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Review article

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Biology and therapeutic targeting of vascular endothelial growth factor A

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Abstract

The formation of new blood vessels, called angiogenesis, is an essential pathophysiological process in which several families of regulators have been implicated. Among these, vascular endothelial growth factor A (VEGFA; also known as VEGF) and its two tyrosine kinase receptors, VEGFR1 and VEGFR2, represent a key signalling pathway mediating physiological angiogenesis and are also major therapeutic targets. VEGFA is a member of the gene family that includes VEGFB, VEGFC, VEGFD and placental growth factor (PLGF). Three decades after its initial isolation and cloning, VEGFA is arguably the most extensively investigated signalling system in angiogenesis. Although many mediators of angiogenesis have been identified, including members of the FGF family, angiopoietins, TGFβ and sphingosine 1-phosphate, all current FDA-approved anti-angiogenic drugs target the VEGF pathway. Anti-VEGF agents are widely used in oncology and, in combination with chemotherapy or immunotherapy, are now the standard of care in multiple malignancies. Anti-VEGF drugs have also revolutionized the treatment of neovascular eye disorders such as age-related macular degeneration and ischaemic retinal disorders. In this Review, we emphasize the molecular, structural and cellular basis of VEGFA action as well as recent findings illustrating unexpected interactions with other pathways and provocative reports on the role of VEGFA in regenerative medicine. We also discuss clinical and translational aspects of VEGFA. Given the crucial role that VEGFA plays in regulating angiogenesis in health and disease, this molecule is largely the focus of this Review.

Sections

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Introduction

Angiogenesis, the growth of blood vessels from the pre-existing vasculature, is a crucial process for embryonic and postnatal development, tissue repair, and reproductive functions, and is implicated in major disease processes, including cancer and intraocular vascular disorders[1]. Angiogenesis involves the proliferation and migration of endothelial cells (ECs), which line blood vessels. EC proliferation can occur through sprouting or intussusceptive mechanisms[2]. During sprouting angiogenesis, ECs specify into proliferating stalk cells and migrating tip cells that guide the nascent sprout (Fig. 1). Sprouting angiogenesis often precedes intussusceptive angiogenesis (also known as splitting angiogenesis), which involves the formation of a central pillar within a capillary that eventually splits into two parallel capillaries[2,3].

ECs derive from a common mesodermal precursor. Yet, single-cell transcriptomic analyses revealed large heterogeneity among ECs consistent with their organ-specific properties and functions such as the regulation of haemostasis, vascular tone, inflammation and angiogenesis[4–7]. Vasculogenesis, the de novo formation of a vascular network from endothelial precursors, takes place primarily during embryonic life[8]. Numerous studies have established the role of multiple signalling pathways in the development of the vasculature, including platelet-derived growth factor B (PDGFB), angiopoietins (ANG1–4), ephrins (EPH), NOTCH and its ligand Delta-like protein 4 (DLL4)[9,10]. However, to date, the family of vascular endothelial growth factors (VEGFs) and their receptor tyrosine kinases (RTKs) are arguably the best-characterized pathways not only during developmental angiogenesis but also in pathological angiogenesis. Moreover, VEGFA has been implicated in the regulation of vascular permeability, a key property of blood vessels in health and disease[10–13] (Fig. 1), as described below.

In this Review, we discuss the biochemical characteristics of VEGFA, the main member of the VEGF family, and its biological roles; however, other members are discussed when appropriate. We provide a description of the VEGF receptors, highlight current therapies targeting this family in cancer and intraocular disorders, and outline possible future directions.

VEGFA and its isoforms

VEGFA (or VEGF hereafter) is the prototypic member of a family that also includes placental growth factor (PLGF, also known as PGF)[14], VEGFB[15], VEGFC[16] and VEGFD[17]. In addition, VEGF family homologs have been described in the genome of the parapoxvirus *Orf* virus – termed VEGFE – and have been implicated in the angiogenesis and inflammation of skin lesions in animals infected with the virus[18,19] (Fig. 2a). A comprehensive phylogenetic analysis of members of the VEGF family has been recently published[20]. The biological and biochemical characteristics of VEGF as well as the regulation of *VEGFA* gene expression is discussed in the next sections.

Native VEGF was isolated based on EC mitogenic activity (Supplementary Box 1). VEGF showed a molecular mass of 45-kDa by SDS-PAGE under non-reducing conditions and 23-kDa under reducing conditions, consistent with a homodimer[21]. Subsequent cDNA cloning revealed that the main VEGF species is a 165-amino acid glycoprotein with sequence homology to the A and B chains of PDGF[22]. *Vegfa* is first expressed in the anterior portion of mouse embryos, where it directs the migration of cells positive for VEGFR1 and VEGFR2 (ref. 23). Indeed, the majority of normal and abnormal cells can produce VEGF. In the adult, VEGF induces proliferation, sprouting, migration and tube formation of ECs[24], and is a potent survival factor for ECs during physiological and tumour angiogenesis[25]. Additionally, VEGF has been reported to have several neuronal and neurodevelopmental roles[26].

VEGF can exist as one of several isoforms generated through alternative exon splicing of a single gene, comprising 8 exons[27,28]. These include $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ and some less common variants such as $VEGF_{145}$ (ref. 29). Early studies showed that the bioavailability and angiogenic activity of the VEGF isoforms is determined by their differential affinity for heparan sulfate proteoglycans (HSPGs) at the cell surface and in the extracellular matrix (ECM)[30] (Fig. 2b). As such, after secretion, $VEGF_{121}$ may diffuse freely in tissues as it lacks HSPG-binding domains, while approximately half of the secreted $VEGF_{165}$ is diffusible, leaving the other half bound to cell-surface HSPGs through exon 7-encoded basic residues. Given its ability to both diffuse in tissues and bind to the cell surface, $VEGF_{165}$ is the most biologically relevant isoform in normal tissues and in tumours (reviewed in ref. 30). The longer forms, such as $VEGF_{189}$ or $VEGF_{206}$, bind HSPGs through two different heparin-binding domains, encoded respectively by exons 6 and 7 and are almost completely sequestered in the ECM, although they can be mobilized following proteolysis[30–32]. Proteolytic processing of $VEGF_{165}$ gives rise to the biologically active $VEGF_{110}$ fragment[31,33], whereas processing by matrix metalloproteinase 3 (MMP3) results in $VEGF_{113}$ (refs. 34,35). An important role of proteolytic processing has also been reported for other members of the VEGF family such as VEGFC and VEGFD[36]. Functionally, mice expressing only $VEGF_{120}$ (homologue of human $VEGF_{121}$) die soon after birth and those that survive show ischaemic cardiomyopathy and multiorgan failure. Mice expressing only $VEGF_{188}$ (human $VEGF_{189}$) display abnormally thin vessel branches and approximately 50% of them die at birth[37]. Mice expressing only $VEGF_{164}$ (human $VEGF_{165}$) are viable and healthy, which underlines the importance of $VEGF_{165}$ as the principal effector of VEGF action[37].

Although the VEGF isoforms are known for their pro-angiogenic effects, inhibitory isoforms have been described. The first to be reported was $VEGF_{165}$b, which results from differential splicing from the end of exon 7 into the 3′ untranslated region of the mRNA[38]. Subsequently, $VEGF_{121}$b, $VEGF_{183}$b, $VEGF_{189}$b and $VEGF_{206}$b were reported[39]. Although these variants were reported to inhibit $VEGF_{165}$ activity in multiple settings[38–41], it was later demonstrated that $VEGF_{xxx}$b proteins are, in fact, weak agonists rather than antagonists, owing to the inability to bind HSPGs or the VEGFR2 co-receptor neuropilin 1 (ref. 42) (see the section VEGF receptors and signal transduction). In addition, evidence has been presented that $VEGF_{165b}$ and related b transcripts do not occur naturally but may represent cloning artefacts[43]. In 2014, a novel anti-angiogenic VEGF isoform was described, VEGFAx, with a 22-amino acid C-terminal extension arising from reading of the canonical stop codon as a serine[44]. VEGFAx was reported to inhibit EC proliferation and reduce tumour growth in xenografts implanted in nude mice[44]. However, later studies found that, contrary to these reports, VEGFAx stimulates EC proliferation, angiogenesis and vascular permeability, although less effectively than wild-type $VEGF_{165}$, likely due to the inability to interact with neuropilin 1 (ref. 45). To date, there is no evidence that VEGFAx is naturally produced.

Regulation of *VEGFA* gene expression

VEGFA gene expression is controlled at transcriptional and translational levels, and numerous factors are involved in its regulation. One of the best-characterized regulators of VEGF release is hypoxia[46], although several non-hypoxic mechanisms have also been established. The next sections provide insights into hypoxia-dependent and hypoxia-independent mechanisms of VEGF regulation.

