFA121 on the cell surface and/or in the extracellular matrix (ECM) renders this isoform highly diffusible. VEGFA189, on the other hand, includes HSPG- and neuropilin-binding domains and is therefore retained in the vicinity of the producer cell. However, protease cleavage of matrix-bound VEGFA189 allows the release of an active, freely diffusible 110-amino-acid fragment⁶. VEGFA165 (which lacks exons 6a and 6b) and VEGFA145 (which lacks exon 6b and 7; see **Supplementary information S1** (figure)) show an intermediate degree of retention. Most cells produce several VEGFA isoforms simultaneously, with 121 and 165 being the predominant isoforms.

The early finding that binding of ¹²⁵I-VEGFA165 to VEGFR2 is enhanced by heparin (commonly used as a substitute for heparan sulphate (HS) in experimental model systems) has been confirmed by recent studies, which show that heparin amplifies signalling by VEGFA165, but not VEGFA121, through VEGFR2 (REF. 87). The role of different VEGF isoforms in retinal vascular development has been studied in mice that selectively express single VEGF isoforms. Interestingly, VEGFA164/164 mice show normal vascular development, whereas VEGFA120/120 mice exhibit severe defects in vascular outgrowth and patterning⁸⁸. Tip cells of sprouting blood vessels have been shown to migrate in response to VEGFA164 gradients, and these

Fenestrated capillaries

Small, permeable blood vessels with circular pores, covered with a thin diaphragm.

Tip cell

The endothelial cell that heads a blood-vessel sprout during angiogenesis.

gradients are shaped by interactions with HSPGs⁸⁹. Biochemical analyses strongly indicated that heparin/HS, through simultaneous binding to growth factors and receptors, increase the half life of the receptor complex by several orders of magnitude⁹⁰. Furthermore, presentation of VEGFA165 to VEGFR2 in *trans*, by HSPGs that are expressed on adjacent pericytes, allows a marked increase in signal amplitude and duration⁹¹. Taken together, these data show that HSPGs affect the localization, duration and, thereby, quality of VEGFR signalling.

Neuropilin-1, a cell-surface glycoprotein that lacks intrinsic catalytic activity and which was initially identified as a receptor for the collapsin/semaphorin family of axonal guiding proteins, binds to VEGFA165, but not to VEGFA121 (REF. 92). Neuropilin-1 is expressed in arteries, whereas the related neuropilin-2 is expressed in venous and lymphatic vessels⁸⁶. However, both neuropilins are expressed in several other cell types, including neuronal cells (see [Supplementary information S2](#) (table)). VEGFA165, as well as other HS-binding protein ligands, might compete with semaphorin 3A for an overlapping binding site on neuropilin-1 (REF. 93). Expression of neuropilin-1 in tumour cells results in enlarged tumours with substantially enhanced tumour angiogenesis⁹⁴, which indicates that neuropilin stabilizes the VEGF/VEGFR signalling complex when expressed on adjacent cells (that is, in *trans*).

Modulation by adhesion molecules. Apart from the molecular interactions that are discussed above, VEGF receptors form complexes with a spectrum of molecules that might or might not function as co-receptors in VEGF signalling. Blood flow and shear stress influence vascular development and remodelling, at least in part through the creation of a mechanosensory complex that involves VEGFR2, platelet-endothelial-cell adhesion molecule-1 (PECAM1) and vascular endothelial (VE)-cadherin, which functions upstream of integrin activation⁹⁵ (FIG. 1c). VE-cadherin, which is located in endothelial-cell adherens junctions, forms a complex with VEGFR2 and the cytoplasmic tyrosine kinases Src and Yes *in vitro* and *in vivo*, which regulates vascular permeability by loosening the junctions⁹⁶ (BOX 2).

Many members of the integrin family of ECM receptors are involved directly or indirectly in regulating endothelial-cell function⁹⁷. In endothelial-cell migration, convergence between growth factors and integrin signalling is essential, for example, in the regulation of focal-adhesion turnover. VEGFs, as well as FGFs, might activate integrins through the induction of the PI3K-AKT/PKB pathway⁹⁷. Integrins might also affect the activity state of RTKs. Seeding of endothelial cells on vitronectin, the ligand for the $\alpha v\beta 3$ integrin, leads to enhanced VEGFR2 activation and signal transduction through the AKT/PKB pathway⁹⁸. On the other hand, knockout of the $\beta 3$ chain in mice leads to elevated expression levels of VEGFR2 (REF. 99). Taken together, these findings highlight the importance of a balanced interplay between angiogenic-growth-factor- and integrin-mediated signal transduction.