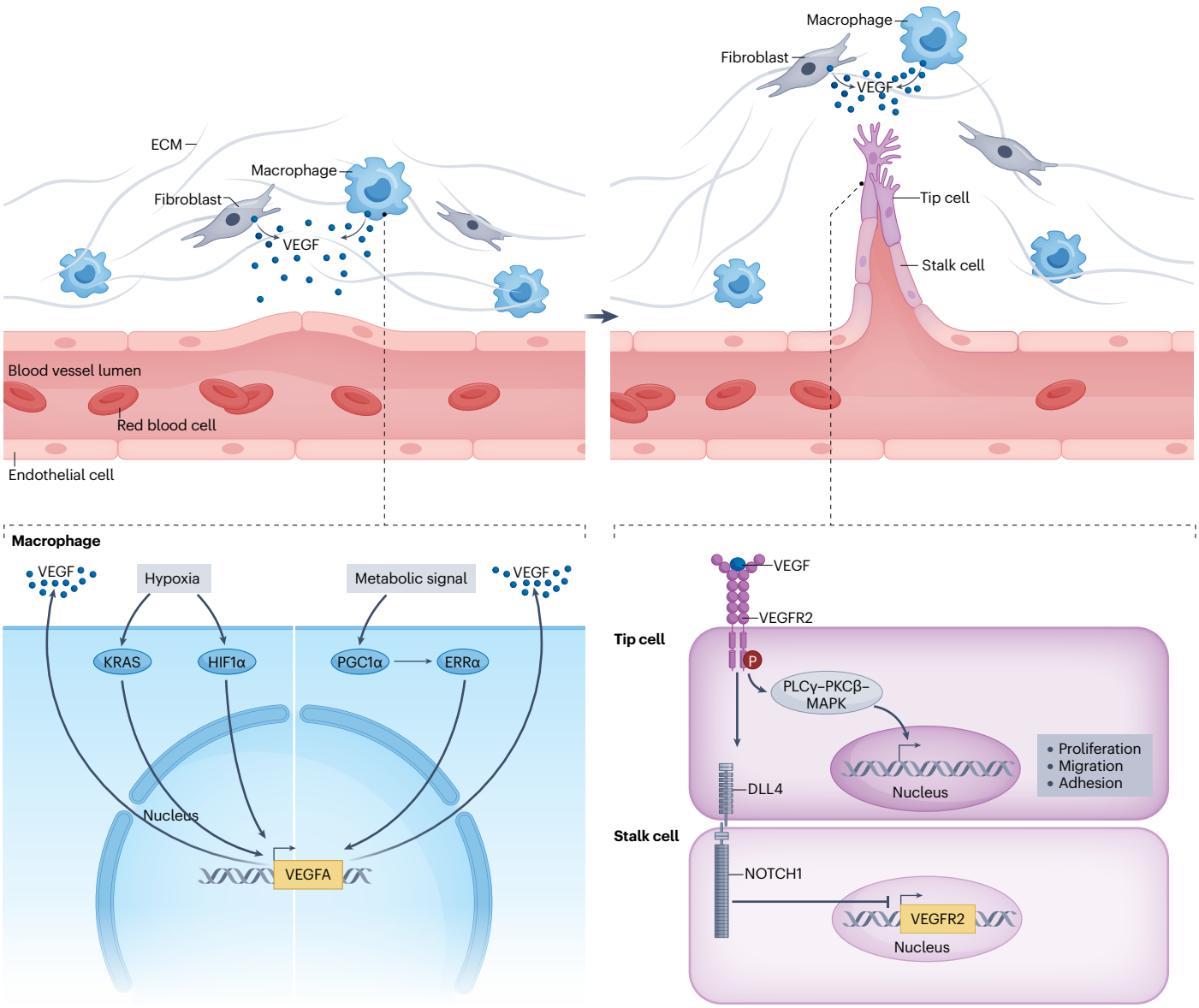

Fig. 1 | Role of VEGF in angiogenesis. During sprouting angiogenesis, cells within the microenvironment, including macrophages and fibroblasts, respond to hypoxia through the translocation of hypoxia-inducible factor 1α (HIF1α) or KRAS to the nucleus, to activate the expression of target genes, including *VEGF*. Metabolic signals can also promote the release of vascular endothelial growth factor (VEGF) in response to peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) through oestrogen-related receptor-α (ERRα). Upon VEGF stimulation, endothelial cells (ECs) specify into tip and stalk cells. Tip cells are a highly motile type of EC that sense the environment for guidance cues and lead the cells of the nascent sprouts through the formation of filopodia. Stalk cells, another type of EC, form the body of the nascent sprout, providing structural support to the nascent capillary. Mechanistically, tip cell formation is dependent on VEGF receptor 2 (VEGFR2) phosphorylation, leading to Delta-like protein 4 (DLL4)-mediated activation of NOTCH1 in adjacent ECs. The activation of NOTCH1 suppresses tip cell behaviour and promotes stalk cell phenotype, which activates several pathways to induce the expression of genes involved in proliferation, migration, adhesion to the extracellular matrix (ECM) and permeability. This activation guides stalk cells into the formation of the nascent sprout, which matures and stabilizes following the recruitment of pericytes and the deposition of ECM. MAPK, mitogen-activated protein kinase; PKCβ, protein kinase Cβ; PLCγ, phospholipase C isoform-γ.

Hypoxia-dependent mechanisms of VEGF regulation

Hypoxic responses in cells and tissues are largely mediated by the family of hypoxia-inducible factor (HIF) transcription factors, which play an integral role in the changes driving cellular adaptation to low oxygen availability[47]. Upon oxygen deprivation, the HIF pathway can activate many target genes, including *VEGFA*[48]. This effect is mediated by the

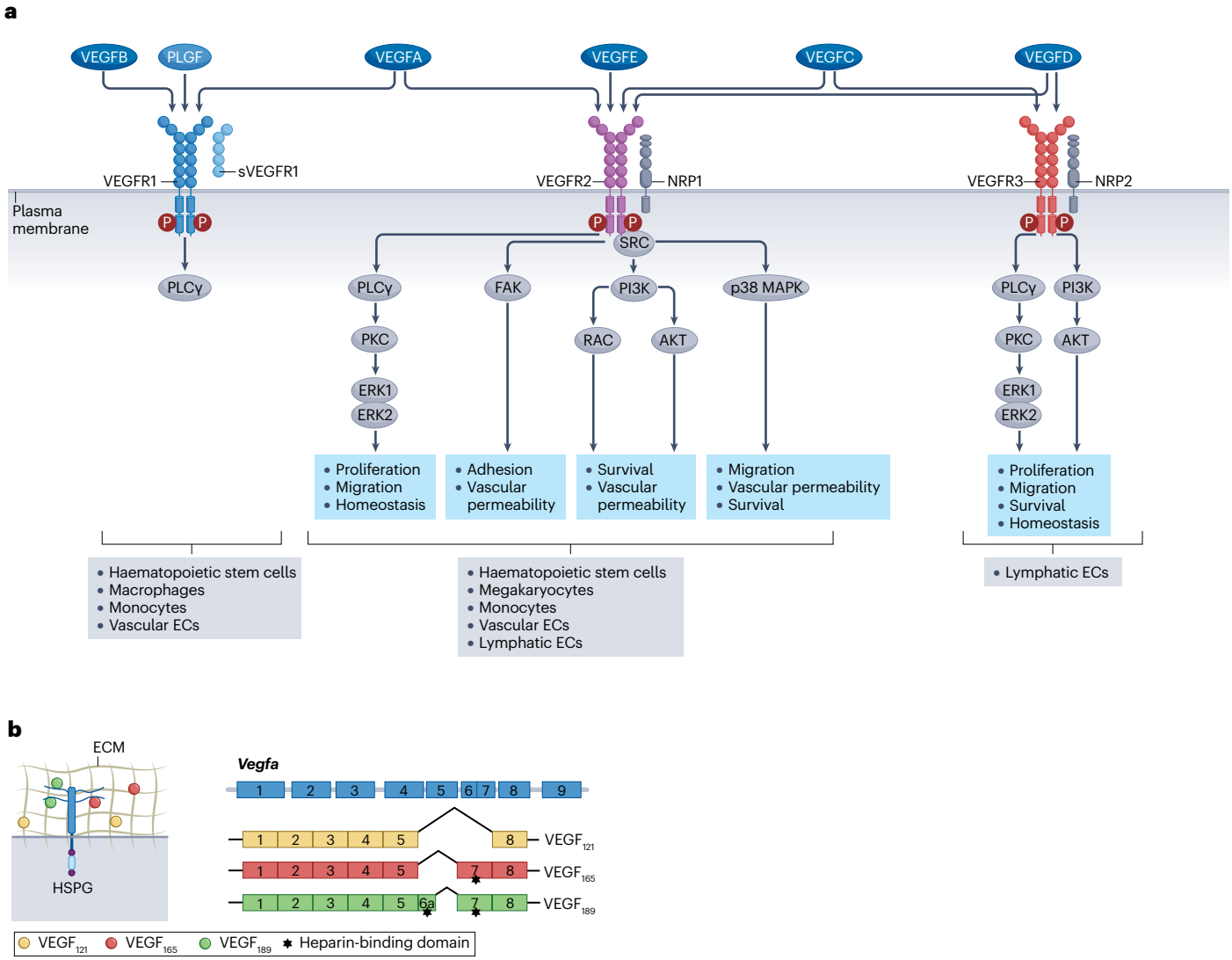

Fig. 2 | VEGF–VEGFR interactions and signal transduction and VEGF isoforms. **a**, Vascular endothelial growth factor B (VEGFB), placental growth factor (PLGF) and VEGFA bind VEGF receptor 1 (VEGFR1) and its soluble form (sVEGFR1) through immunoglobulin-like domain 2 (D2) and D3. Upon VEGF binding, phosphorylation of Tyr1169 in VEGFR1 is weak and only slightly activates the phospholipase C isoform-γ (PLCγ)–protein kinase C (PKC) pathway for mitogen-activated protein kinases (MAPK). VEGFR1 is expressed in vascular endothelial cells (ECs), haematopoietic stem cells, immune cells and even in certain tumour cells (not shown). VEGFA can also bind neuropilin 1 (NRP1) and VEGFR2, which can also be activated through binding of VEGFE and the processed forms of VEGFC and VEGFD. Following VEGFR2 stimulation, several pathways are activated. The PLCγ–PKC pathway activates extracellular-signal-regulated kinase 1 (ERK1) and ERK2 to control proliferation, migration, and homeostasis and, through focal adhesion kinase (FAK), it regulates adhesion to the extracellular matrix (ECM) and vascular permeability. Phosphoinositide 3-kinase (PI3K) controls survival and permeability through the activation of two different signalling cascades, which are mediated through SRC activation: AKT and RAC. Moreover, p38 MAPK activation is involved in migration, permeability and survival. VEGFR2 is expressed in haematopoietic stem cells, megakaryocytes and vascular and lymphatic ECs. VEGFC and VEGFD additionally bind VEGFR3 and NRP2, expressed in lymphatic ECs. VEGFR3 is key to the control of lymphatic EC proliferation, survival, migration and homeostasis, which are regulated through the activation of PLCγ–PKC and PI3K pathways. **b**, Alternative exon splicing of the *Vegfa* gene generates various VEGF isoforms, with differential affinity for heparan sulfate proteoglycans (HSPGs) at the cell surface and in the ECM. $VEGF_{121}$ lacks HSPG-binding domains and diffuses freely in tissues. Approximately half of $VEGF_{165}$, the main VEGF isoform, is diffusible while the other half binds to cell-surface HSPGs through exon 7-encoded sequences. The longer forms, such as $VEGF_{189}$ or $VEGF_{206}$ (not shown), bind HSPGs through two different heparin-binding domains encoded by exon 6 and exon 7 and are almost completely sequestered by HSPGs in the ECM.

binding of HIF1α or HIF2α to a highly conserved hypoxia response element (HRE) on the *VEGFA* gene[49,50]. Under normoxic conditions, HIF is recognized by the von Hippel-Lindau (VHL) tumour suppressor protein, resulting in polyubiquitylation and proteosomal degradation[51]. Inactivating mutations in *VHL*, such as those occurring in VHL syndrome or renal cell carcinomas, result in inefficient degradation of HIF and in *VEGFA* upregulation in normoxic conditions[51]. HIF-regulated *VEGFA* gene expression executes specific angiogenic programmes

by increasing vascular permeability, EC proliferation, migration and adhesion, and tube formation[52–54]. Additionally, hypoxia-induced VEGF production is a driving force for the development of new vessels during embryonic development in addition to being implicated in the vascularization of solid tumours[55]. However, deletion of the HRE in the mouse *Vegfa* promoter did not result in embryonic lethality even in the homozygous state[56]. This is in contrast to embryonic lethality following inactivation of a single *Vegfa* allele[57,58], suggesting that HIF is not required for VEGF-dependent embryonic vasculogenesis and angiogenesis, although hypoxia has been strongly associated with embryonic development[59]. However, in mice with HRE deletion, there was a marked reduction in *Vegfa* mRNA upregulation in ocular models of acute hypoxia compared to wild-type mice, verifying the functional consequences of the deletion in other settings[60]. This apparent paradox suggests that not only the role of HIF in regulating VEGF expression may be context dependent but also that HIF-independent pathways can regulate VEGF expression. Indeed, a significant upregulation of VEGF has been shown in cancer cells in response to hypoxia, even after *Hif1a* knockdown by small-interfering RNA (siRNA)[61].