Future directions

The challenge in studying the signal-transduction properties of the VEGF/VEGFR family is in providing a proper biological context. Both the basement membrane and the density of supporting perivascular cells fundamentally affect endothelial-cell morphology and function, and therefore probably VEGF/VEGFR biology. This is indicated by the phenotype of vessels in diseases such as diabetes (thickened basement membrane, for example, in the kidney glomeruli) and cancer (reduced pericyte coating). Blood flow is another important aspect of endothelial-cell function *in vivo*, which generally is lacking in cell models that are employed in VEGFR signal-transduction analyses. Blood flow might activate VEGFRs in a ligand-independent manner (by promoting the activation of mechanosensory complexes) (FIG. 1), which implies its crucial influence. Further complexity is provided by the VEGF co-receptors, such as HSPGs and neuropilins, which might be expressed at different densities or, in the case of HS, be differently sulphated. These variables have direct consequences for the strength, and therefore the durability, of the interaction between co-receptors and the VEGFR signalling complex.

An important question is whether binding of VEGF to co-receptors such as neuropilins initiates signal transduction independently of the VEGFRs. Neuropilins lack intrinsic catalytic activity, but might associate with the plexin family of transmembrane proteins, resulting in signal transduction through the activation of small G proteins and regulation of the cytoskeleton¹⁰⁰. Semaphorins bind to neuropilins, resulting in plexin-dependent signalling. Whether binding of VEGF to neuropilins leads to engagement of plexins in a similar manner is currently unknown. Several reports in the literature have shown neurotrophic and neuroprotective effects of VEGF, and it remains to be determined whether these effects are dependent on VEGFR2 or, as suggested by Cheng *et al.*¹⁰¹ and others, transduced through the binding of VEGFs to neuropilins.

An emerging pattern in many biological responses to VEGF is the contribution by more than one type of VEGF receptor, which shows the crucial role of balanced signalling. This is particularly true for vascular permeability, which is a complicating aspect of increased production of VEGF, for example, in malignancies. Vascular permeability is most probably mediated by several mechanisms, which involve loosening of cell-cell junctions and mechanisms for transendothelial passage that might differentially engage the VEGFRs or require coordinated VEGFR signalling. Therefore, to be relevant for the *in vivo* situation, VEGFR signal-transduction models have to provide a context for potential communication between different VEGF receptors at the plasma membrane (through heterodimerization) or intracellularly (through induction of positively or negatively regulating signal-transduction pathways).

Considering all of the complicating aspects of VEGFR biology that are described above, one might wish to declare traditional biochemical approaches to VEGFR biology as a wasted effort. However, there is undeniable power in, for example, knowledge about

Pericytes

Cells that surround small blood vessels, particularly numerous around post-capillary venules.

Collapsin/semaphorin

Transmembrane or secreted molecules that guide the path of growing nerve axons.

phosphorylation-site use, especially when complemented with *in vivo* studies (exemplified by the work of Sakurai *et al.*⁵⁹). It is remarkable that use of phosphorylation sites for all three VEGFRs is dynamic, as described above, and might be regulated by the type of ligand (in the case of VEGFR1 (REF. 56)), by the activity state of the cell (VEGFR2 (REF. 69)) and by heterodimerization (VEGFR3 (REF. 15)). There has been an impressive development in the recent years of clinical therapies that are aimed to enhance or suppress VEGF/

VEGFR function. That even relatively short-term suppression of VEGFR function might lead to adverse effects on normal blood vessels is obvious from studies in mouse models. It is a very interesting perspective that future fine tuning of VEGF-based therapies would incorporate insights into the basic mechanism of action of the VEGFRs and focus on the elimination or the enhancement of relevant phosphorylation sites or signalling pathways that are crucial in particular aspects of endothelial-cell biology.

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Competing interests statement

The authors declare no competing financial interests.

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