One of the HIF-independent, hypoxia-dependent mechanisms for the regulation of VEGF expression is the *KRAS* oncogene-mediated activation of *VEGFA*, whereby mutated *KRAS* augmented the hypoxic induction of *VEGFA*, and this was observed in wild-type and *HIF1A*-knockdown colon cancer cells[61]. In fact, several mechanisms for *RAS*-mediated regulation of *VEGFA* in hypoxia have been proposed. Hypoxia can activate multiple RAS effector pathways, including extracellular-signal-regulated kinase, JUN N-terminal kinase (JNK), p38 mitogen-activated protein kinases (MAPK), protein kinase B (PKB, also known as AKT) and RHO[61]. AKT is a major downstream target of phosphoinositide 3-kinase (PI3K), and inhibition of PI3K strongly downregulated the hypoxic induction of VEGF[62]. Activated RAS can also control VEGF protein activity through stimulation of the expression of several proteases, including MMPs, which activate VEGF protein secretion, thereby increasing its extracellular levels[63]. Previous studies have also shown that *VEGFA* transcription can be regulated by NF-κB, which is another important transcription factor that can mediate hypoxic responses. However, analyses of the VEGF promoter have not identified κB binding sites[27,64], and NF-κB may regulate *VEGFA* indirectly through other transcription factors. Numerous other growth factors, including epidermal growth factor, transforming growth factor-α (TGFα), TGFβ, keratinocyte growth factor, insulin-like growth factor 1, fibroblast growth factor (FGF) and PDGF, upregulate *VEGFA* mRNA expression, suggesting that release of such factors cooperates with local hypoxia in regulating VEGF release in the microenvironment[24]. Additionally, inflammatory cytokines, such as IL-1α, IL-1β, IL-6 and IL-8, induce *VEGFA* expression in several cell types[65,66].

Hypoxia-independent mechanisms of VEGF regulation

Various reports have also described hypoxia-independent pathways involved in VEGF regulation. Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), a potent metabolic sensor and regulator, has been shown to directly activate *VEGFA* expression, independently of hypoxia, through oestrogen-related receptor-α (ERRα)[67]. Additionally, PGC1α expression is upregulated in cultures of brown adipocytes, and its expression is transiently increased at the time when the pro-angiogenic factors are increased during cold acclimation. These changes are independent of hypoxic regulation, suggesting that adipose tissue undergoes hypoxic-independent angiogenesis[68]. Oxidative stress has also been proposed as a regulator of *VEGFA* expression in adipocytes[69], which suggests a common hypoxia-independent mechanism to regulate adipose tissue-dependent angiogenesis.

VEGF receptors and signal transduction

The VEGF family of ligands bind in a partially overlapping pattern to three RTKs (VEGFR1, VEGFR2 and VEGFR3) as well as two co-receptors (neuropilin 1 and neuropilin 2)[70]. VEGFR1 (also known as FLT1) and VEGFR2 (also known as KDR or FLK1 in mice) have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain and are expressed mainly on ECs and haematopoietic cells, although a lower level of expression has been reported also in other cell types[70,71]. VEGFR3 (also known as FLT4) has a similar structure but is proteolytically cleaved in the extracellular domain during synthesis, resulting in the generation of a disulfide bridge between the two resulting polypeptides[72]. VEGFR3 is mainly expressed in lymphatic ECs. In addition, neuropilin 1 and neuropilin 2 are type 1 transmembrane glycoprotein co-receptors for VEGFs and their respective VEGFRs, and are expressed in both vascular and lymphatic ECs[73]. VEGFs bind to the VEGFRs and co-receptors on the cell surface and stimulate cellular responses through receptor dimerization and transphosphorylation[74] (Fig. 2a). The following subsections provide a more detailed description of each receptor and its regulation.

VEGFR1

VEGFR1 is perhaps the least understood of the VEGF receptors, although recent studies emphasize important and unexpected roles. VEGFR1, initially known as FLT1, was cloned in 1990 as an orphan RTK, with expression in highly vascularized organs such as the lungs[71], which raised the possibility that FLT1 might be a candidate VEGF receptor[75]. However, the lack of detectable tyrosine phosphorylation after VEGF stimulation seemed initially inconsistent with this hypothesis[75]. Contemporaneous in situ ligand-binding studies with radioactive VEGF suggested some overlap between the distribution of VEGF-binding sites and *Flt1* mRNA expression in tissues[76], an observation that led to testing whether VEGF might bind Flt1-expressing cells – high-affinity binding was observed, confirming that Flt1 was indeed a receptor for VEGF[75]. These early studies anticipated two key features of VEGFR1: it is a poor signalling receptor but a very effective VEGF binder[77]. VEGFR1 appears not to be phosphorylated on consensus positive regulatory tyrosine residues present in the activation loop of most tyrosine kinases, which could explain its low level of kinase activity[78]. Furthermore, a motif in the juxtamembrane region of VEGFR1 but not of VEGFR2 represses PI3K activation and EC migration[79]. Based on these observations, it was proposed that VEGFR1 might not primarily be a receptor transmitting a mitogenic signal but rather a 'decoy' receptor[80]. There is now agreement that VEGFR1 is able to regulate the activity of VEGF on the vascular endothelium in a negative fashion by sequestering and rendering this factor less available to VEGFR2 (ref. 80). Early structure/function studies have shown that deletion of immunoglobulin-like domain 2 (D2) abolished VEGF or PLGF binding to VEGFR1. Replacing D2 of VEGFR3 with D2 of VEGFR1 conferred on VEGFR3 the ligand specificity of VEGFR1 and abolished the ability of the mutant VEGFR3 to bind VEGF-C, indicating that D2 is essential for ligand binding in both receptors[81]. Additionally, X-ray crystallography studies have shown that VEGFR1 binds, through D2 and D3, to other ligands, VEGFB and PLGF, which do not bind VEGFR2 (refs. 81–85).

VEGFR1 exists either as full length or as an alternatively spliced form. The full-length form is membrane bound and is expressed in

vascular ECs and in a spectrum of non-ECs, including haematopoietic stem cells (HSCs), macrophages and monocytes and even in certain tumour cells[85]. The major alternatively spliced variant encodes a soluble form known as sFLT1 or sVEGFR1, which consists of the first six immunoglobulin-like domains, and exhibits high-affinity binding to VEGF, PLGF and VEGFB[86]. sVEGFR1 is highly expressed in normal placenta and increased plasma levels of sVEGFR1 have been detected in pregnant women diagnosed with pre-eclampsia (hypertensive syndrome of pregnancy)[87,88], as discussed later.

Functionally, VEGFR1 plays important roles in the regulation of angiogenesis during embryonic development as well as in monocyte and macrophage migration. This was established through the generation of *Flt1*-knockout mouse embryos, which died at embryonic day (E)8.5 due to an overgrowth of ECs and disorganized blood vessels[89]. To examine whether the tyrosine kinase domain of VEGFR1 was essential to the function of this receptor, a mutant mouse strain that lacks the tyrosine kinase domain of VEGFR1 was generated, that is, $Flt1^{TK-/-}$ mice. Surprisingly, the mutant mice were healthy with an almost normal vascular system, indicating that the role of VEGFR1 during vascular development was exerted by the extracellular domain and was therefore largely independent of its tyrosine kinase activity[90]. However, $Flt1^{TK-/-}$ mice showed slower tumour growth, less metastasis and a milder inflammatory reaction in a rheumatoid arthritis model. The latter was shown to be mediated by bone marrow-derived VEGFR1-positive cells, where VEGFR1 appears to be essential for their infiltration into tissues[91,92]. Furthermore, VEGFR1 as noted above has been shown to be expressed in certain tumour cell lines and to mediate their growth in response to VEGF and PLGF[93,94]. Later studies provided evidence for a non-mitogenic function of VEGFR1 in liver sinusoidal ECs (LSEC). VEGFR1 activation achieved with PLGF or a VEGFR1-selective VEGF mutant (VEGFR1 agonists), resulted in the paracrine release of hepatocyte growth factor (HGF), IL-6 and other hepatotropic molecules by LSEC, resulting in hepatocyte proliferation when co-cultured with LSECs[95]. Such a mechanism protected the liver from damage induced by the hepatotoxin CCl_4 despite the inability of a VEGFR1 agonist to induce LSEC proliferation. These findings suggest that one of the functions of VEGFR1 signalling in the vascular endothelium is the paracrine release of tissue-specific growth and survival factors, possibly in a vascular bed-specific fashion[95].

VEGFR2

VEGFR2 is a 200–230-kDa receptor for VEGFA and the viral protein VEGFE[96,97]. Additionally, VEGFR2 can bind VEGFC and VEGFD following proteolytic processing of the full-length proteins[36,98]. VEGFR2 is expressed in both vascular and lymphatic ECs, although low levels of expression have been documented in other cell types[99]. $Vegfr2^{-/-}$ embryos die by E8.5–9.5, exhibiting defects in endothelial and haematopoietic precursors, indicating that the receptor is crucial for vascular development[100]. VEGF binds to D2 and D3 of VEGFR2, resulting in tyrosine autophosphorylation in its kinase domain and receptor dimerization[101,102].

VEGFR2 is the major mediator of the physiological and pathological effects of VEGF. VEGFR2 mediates EC proliferation, migration and arterial fate specification, largely through RAS-independent activation of the extracellular-signal-regulated kinases 1 and 2 (ERK1/2)–MAPK pathway[13,103]. VEGFR2 also activates PI3K, resulting in the activation of several intracellular molecules such as AKT and the small GTP-binding protein RAC. The AKT pathway regulates cellular survival by inhibiting pro-apoptotic pathways such as B cell lymphoma 2 (BCL-2)-associated death promoter homologue (BAD) and caspase 9 (ref. 104). The small GTP-binding protein RAC has been implicated in regulating vascular permeability and cellular migration. Indeed, the small GTPases RHO, CDC42 and RAC affect many cellular processes in ECs, including cytoskeletal organization, cell morphology, adhesion, migration and junctional integrity[105].

Several potential phosphorylation sites are present in VEGFR2 but the best characterized are Tyr1173 (1175 in humans) and Tyr949 (951 in humans). Generation of knock-in mice in which Tyr1173 was substituted by a Phe elucidated the essential role of this residue in VEGFR2-mediated signal transduction and biological effects. Mice homozygous for Phe1173 died in utero around E8.5–9.5, virtually phenocopying *Vegfr2*-null mice[106]. Such a crucial role for Tyr1173 likely reflects the fact that it is a binding site of phospholipase C isoform-γ (PLCγ), and is thus important for VEGF-dependent PLCγ–protein kinase C (PKC)–MAPK activation, leading to DNA replication in ECs[107,108]. The β-isoform was reported to be the major PKC isoform implicated in VEGF-induced mitogenesis[107,108]. Additionally, *Plcg1*-null mice die at approximately E9.0 because of the absence of vasculogenesis and haematopoiesis, reminiscent of *Vegfr2*-null mice[109]. The significance of Tyr949 is described in the section on vascular permeability, given its involvement in regulating this function.

Earlier studies provided evidence that RTK endocytosis plays an important role in ensuring spatial restriction of signalling by enabling localized intracellular responses, for example, cytoskeleton rearrangements, in order to achieve accurate directionality in the response to migratory stimuli[110]. Indeed, much evidence indicates that endocytosis is not only a pathway for receptor degradation in lysosomes but also an important step to control cell signalling events triggered by numerous RTKs, including VEGFR2 (ref. 111). It has been reported that VEGF-induced, clathrin-dependent VEGFR2 endocytosis is important for intracellular activation of VEGFR2 signalling[112,113]. Additionally, over the last several years, numerous studies have described the significance of clathrin-dependent and clathrin-independent VEGFR2 endocytosis in regulating such events as EC migration in response to specific cues or tip cell induction in various physiological and pathological circumstances[113–116].

VEGFR3

Given the more prominent role of VEGFR3 in lymphangiogenesis, only a few points are made here. For a detailed description, the reader is referred to some comprehensive reviews discussing this receptor[10,98]. VEGFR3 is a 195-kDa high-affinity receptor for VEGFC and VEGFD. $Vegfr3^{-/-}$ mice die at E9.5 due to abnormal vessel organization, leading to fluid accumulation and cardiovascular failure[117]. These results suggest that VEGFR3 plays a critical role in the proliferation and differentiation of vascular ECs at an early embryonic stage. In the adult, VEGFR3 expression is detected primarily in lymphatic ECs and, to some extent, on quiescent vascular ECs[118]. Moreover, VEGFR3 is expressed in blood and lymphatic ECs from several cancer types and blocking VEGFR3 signalling leads to suppression of tumour lymphangiogenesis and lymph node metastasis. Studies confirmed the structural similarities between VEGFR1 and VEGFR3, in particular the prominent role of D2 and D3 in ligand binding in both receptors[81,119]. A chimaeric VEGFR3–Fc fusion protein was shown to bind VEGFC and to inhibit lymphangiogenesis and metastasis in transgenic mice[120,121]. Activation of VEGFR3 through homodimerization induces proliferation, migration and survival in lymphatic ECs through induction of PI3K and stimulation of AKT pathways. VEGFR3 can bind both the full-length and proteolytically processed forms of VEGFC and VEGFD, whereas VEGFR2, as noted, is only

capable of binding the proteolytically processed forms. Therefore, the availability of the ligands indirectly modulates the signal transduction capacities of the two receptors[122].

Neuropilins

Earlier studies indicated that some tumours and ECs express cell-surface VEGF-binding sites for $VEGF_{165}$ (but not $VEGF_{121}$) distinct in affinity and molecular mass from the VEGF RTKs[123]. Subsequently, a VEGF isoform-specific receptor was identified as neuropilin 1 (ref. 124), a molecule that had been previously shown to bind the semaphorin family and was implicated in neuronal guidance (reviewed in ref. 73). Neuropilin 1 and neuropilin 2 are transmembrane glycoproteins of 120 kDa with a highly conserved structure, divided into four domains[125]. Neuropilins act as co-receptors for specific isoforms of VEGF. In contrast to $VEGF_{165}$, $VEGF_{121}$ has weak or undetectable binding to neuropilin 1, leading to the hypothesis that the residues required for neuropilin 1 binding reside in exon 7-encoded sequences. However, crystal structure studies indicated that exon 8-encoded sequences, present also in $VEGF_{121}$, are the site of direct interaction between VEGF and neuropilin 1, although exon 7 sequences are required to extend this region to effectively gain access to the neuropilin 1 dimer[126]. More recent studies have suggested additional levels of complexity in the interaction between neuropilin and VEGF isoforms[127].

Neuropilin 1 is expressed in all ECs, neuronal progenitors and macrophages, whereas neuropilin 2 is mainly expressed in lymphatic ECs[128]. In both cases, neuropilins require an additional transmembrane molecule to exhibit biological activity. VEGFRs are the major co-receptor for VEGF-mediated activities. Notably, neuropilin 1 is known to form complexes with $VEGFA_{165}$ and VEGFR2 to promote angiogenic signalling, while neuropilin 2 preferentially binds weakly to VEGFC and VEGFR3 to facilitate lymphangiogenic signalling[129]. In vitro studies showed that $VEGF_{165}$ binding to neuropilin 1 enhances VEGFR2 signalling and increases EC proliferation and migration, possibly due to the increased affinity and binding kinetics of $VEGF_{165}$ for neuropilin 1 as compared to VEGFR2 (ref. 130). This was also shown through the generation of transgenic mice in which VEGF binding to neuropilin 1 is disrupted. Surprisingly, these mice had milder angiogenic defects during embryonic development as compared to *Nrp1*-knockout mice[131], which suggested that $VEGF_{165}$ binding to neuropilin 1 is dispensable for embryonic angiogenesis but may be important for postnatal angiogenesis. Given that neuropilin 1 is a non-catalytic transmembrane protein, it was then proposed as a co-receptor to transduce downstream signals through interaction with VEGFR2 (ref. 124).

Biological roles of VEGFA

VEGFA has many functions in physiology and disease (Figs. 3 and 4). We discuss these in the following subsections.

Physiological angiogenesis

Angiogenesis is a critical process during embryonic and fetal development that occurs in the adult only during skeletal growth, tissue regeneration, reproductive organ angiogenesis and pregnancy. As noted, VEGF binding to VEGFR2 stimulates EC proliferation, primarily through the PLCγ–PKC–MAPK signalling pathway[103,108].

Developmental angiogenesis. The role of VEGF in development is emphasized by embryonic lethality in mice after loss of a single *Vegfa* allele, which is caused by impaired angiogenesis and perturbed formation of blood islands[57,58]. During embryonic development, the common precursor for endothelial and haematopoietic cells, that is, haemangioblasts, in the posterior primitive streak are marked by the expression of *Flk1* (*Vegfr2*). Haemangioblasts migrate from the posterior primitive streak into the yolk sac, where they aggregate into blood islands that fuse to generate a primary capillary plexus in a process mediated by VEGF, Sonic hedgehog (SHH) and Notch signalling[12,132]. Following vascular remodelling, nascent vessels are stabilized through the recruitment of smooth muscle cells and pericytes, also known as mural cells[133]. Pericytes share a basement membrane with microvascular ECs and their reciprocal interactions are crucial for EC barrier function and growth regulation[134]. Pericyte losses or abnormalities are implicated in tumour angiogenesis, metastasis and hyperpermeability conditions[133–135].

Next, specification of arterial versus venous fate (arteriovenous specification) is dependent on several pathways, including EPHB2, DLL4–NOTCH and VEGF[136–138]. VEGF has also been implicated in arteriovenous specification, whereby VEGF induces EPHB2 expression, and hence promotes arterial specification[139]. Additionally, spatial localization of VEGF, through binding to VEGFR1, determines proper localization of vessel formations[140–142]. Sprouting angiogenesis is then responsible for the remodelling and expansion of this network[143]. Tip cells, at the leading edge of a nascent vessel, extend filopodia expressing VEGFR2 and respond to a gradient of heparin-binding VEGF[144]. Stalk cells form the backbone of the vessel (Fig. 3a, left). DLL4 is downstream of VEGF and acts as a negative feedback regulator that restrains VEGF signalling on ECs, shifting the balance towards stalk cells[144]. Inhibition of DLL4–NOTCH signalling results in hyper-sprouting, with disorganized and poorly perfused blood vessels[145,146]. In the absence of pro-angiogenic stimuli, such as in adult resting vasculature, ECs are retained in a quiescent state and EC homeostasis is maintained by low-level VEGF signalling[147].

Corpus luteum development and pregnancy. During the ovarian cycle, under the influence of gonadotropins, granulosa cells in the developing follicle undergo differentiation into progesterone-producing corpus luteum, a process accompanied by intense angiogenesis (Fig. 3a, middle). The expression of *Vegfa* mRNA is strongly correlated with luteal angiogenesis in multiple species[11]. Using the abovementioned VEGFR1 construct, it was shown that VEGF is essential for angiogenesis in the corpus luteum[148]. VEGF has also been implicated in uterine angiogenesis and vascular remodelling in pregnancy[149]. Additionally, numerous studies have shown that VEGF inhibitors block angiogenesis and growth in endometriotic lesions[150]. Endocrine-gland-derived VEGF (EG-VEGF, also known as PROK1) is also an EC mitogen with selectivity for the endothelium of steroidogenic glands and is expressed in normal human ovaries in a pattern complementary to VEGF[151,152]. EG-VEGF, together with VEGF, has been implicated in the excessive angiogenesis and ovarian tissue growth associated with polycystic ovary syndrome, a leading cause of infertility[152].

The VEGF pathway is also implicated in pre-eclampsia, a pregnancy disorder characterized by hypertension and proteinuria, which increases the risk of perinatal morbidity and mortality in mothers and fetuses[153]. Levels of sFLT1 (sVEGFR1) are increased in women with pre-eclampsia before clinical symptoms are observed, suggesting a correlation between serum sFLT1 levels and development of pre-eclampsia[88]. Presently, high serum levels of sFLT1 represent an established diagnostic and prognostic marker of pre-eclampsia as well as a promising therapeutic target for this disease[154]. Although initial therapeutic efforts attempted to remove sFLT1 (ref. 155) or administer

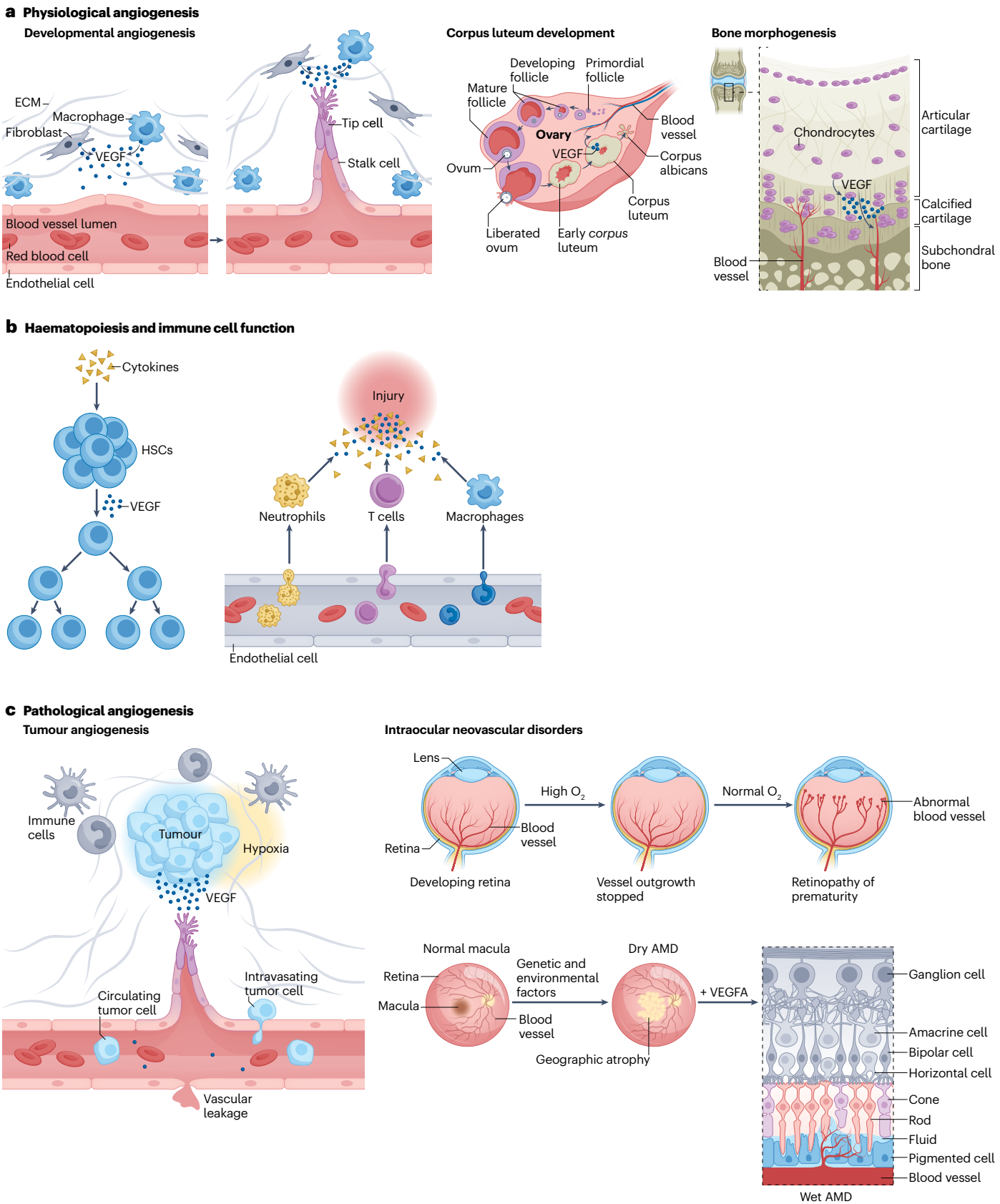
a Physiological angiogenesis
Developmental angiogenesis
ECM
Macrophage
Fibroblast
VEGF
Blood vessel lumen
Red blood cell
Endothelial cell
Tip cell
Stalk cell
Corpus luteum development
Developing follicle
Primordial follicle
Mature follicle
Ovary
Blood vessel
VEGF
Corpus albicans
Ovum
Corpus luteum
Liberated ovum
Early corpus luteum
Bone morphogenesis
Articular cartilage
Chondrocytes
VEGF
Calcified cartilage
Subchondral bone
Blood vessel
b Haematopoiesis and immune cell function
Cytokines
HSCs
VEGF
Injury
Neutrophils
T cells
Macrophages
Endothelial cell
c Pathological angiogenesis
Tumour angiogenesis
Immune cells
Tumour
Hypoxia
VEGF
Circulating tumor cell
Intravasating tumor cell
Vascular leakage
Intraocular neovascular disorders
Lens
Retina
Blood vessel
Developing retina
High O2
Vessel outgrowth stopped
Normal O2
Abnormal blood vessel
Retinopathy of prematurity
Normal macula
Retina
Macula
Blood vessel
Genetic and environmental factors
Dry AMD
Geographic atrophy
+ VEGFA
Ganglion cell
Amacrine cell
Bipolar cell
Horizontal cell
Cone
Rod
Fluid
Pigmented cell
Blood vessel
Wet AMD

Fig. 3 | Biological effects of VEGF. a, Examples of physiological functions of vascular endothelial growth factor (VEGF)-mediated angiogenesis. VEGF is important for embryonic and postnatal angiogenesis. VEGF released from a variety of cell types, including stromal cells, promotes sprouting through tip and stalk cell formation, as described in the text, essential for tissue maintenance and homeostasis during embryonic development and adult life (left). In response to pituitary gonadotrophins, granulosa cells in the developing follicle within the ovaries undergo differentiation into progesterone-producing corpus luteum, a process accompanied by VEGF upregulation and intense angiogenesis (middle). During bone morphogenesis, VEGF secreted by chondrocytes promotes vascularization and survival of chondrogenic and osteogenic cells. These cells then promote cartilage matrix degradation and promote ossification. Moreover, the newly formed blood provides support to progenitors for the specific cell types that form bone and marrow (right). **b**, VEGF function in the haematopoietic system and immune cell function. Haematopoietic stem cells (HSCs) have been shown to secrete VEGF in response to cytokine stimulation, which induces the proliferation and migration of these cells. Additionally, VEGF is chemotactic for immune cells such as macrophages, neutrophils or T cells, recruiting these cells to the sites of injury. **c**, Examples of pathological functions of VEGF-mediated angiogenesis. During tumour progression, the hypoxic microenvironment promotes the release of cytokines, chemokines and growth factors, including VEGF, that interact with the surrounding endothelial cells to promote tumour angiogenesis. Persistently high VEGF levels can lead to the formation of abnormal and leaky neovessels, which facilitates tumour cell intravasation and metastasis (left). VEGF is also involved in the development of abnormal blood vessels in the retina. This occurs, for example, in retinopathy of prematurity, when the eye returns to normoxia following exposure to high oxygen to provide adequate oxygenation to premature infants with immature lungs (right). As described in the main text, return to normoxia can be followed by VEGF upregulation and pathological retinal angiogenesis. The newly formed vessels are leaky and abnormal, which may lead to an inflammatory cascade resulting, for example, in fibrosis and retinal detachment from its normal connection to blood vessels (top). In the adult, VEGF promotes the neovascularization that is responsible for the development of proliferative retinopathies or wet age-related macular degeneration (AMD) (bottom). Early-stage or dry AMD is characterized by the formation of drusen and abnormalities in the retinal pigment epithelium. Late-stage AMD, or wet AMD, is characterized by choroidal neovascularization in the macula, a photoreceptor-rich area in the central portion of the retina that is responsible for central vision. In these conditions, VEGF is upregulated, leading to the development of pathological retinal neovascularization in the fenestrated endothelium of the choroid that can cause blindness. ECM, extracellular matrix.

PLGF[156], novel approaches involve the modulation of its expression through siRNA[157]. A recent study reported the use of siRNA technology against sFLT1 as a therapy for pre-eclampsia. siRNA selectively targeting *FLT1* mRNAs without affecting full-length FLT1 was delivered to pregnant mice, resulting in reduced circulating sFLT1. In a baboon pre-eclampsia model, siRNA suppressed sFLT1 overexpression and reduced blood pressure, suggesting that siRNA-based modulation of sFLT1 could be a treatment for women with pre-eclampsia.

Bone morphogenesis. Much research has shown that VEGF has several roles in bone development, promoting vascularization during fetal bone formation and regulating the survival and activity of chondrogenic and osteogenic cells[158] (Fig. 3a, right). Early studies, using a sVEGFR1 construct, showed that VEGF produced by chondrocytes is crucial for vascular invasion of the metaphysis (the wide portion of a long bone, containing the area that grows during childhood; also known as growth plate), cartilage resorption and primary ossification of long bones[159]. Counterintuitively, VEGF inactivation resulted in expansion of the hypertrophic chondrocyte zone due to failure of these cells to undergo apoptosis, indicating that the blood vessels carry key signals required for correct growth plate morphogenesis[159]. In this context, a new capillary subtype that mediates growth of the bone vasculature, maintains perivascular osteoprogenitors and couples angiogenesis to osteogenesis was identified[160,161]. Subsequent studies provided further evidence for the requirement of $VEGF_{165}$ for bone morphogenesis, which plays a role not only in mediating bone vascularization but also in allowing normal differentiation of hypertrophic chondrocytes, osteoblasts, ECs and osteoclasts[162]. Chondrocyte-secreted VEGF also seems to be indispensable for the promotion of angiogenesis around the epiphysis (the region of the long bone that forms the joint) of long bones and to facilitate secondary ossification. Additionally, VEGF has been shown to promote bone repair by promoting angiogenesis and bone turnover through the differentiation of osteoblasts and osteoclasts[163].

Haematopoiesis and immune cell functions

Haematopoiesis is a tightly regulated process that involves multiple growth factors, cytokines and chemokines acting to regulate and maintain HSCs. HSCs have been shown to secrete VEGF in response to cytokine stimulation to enable transendothelial migration during progenitor cell mobilization into the circulation[164]. Accordingly, VEGFR2 is expressed on a subset of multipotent HSCs[165] (Fig. 3b). In experiments where VEGF-depleted bone marrow was injected into lethally irradiated mice, VEGF-deficient HSCs from the bone marrow failed to repopulate lethally irradiated hosts, suggesting that VEGF, through a cell-autonomous mechanism, is involved in HSC survival[166]. Interestingly, HSC survival was rescued through the expression of *PLGF* (also known as *PGF*) in VEGF-deficient bone marrow cells, demonstrating the role of VEGFR1 in this process. In agreement with this, inhibition of VEGFR1 but not of VEGFR2 using monoclonal antibodies blocked HSC cycling, differentiation and haematopoietic recovery after irradiation, resulting in increased lethality[167]. Given the importance of HSCs in haematopoietic malignancies, VEGF secreted by HSCs has also been implicated in tumour progression, both as an autocrine and paracrine signal, at least in some blood cancers[168].

VEGF can also modulate innate and adaptive immune responses directly or indirectly through immune cells. VEGF can stimulate chemotaxis of inflammatory cells, including macrophages, neutrophils, dendritic cells, myeloid-derived suppressor cells and T cells, since immune cells express VEGFRs[169]. Innate immune cells such as dendritic cells, neutrophils and macrophages can produce chemokines and cytokines that suppress tumour angiogenesis. However, under certain conditions, these cells can promote angiogenesis by secreting, for example, VEGF, IL-10, IL-17, Bv8 (also known as PROK2) and MMP9 (ref. 170). Indeed, neutrophils can aid in the acquisition of resistance in response to anti-angiogenic therapy in multiple cancer models[171,172].

Pathological angiogenesis

Pathological angiogenesis involves a persistent and unresolved angiogenic cascade that leads to the formation of leaky and abnormal vessels, often causing failure of the establishment of adequate vascular perfusion[3]. As noted above, VEGF is important in the molecular pathogenesis of tumour development and in several blinding eye diseases, including age-related macular degeneration (AMD), diabetic retinopathy and retinal vein occlusion. As such, several therapies that target the

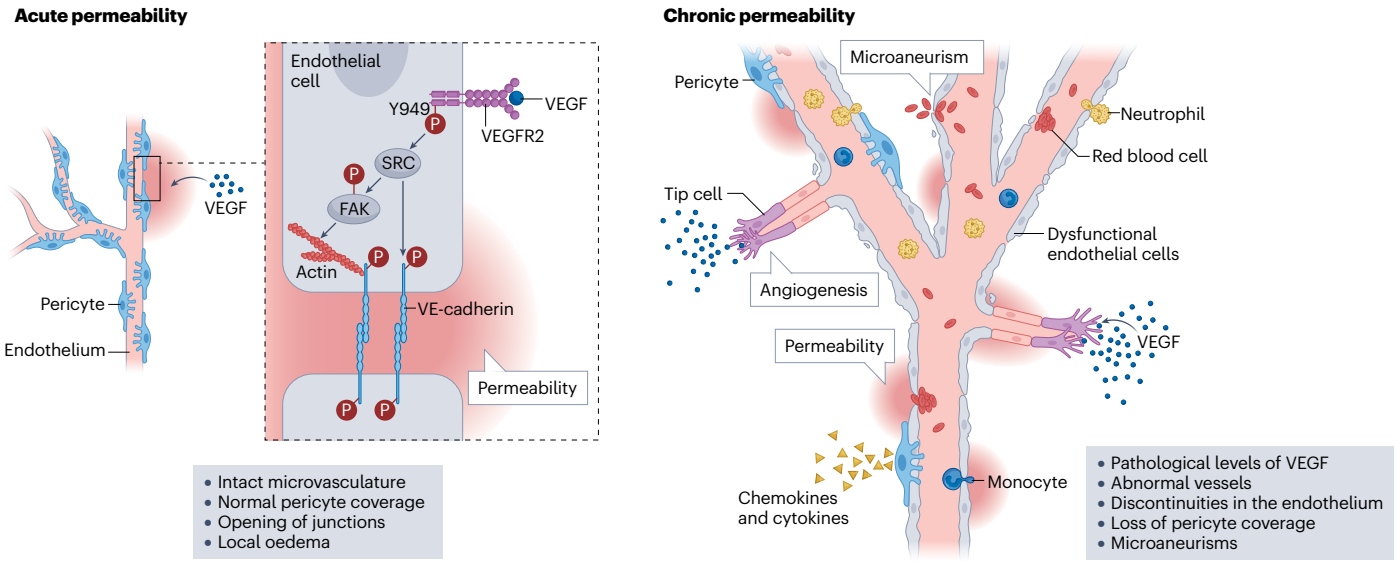

Fig. 4 | Complex effects of VEGF on vascular permeability. Vascular endothelial growth factor (VEGF) is responsible for acute as well as chronic vascular hyperpermeability. An early response to VEGF in the intact endothelium of capillaries and post-capillary venules is mediated by phosphorylation of VEGF receptor 2 (VEGFR2) Tyr949, resulting in the activation of SRC, and leading in turn to VE-cadherin phosphorylation, actin rearrangement and transient opening of inter-endothelial junctions (see the main text), which results in a transient increase in permeability. However, if VEGF stimulation persists, activation of the proliferative pathways results in growth of blood vessels, which frequently fail to mature, are dilated and have a discontinued and dysfunctional endothelium, reduced pericyte coverage and microaneurisms. These features result in persistently high permeability, with increased inflammatory cell extravasation and bleeding – the hallmarks of pathological vessels in tumours and other disorders. Chronic vascular permeability is a key feature of tumour-associated blood vessels and facilitates metastasis, In other conditions, such as age-related macular degeneration or diabetic retinopathy, oedematous fluid and haemorrhages originating from the abnormally leaky vessels impair vision and reduce the amount of oxygen that reaches the tissue, resulting in further damage to the retina. FAK, focal adhesion kinase.

VEGF family and its receptors have been developed for the treatment of these diseases (Table 1).

Tumour angiogenesis. The hypoxic tumour microenvironment (TME) is driven by the transcriptional activity of HIFs, which can upregulate *VEGFA* to induce tumour angiogenesis, a key hallmark of cancer progression[173]. Moreover, hypoxia can induce VEGF release from other cells in the TME, including myeloid cells and cancer-associated fibroblasts[47]. These cells promote tumour angiogenesis potentially also through release of alternative pro-angiogenic mediators, including FGF2, PLGF, tumour necrosis factor (TNF) and Bv8 (ref. 170) (Fig. 3c, left). Additionally, these cells release proteases, such as MMPs, that can release ECM-sequestered pro-angiogenic molecules[174]. However, the unbalanced exposure of tumour ECs to VEGF and other pro-angiogenic molecules leads to failed maturation of the newly formed vascular networks, resulting in disorganized, leaky vessels that lack mural cell coverage (see section on vascular permeability and Fig. 4). The high interstitial pressure associated with such vessels can reduce the efficacy of therapeutics due to their inability to reach the tumour[175,176]. Interestingly, myeloid cells have been associated with refractoriness to anti-VEGF therapies[177]. Briefly, refractory tumours were associated with a significant increase in the frequency of tumour-infiltrating myeloid cells, and recruitment of these cells was sufficient to confer refractoriness to anti-VEGF treatment. Mechanistically, myeloid cells of the neutrophil lineage[178] could enable the escape to anti-VEGF through the activation of other pro-angiogenic molecules such as Bv8 (refs. 179,180). Interestingly, no apparent contribution of the adaptive immune system was identified in these models, although the same myeloid cells have been characterized as immunosuppressive in other tumour models through the ability to inhibit T cell-mediated functions[181,182], consistent with their multiple and complex roles in tumorigenesis[178]. However, other cells of the TME could also be contributing to anti-VEGF escape. For example, cancer-associated fibroblasts have been reported to produce stromal cell-derived factor 1 (SDF1)[183] and PDGFC[184]. Moreover, tumours have developed additional strategies to promote blood vessel growth, including vessel co-option, where tumour cells hijack the existing vasculature to support growth and metastasis[185]. Vascular mimicry, in which cancer cells form blood-filled channels lined by tumour cells[186], has also been proposed as an escape mechanism. However, this hypothesis remains controversial due to the difficulty of reconciling the poor blood flow in such channels with enhanced tumour growth[186]. Nevertheless, despite the potential redundancy of mediators, anti-VEGF therapies have been effective at blocking the development of blood vessels within tumours not only in mice but also in human patients (Box 1).

Intraocular neovascular disorders. The role of VEGF in the normal development of the eye vasculature is well established[187]. Additionally, *VEGFA* overexpression is the driving force for retinopathy of prematurity, a potentially blinding condition observed in preterm infants with immature lungs and exposed to oxygen therapy to ensure adequate tissue oxygenation (reviewed in refs. 12,188,189). High oxygen saturation can suppress HIF-dependent *VEGFA* expression in the retina, resulting in blood vessel regression. Return to normoxic conditions

can lead to retinal hypoxia due to inadequate perfusion, followed by VEGF upregulation, which promotes retinal angiogenesis. The newly formed vessels are leaky and abnormal, leading to an inflammatory cascade potentially resulting in fibrosis, traction, and detachment of the retina from its normal connection with blood vessels[190] (Fig. 3c, right). However, the role of VEGF in the retina is not limited to pathological conditions during development. Elevated VEGF levels were measured in the eye fluids from patients with proliferative retinopathies secondary to diabetes and other ischaemic conditions such as retinal vein occlusion[191,192]. Patients with proliferative diabetic retinopathy experience retinal vascular damage as a consequence of hyperglycaemia, which causes pericyte loss and capillary ischaemia. In these conditions, VEGF is upregulated, leading to the development of pathological retinal neovascularization[188]. Additionally, VEGF is expressed in the retinal pigment epithelium (RPE) and plays a critical role in the normal development of the choriocapillaris[193–195], the fenestrated capillary layer that nourishes RPE and the photoreceptors[196]. Furthermore, *VEGFA* is highly expressed in RPE cells and stromal cells in neovascular AMD (nAMD), the leading cause of vision loss in the adult population[197,198]. Early-stage AMD is characterized by the formation of drusen (fatty protein deposits) and abnormalities in the RPE. Late-stage AMD can be neovascular (also known as wet) or non-neovascular (known as atrophic or dry). nAMD, which accounts for 10% of all cases but is responsible for 80% of AMD-associated blindness, is characterized by choroidal neovascularization in the macula, a photoreceptor-rich area in the central portion of the retina that is responsible for central vision[198]. Much evidence indicates that VEGF plays a key role in the development of nAMD[197]. As such, anti-VEGF therapy has been effective not only for the treatment of patients with ischaemic retinal disorders[197] but also for patients with nAMD (Box 2). Currently, anti-VEGF is the most effective treatment for nAMD[199,200]. After anti-VEGF therapeutics became available, the leading cause of vision loss secondary to AMD is no longer nAMD but rather geographic atrophy, a key feature of advanced dry AMD[7].

Vascular permeability

Permeability is an essential property of the microvasculature since it enables exchanges of solutes between blood and tissues. However, excessive vascular permeability is a key aspect of a variety of pathological conditions, including cancer and ocular vascular disorders[201,202]. Numerous studies have described structural abnormalities associated with high leakiness, including reduced pericyte coverage, tortuosity (irregular twists and turns that reflect defective remodelling in vessels), microaneurisms, thinning of the endothelium and others[135,175,203] (Fig. 4).

A potential role for VEGF in vascular permeability was suggested in the early 1980s, when VPF was identified as a tumour-secreted protein that increased vascular permeability as assessed by Evans Blue extravasation after injection in the guinea pig skin (Miles assay)[204]. This effect was very rapid, although transient. It was hypothesized that VPF-mediated increases in vascular permeability are crucial for tumour angiogenesis[205]. According to this hypothesis, plasma extravasation activates the clotting pathways, leading to the formation of a fibrin gel that promotes migration of tumour cells, inflammatory cells and ECs[205].

Elucidation of the signalling pathways that mediated the effects of VEGF in the Miles assay enabled the testing of these hypotheses. Earlier studies reported that specific members of the SRC family of kinases, in particular *Fyn* and *Src* (also known as *Zap70*) are required for the permeability-enhancing effects but not for the pro-angiogenic effects of VEGF[206]. Subsequent studies have shown that phosphorylation of Tyr949 in VEGFR2 is key to VEGF-induced vascular permeability[207]. Phosphorylated Tyr949 (pTyr949) interacts with the T cell-specific adaptor protein (TSAd)[208], which triggers formation of complexes between SRC and VE-cadherin, leading to transient opening of inter-endothelial junctions[209]. SRC-dependent phosphorylation of focal adhesion kinase (FAK) is also required for VE-cadherin-mediated EC junctional breakdown and vascular leakage[210]. Mice in which Tyr949 was substituted with Phe (Tyr949Phe/Tyr949Phe) had almost no response to VEGF in a Miles assay but had no detectable developmental or adult abnormalities, suggesting that this function of VEGF does not play major homeostatic roles[207]. Interestingly, tumours implanted in Tyr949Phe/Tyr949Phe mice had similar growth rates to those in wild-type mice, and angiogenesis was not inhibited, although some reduction in tumour oedema was noted, especially at early time points. A reduction in metastasis was also reported, consistent with

Table 1 | FDA-approved anti-angiogenic drugs for the treatment of cancer and intraocular neovascular disorders

Drug	FDA approval	Application
Tumour angiogenesis		
Ramucirumab (Cyramza)	2014	First-line GC, NSCLC, mCRC, HCC
Aflibercept (Zaltrap)	2011	Second-line mCRC
Bevacizumab (Avastin)	2004	First-line mCRC, NSCLC, GBM, mRCC, mCC, mOC
Tyrosine kinase inhibitors		
Axitinib	2019	First-line mRCC
Cabozantinib	2012	First-line mRCC, MTC; second-line HC
Lenvatinib	2018	First-line HCC; second-line TC
Nintedanib	2020	Second-line NSCLC
Pazopanib	2009	First-line mRCC
Regorafenib	2017	First-line mCRC; second-line HCC
Sorafenib	2005	First-line mRCC, HCC, TC
Sunitinib	2017	First-line mRCC
Vandetanib	2011	First-line MTC
Intraocular neovascular disorders		
Faricimab (Vabysmo)	2022	nAMD, DME
Brolucizumab (Beovu)	2019	nAMD, DME
Aflibercept (Eylea)	2011	nAMD, DR, DME, RVO
Ranibizumab (Lucentis)	2006	nAMD, DR, DME, RVO
Pegaptanib (Macugen)	2004	nAMD
Bevacizumab (Avastin)	Used off-label	nAMD, DME, RVO

FDA approval year refers to the first indication for which each drug was approved. DME, diabetic macular oedema; DR, diabetic retinopathy; GC, gastric cancer; GMB, glioblastoma multiforme; HCC, hepatocellular carcinoma; mCC, metastatic cervical cancer; mCRC, metastatic colorectal cancer; mOC, metastatic ovarian cancer; mRCC, metastatic renal cell carcinoma; MTC, medullary thyroid cancer; nAMD, neovascular age-related macular degeneration; NSCLC, non-small-cell lung cancer; RVO, retinal vascular occlusion; TC, thyroid cancer.

Box 1

Anti-angiogenic therapy in cancer

The consistently high expression of vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) in human tumours and numerous studies in mouse models showing robust tumour suppression using VEGF pathway inhibitors provided a rationale for targeting this pathway[263]. The generation of antibodies against VEGF for the treatment of cancer started in the early 1990s when VEGF inhibition with a monoclonal antibody resulted in reduced tumour growth in several cancer models[264,265]. Later, this antibody was humanized, resulting in bevacizumab, a potent inhibitor suitable for clinical trials[266]. Bevacizumab was first approved by the FDA for the treatment of advanced colon cancer and has since been approved for the treatment of several other cancer types[263]. Today, bevacizumab has 12 FDA approvals. Therapies against other members of the VEGF family have also been attempted such as anti-placental growth factor (anti-PLGF) antibodies. However, although initial results showed promising inhibition of tumour growth and metastasis[267], anti-PLGF antibodies exhibited no significant effect on tumour angiogenesis in several tumour models[268]. VEGFRs have also been targeted for the development of anti-angiogenic therapies, either as biologics such as monoclonal antibodies, which competitively bind the ligands of the VEGF family, or as tyrosine kinase inhibitors, which block the activation of several pathways downstream of VEGFRs. VEGFR1 has been exploited for the generation of aflibercept, a chimaeric protein that contains the ligand-binding domain of human VEGFR1 (immunoglobulin-like domain 2 (D2)) and D3 of VEGFR2 fused to the Fc portion of human IgG_1. Aflibercept acts as a decoy receptor that is able to trap all isoforms of VEGF, PLGF and VEGFB[217]. Aflibercept was approved by the FDA for the treatment of second-line colorectal cancer in combination with chemotherapy[263]. Later, ramucirumab, a human anti-VEGFR2 antibody, was approved for the treatment of gastric cancer, non-small-cell lung cancer, colorectal cancer and hepatocellular carcinoma[269]. Ramucirumab inhibited the mitogenic effects of VEGF on human endothelial cells. It also downregulates VEGFR2 expression[270]. Several VEGFR tyrosine kinase inhibitors have also been developed, which are usually less specific and block multiple kinases. For example, axitinib, cabozantinib, lenvatinib, nintedanib, pazopanib, regorafenib, sorafenib, sunitinib and vandetanib have been approved by the FDA for the treatment of metastatic renal cell carcinoma (RCC), among others[263].

However, the off-target effects and high toxicity of these inhibitors poses clinical challenges, which frequently prevent long-term use[271]. Many patients develop resistance, potentially as a result of the activation of other pro-angiogenic pathways, including fibroblast growth factors (FGFs), angiopoietins and platelet-derived growth factors (PDGFs)[7]. Still, combinatorial therapies targeting VEGF and some of these pathways, such as the angiopoietin–Tie2 pathway, did not result in enhanced benefits. Conversely, combining immune-checkpoint inhibitors, such as anti-PD1, anti-PDL1 or anti-CTLA4, with anti-VEGF agents has achieved considerable benefits. Indeed, some combinations have been approved by the FDA for the treatment of several tumour types, including RCC and non-small-cell lung cancer, although the mechanisms underlying such additive benefits are multifactorial (reviewed in refs. 7,258,272). A particularly interesting example is the combination of atezolizumab (anti-PDL1) and bevacizumab for the treatment of hepatocellular carcinoma[257]. While monotherapy with immune-checkpoint inhibitors failed to provide a survival benefit, the bevacizumab plus atezolizumab combination increased patient survival and is presently a standard of care for this malignancy[258,259]. Strong additivity has also been observed with lenvatinib and pembrolizumab in endometrial cancer[273]. Additionally, inactivating mutations in the *VHL* gene, which result in increased expression of hypoxia-inducible factor (HIF) target genes, including *VEGFA*, occur in von Hippel-Lindau (VHL) syndrome and, frequently, in RCC. Recently, belzutifan[274], a highly specific HIF2α inhibitor, has been approved by the FDA for therapy of VHL syndrome and RCC with mutant *VHL*, potentially expanding the possibility of combinations of anti-VEGF agents with novel therapies. However, treatment of patients with RCC with belzutifan resulted in acquired resistance to the drug due to several mutations[275], suggesting the need for the development of additional HIF inhibitors or combinations for patients with RCC with mutant *VHL*.

SRC kinase inhibition studies[206]. Similarly, in models of intraocular neovascularization, a reduction in leakage but not in angiogenesis was described in Tyr949Phe/Tyr949Phe mice compared to wild type[207,211]. Recently, it was reported that targeting syndecan 2, a transmembrane HSPG implicated in a variety of biological processes[212,213], including VEGF-dependent vascular sprouting in the zebrafish[214], inhibits VEGF-induced permeability (but not angiogenesis) in the mouse through selective dephosphorylation of Tyr949 (ref. 215). In a model of acute hypoxia resulting from middle cerebral artery occlusion, inhibiting syndecan 2 with antibodies was reported to reduce tissue damage[215], consistent with earlier studies in which a VEGFR1–Fc fusion protein was used[216]. Although inhibitors that fully block the VEGF pathways have been reported to have, in general, more robust effects[177,217–219], it has been argued that selective targeting of the pTyr949-dependent pathway may be preferable in order to avoid the potential toxicities of full VEGF blockade[207,211]. It is noteworthy that the pTyr949-dependent pathway is not the only pathway implicated in VEGF-induced permeability. Earlier studies reported suppression of both permeability and EC proliferation by PKCβ inhibitors[107,220]. As noted, PKCβ is implicated in the VEGFR2 pTyr1173-dependent pathway[108]. These effects can be reconciled by the notion that uncontrolled VEGF induces growth of pathological vessels having multiple abnormalities that render them inherently leaky, largely accounting for the high permeability of these vessels[221]. Indeed, treatment of tumour-bearing mice with VEGF-neutralizing antibodies resulted in the reduction of vascular permeability, an effect that was mediated by significant remodelling of the abnormal vasculature[222]. In summary, while the acute effects of VEGF on vascular permeability are dependent on the Tyr949 pathway,

chronic permeability appears critically dependent on engagement of the proliferative pathways.

Angiogenesis in regenerative medicine

Regenerative medicine is the branch of medicine that uses stem cell therapy and tissue engineering to lead healing or replacement of damaged tissues or organs[223]. In this context, the development of blood vessels is key to the successful delivery of nutrients and oxygen to tissues or organs. Moreover, induction of angiogenesis is required for the successful development and integration of blood vessels into transplanted tissues. As such, therapeutic angiogenesis, that is, the administration of angiogenic factors, delivered as recombinant proteins or by gene therapy, is a promising strategy for the treatment of disorders with insufficient blood perfusion such as peripheral vascular disease or coronary artery disease[224] (Fig. 5a). VEGF has been implicated as a mediator of wound healing[225]. Studies in various preclinical models, including a porcine model of chronic myocardial ischaemia thought to be clinically relevant, reported dramatic benefits, including reduction in infarct size, after administration of low amounts of VEGF, thus encouraging clinical trials[226,227]. However, trials testing coronary infusion of $VEGF_{165}$ in patients with myocardial ischaemia failed to show improvements compared to placebo[228]. The reasons for such disappointing results have been extensively debated but, among various possibilities, it is conceivable that young and healthy animals inadequately modelled the extensive atherosclerotic disease observed in patients[229].

New approaches are being attempted, including the construction of tissue-engineered blood vessels (TEBVs) (Fig. 5b). TEBVs serve as vascular substitutes for bypass grafts and can potentially be applied for the treatment of diseases such as atherosclerosis[230]. The structural scaffold for the generation of TEBVs can be made from synthetic materials or from animal biopsies, in which ECs are cultured. To support vascular formation, VEGF (or another growth factor) was encapsulated within the scaffolds, incorporated through genetically engineered domains, or bound electrostatically to synthetic and natural polymers[231]. Another method for the generation of blood vessels involves stem cell-based therapies (Fig. 5c). Adult, embryonic and induced pluripotent stem cells could have the potential to differentiate into ECs and secrete angiogenic factors in the surrounding tissues[232,233]. However, challenges in delivery methods and immune responses remain significant problems.

Pro-angiogenic strategies also involve the activation of the unfolded protein response (UPR) pathway. The UPR is activated when unfolded proteins accumulate in the endoplasmic reticulum, and several pathways are involved during the process, including the PERK, IRE1α (encoded by *Ern1*) and ATF6 pathways[234]. Modulating these

Box 2

Anti-angiogenic therapy in eye diseases

In 1948, the existence of a diffusible angiogenic factor (factor X) in the eyes of patients with diabetic retinopathy was hypothesized[276]. In 1994, high vascular endothelial growth factor (VEGF) levels were measured in eye fluids from patients with proliferative retinopathies secondary to diabetes and other conditions[191,192]. Additionally, high *VEGFA* expression was reported in tissues associated with neovascular age-related macular degeneration (AMD) (reviewed in ref. 276), and VEGF inhibitors were shown to suppress angiogenesis in models of retinal ischaemia and choroidal neovascularization[276]. This body of evidence provided a rationale for testing anti-VEGF agents in intraocular neovascular disorders. Indeed, several anti-VEGF agents were tested and then approved for the treatment of these diseases. These initially included pegaptanib, an aptamer, and ranibizumab, a humanized Fab antibody fragment targeting all isoforms of VEGF[7,277]. Later, aflibercept was approved by the FDA for the treatment of neovascular AMD, diabetic macular oedema and retinal vein occlusion. However, despite the prediction that aflibercept would reduce the number of intravitreal injections needed for the treatment of patients with neovascular AMD, recent studies suggest that it is not the case[278]. Bevacizumab is widely used off-label for the treatment of neovascular AMD, diabetic macular oedema and retinal vein occlusion, although it only has FDA approval for the treatment of cancer. Although administration of soluble VEGF receptor 1 (sVEGFR1) through adenoviral delivery in mice reported dramatic retinal atrophy[279], subsequent studies employing multiple approaches to inhibit VEGF action in the retina did not show such negative effects[280]. There is a need to develop agents with longer-lasting therapeutic effects after intraocular injection, with a number of approaches to this end having been attempted[7]. For example, heparin-binding VEGFR1 variants have been recently shown to have greater efficacy in mouse models of neovascularization than standard of care owing to higher retention in the eye[281]. Other anti-VEGF agents that have been recently approved by the FDA as therapeutics for multiple intraocular neovascular disorders include brolucizumab, a single-chain variable fragment that targets VEGFA, and faricimab, a bispecific antibody that targets VEGFA and angiopoietin 2 (ref. 7). Unexpectedly, recent studies have identified serious side effects after administration of brolucizumab in the form of a rare retinal vasculitis or retinal vascular occlusion that may lead to blindness, resulting from a high-affinity immune response[282]. Therefore, despite remarkable clinical efficacy[283], this drug is rarely used today. Other agents, including DARPins (genetically engineered antibody mimetic proteins exhibiting highly specific and high-affinity target protein binding), biosimilars of approved anti-VEGF agents or gene therapy with agents targeting VEGF, are being investigated for neovascular AMD[7]. Moreover, several efforts are currently focused on optimizing the delivery method of anti-VEGF therapies to reduce the number of injections needed in patients with neovascular AMD[7,281]. Given the important role of VEGFR3 not only in lymphangiogenesis but also in tumour progression, interest in developing anti-VEGFR3 therapies has increased. One such therapy is OPT-302, a soluble receptor consisting of domains 1–3 of VEGFR3, fused to Fc IgG, that neutralizes VEGFC and VEGFD. In a phase II study, this molecule has shown potential clinical benefits in patients with wet AMD in combination with ranibizumab[284].

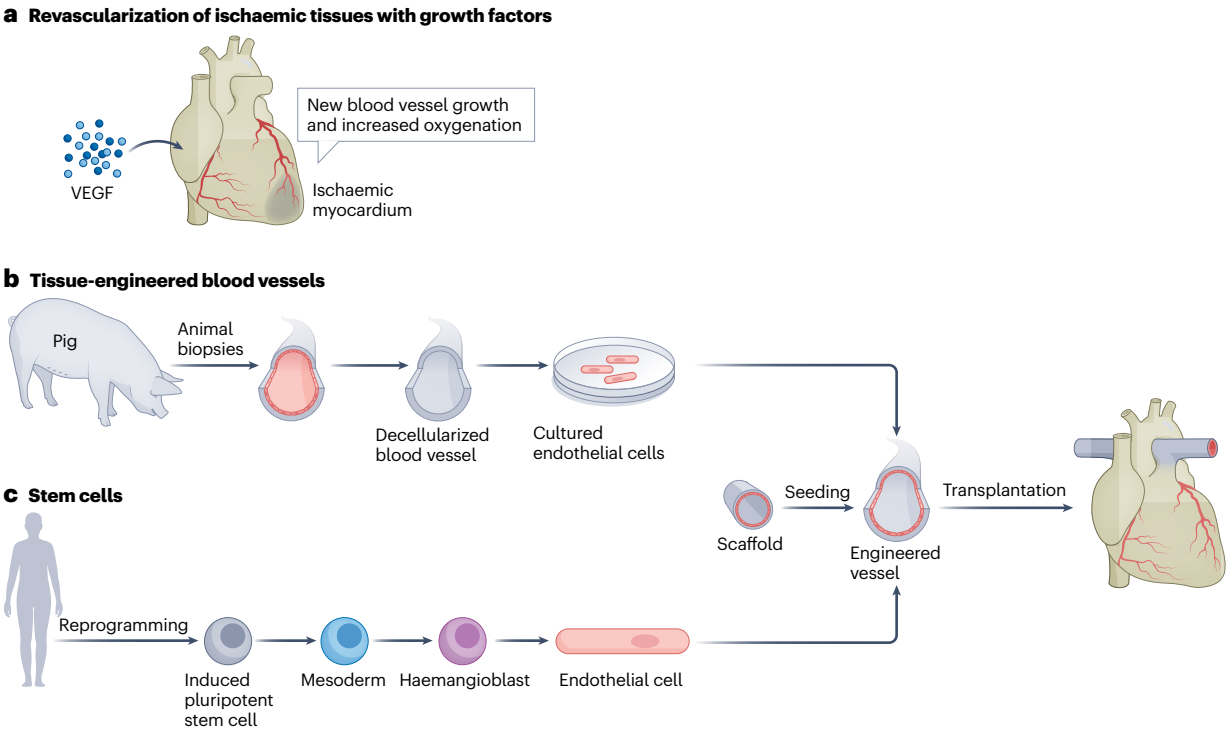

Fig. 5 | Role of VEGF in regenerative medicine. Several strategies have been attempted for the development of blood vessels within tissues. **a**, To promote revascularization of the ischaemic myocardium, treatment with growth factors, such as vascular endothelial growth factor (VEGF), has been attempted. **b**, New approaches are being tried, including the construction of tissue-engineered blood vessels (TEBVs). TEBVs can be made from synthetic materials or from animal biopsies in which, following decellularization, endothelial cells are cultured with the support of VEGF. **c**, Another method for the generation of blood vessels within tissues involves stem cell-based therapies, in which the mesoderm differentiates into the common precursor for endothelial and haematopoietic cells, that is, haemangioblasts, and then acquires endothelial cell properties to promote angiogenesis in the surrounding tissues. TEBVs and stem cell-based therapies could be incorporated into organs-on-chips, which are systems containing engineered or natural miniature tissues grown inside microfluidic chips. These devices provide the capability of recapitulating the in vivo vasculature, reducing the need for the use of animal models.

pathways is currently a focus of regenerative medicine[235]. The first evidence suggesting a role of the UPR during angiogenesis was shown through *Ern1*-knockout studies. *Ern1*$^{-/-}$ mice die at E12.5 and display severe developmental deficiencies[236]. Later studies showed that loss of IRE1α led to a reduction in VEGF and severe dysfunction of the placenta, suggesting a role for this pathway in fetal development by controlling angiogenesis in the placenta[237]. In the adult, hypoxia and nutrient deprivation drive both the activation of UPR pathways and angiogenesis. Activation of the UPR also contributes to the survival effect of VEGF on ECs by positively regulating mTORC2-mediated phosphorylation of AKT[238]. By contrast, a transcription factor downstream of PERK, IRE1α and ATF6 pathways, CCAAT/enhancer binding protein homologous protein (CHOP), transcriptionally represses endothelial nitric oxide synthase (eNOS) and NO production, which supports angiogenic sprouting, permeability and blood flow[239]. As such, depletion of CHOP promotes angiogenesis. Moreover, recent studies have shown that activation of the UPR may have therapeutic benefits. The hexosamine D-mannosamine (ManN) was reported to stimulate EC growth and angiogenesis through activation of the UPR pathways[240]. Briefly, ManN inhibited glycosylation in ECs, leading to activation of the UPR and stimulation of pro-angiogenic signalling pathways such as the JNK pathway. Of note, as opposed to the previously established roles of UPR in the control of VEGF, the effects of ManN on ECs were independent of VEGFR2 (ref. 240). Nevertheless, ManN provides a link between EC metabolism and endoplasmic reticulum stress in the regulation of angiogenesis[7]. Therefore, targeting the different arms of the UPR might be useful for the treatment of cancers and retinopathies[241,242].

Angiogenesis and ageing

Angiogenesis is impaired during ageing, which is the main risk factor for cardiovascular diseases. Older individuals have been shown to have reduced capillary density and angiogenesis in response to ischaemia[243]. This can be explained through the association between angiogenesis-related and ageing-related pathways[244]. For example, EC senescence can be inhibited by NO, whereby eNOS promotes telomerase activity[245]. eNOS function can also be influenced by a redox imbalance in ECs, which occurs in response to ageing[246]. HIF1α is also impaired during ageing, although the exact mechanisms remain elusive. Interestingly, exercise appears to re-establish the activity of HIF1α in aged animals whereas, in sedentary aged animals, multiple mechanisms contribute to reducing HIF1α activity[247].

Recent studies suggest that VEGF signalling insufficiency, caused by increased production of sVEGFR1, drives physiological ageing across multiple organs[248]. VEGF supplementation, through gain-of-function mouse models or adeno-associated virus-assisted

transduction, provided protection from age-related capillary loss, compromised perfusion and reduced tissue oxygenation[248]. Hallmarks of ageing, such as mitochondrial dysfunction, compromised metabolism, EC senescence, and age-related increase in the levels of pro-inflammatory markers in blood and tissues (inflammageing), were decreased in VEGF-treated mice, suggesting that VEGF signalling impairment is an important driver of organ ageing. However, further studies are required to determine whether these intriguing findings can be extended to humans[249]. Nevertheless, a fine balance of VEGF levels is critical in development, physiology and pathology, raising questions about the potential risks of long-term VEGF administration. For instance, VEGF overexpression in aged mice has been reported to result in cardiac hypertrophy[250].

Conclusions and perspectives

Decades of research have established VEGFA as a key regulator of angiogenesis[7]. While VEGF is strongly implicated in normal development[251], wound healing[225] and reproductive processes[148], its role in adult organ homeostasis has been less clear. Although previous studies reported that FGFR signalling is essential for EC integrity and organ homeostasis[252], this conclusion has been challenged[253]. Indeed it is more likely that VEGFR signalling is key to endothelial maintenance and homeostasis[254]. Recent studies have established, through EC-specific VEGFR deletions, that the VEGF family is essential for the maintenance of adult organ homeostasis[255]. Additionally, while VEGF has been shown to be pro-angiogenic in most contexts, the existence of anti-angiogenic isoforms has also been reported. Nevertheless, these isoforms are weak agonists rather than antagonists, and even their very existence is disputed[43]. By contrast, full-length VEGFR1 and sVEGFR1 have emerged as key negative regulators of VEGF signalling and, through their role as decoy receptors, they sequester VEGF and make it less available for VEGFR2 activation. In particular, sVEGFR1 has been implicated in the pathogenesis of pre-eclampsia and, more recently, of ageing. Furthermore, recent studies have reported that treatment with an anti-VEGFR1 antibody, which displaces VEGF bound to VEGFR1, improves muscle function and angiogenesis in a mouse model of Duchenne muscular dystrophy[256]. In this case, anti-VEGFR1 administration made sequestered VEGF more available to bind VEGFR2 as postulated in early studies[80], further emphasizing the therapeutic significance of this receptor.

Twenty years after its initial FDA approval for colorectal cancer, anti-VEGF remains a widely used anticancer approach and a particularly effective treatment for intraocular neovascular disorders (Boxes 1 and 2). However, several challenges still need to be addressed, including resistance and delivery methods. Additionally, the lack of predictive biomarkers for appropriately selecting patients and/or assessing anti-VEGF responsiveness has hindered the implementation of anti-VEGF therapeutics (reviewed in ref. 7). Nevertheless, bevacizumab and other anti-VEGF agents have proven their utility in multiple cancer types and, over the last few years, in combination with immune-checkpoint inhibitors, have advanced the treatment of difficult-to-treat malignancies[257–259].

The major clinical setbacks of VEGF-based approaches have been in therapeutic angiogenesis (promoting angiogenesis to increase tissue oxygenation). Despite the fact that preclinical studies predicted striking efficacy[226,227], clinical trials in patients with myocardial ischaemia failed to show any benefit[228]. It is tempting to speculate that 'angiocrine' factors, which are released by ECs in a tissue-specific or organ-specific manner, may be helpful[95,260,261]. Additionally, growth or survival factors selective for specific ECs have been described, which may be instrumental to the development of organ-specific or tissue-specific therapies[151,262]. Finally, UPR activation might offer an alternative pro-angiogenic approach by linking EC metabolism to endoplasmic reticulum stress[240].

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Both authors wrote the article and critically discussed the contents.

Competing interests

N.F. is a co-founder of Theia Therapeutics and NVasc. L.P.-G. declares no competing interests.

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