Published in final edited form as:
Nature. 2011 May 19; 473(7347): 298–307. doi:10.1038/nature10144.

Molecular mechanisms and clinical applications of angiogenesis

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Abstract

Blood vessels deliver oxygen and nutrients to every part of the body, but also nourish diseases such as cancer. Over the past decade, our understanding of the molecular mechanisms of angiogenesis (blood vessel growth) has increased at an explosive rate and has led to the approval of anti-angiogenic drugs for cancer and eye diseases. So far, hundreds of thousands of patients have benefited from blockers of the angiogenic protein vascular endothelial growth factor, but limited efficacy and resistance remain outstanding problems. Recent preclinical and clinical studies have shown new molecular targets and principles, which may provide avenues for improving the therapeutic benefit from anti-angiogenic strategies.

Blood vessels arose in evolution to allow haematopoietic cells to patrol the organism for immune surveillance, to supply oxygen and nutrients and to dispose of waste. Vessels also produce instructive signals for organogenesis in a perfusion-independent manner (Box 1). Although beneficial for tissue growth and regeneration, vessels can fuel inflammatory and malignant diseases, and are exploited by tumour cells to metastasize and kill patients with cancer. Because vessels nourish nearly every organ of the body, deviations from normal vessel growth contribute to numerous diseases. To name just a few, insufficient vessel growth or maintenance can lead to stroke, myocardial infarction, ulcerative disorders and neurodegeneration, and abnormal vessel growth or remodelling fuels cancer, inflammatory disorders, pulmonary hypertension and blinding eye diseases[1,2].

Several modes of vessel formation have been identified (Fig. 1). In the developing mammalian embryo, angioblasts differentiate into endothelial cells, which assemble into a vascular labyrinth — a process known as vasculogenesis (Fig. 1b). Distinct signals specify

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arterial or venous differentiation[3]. Subsequent sprouting ensures expansion of the vascular network, known as angiogenesis (Fig. 1a). Arteriogenesis then occurs, in which endothelial cell channels become covered by pericytes or vascular smooth muscle cells (VSMCs), which provide stability and control perfusion. Tissues can also become vascularized by other mechanisms, but the relevance of these processes is not well understood. For example, pre-existing vessels can split by a process known as intussusception, giving rise to daughter vessels (Fig. 1c). In other cases, vessel co-option occurs, in which tumour cells hijack the existing vasculature (Fig. 1d), or tumour cells can line vessels — a phenomenon known as vascular mimicry (Fig. 1e). Putative cancer stem-like cells can even generate tumour endothelium[4] (Fig. 1f). Although debated, the repair of healthy adult vessels or the expansion of pathological vessels can be aided by the recruitment of bone-marrow-derived cells (BMDCs) and/or endothelial progenitor cells to the vascular wall. The progenitor cells then become incorporated into the endothelial lining in a process known as postnatal vasculogenesis. Collateral vessels, which bring bulk flow to ischaemic tissues during revascularization, enlarge in size by distinct mechanisms, such as the attraction and activation of myeloid cells[5].

The revascularization of ischaemic tissues would benefit millions, but therapeutic angiogenesis remains an unmet medical need. Instead, more success has been achieved by targeting the vascular supply in cancer and eye diseases[6]. In this Review, we describe key molecular targets in angiogenesis and discuss the clinical experience with the most widely used class of anti-angiogenic agent — blockers of vascular endothelial growth factor (VEGF, also known as vascular permeability factor or VPF). Rather than providing an encyclopaedic survey, we focus on some of the recently discovered mechanisms and principles, and on targets with translational potential.

Vessel branching, maturation and quiescence

We first provide the current view of the sequential steps of vessel branching (quiescence, activation and resolution), before discussing the molecular players involved in more depth (Fig. 2). In a healthy adult, quiescent endothelial cells have long half-lives and are protected against insults by the autocrine action of maintenance signals such as VEGF, NOTCH, angiopoietin-1 (ANG-1) and fibroblast growth factors (FGFs). Because vessels supply oxygen, endothelial cells are equipped with oxygen sensors and hypoxia-inducible factors — such as prolyl hydroxylase domain 2 (PHD2) and hypoxia-inducible factor-2α (HIF-2α), respectively — which allow the vessels to re-adjust their shape to optimize blood flow. Quiescent endothelial cells form a monolayer of phalanx cells with a streamlined surface, interconnected by junctional molecules such as VE-cadherin and claudins. These endothelial cells are ensheathed by pericytes, which suppress endothelial cell proliferation and release cell-survival signals such as VEGF and ANG-1. Endothelial cells and pericytes at rest produce a common basement membrane.

When a quiescent vessel senses an angiogenic signal, such as VEGF, VEGF-C, ANG-2, FGFs or chemokines, released by a hypoxic, inflammatory or tumour cell, pericytes first detach from the vessel wall (in response to ANG-2) and liberate themselves from the basement membrane by proteolytic degradation, which is mediated by matrix

metalloproteinases (MMPs) (Fig. 2a). Endothelial cells loosen their junctions, and the nascent vessel dilates. VEGF increases the permeability of the endothelial cell layer, causing plasma proteins to extravasate and lay down a provisional extracellular matrix (ECM) scaffold. In response to integrin signalling, endothelial cells migrate onto this ECM surface. Proteases liberate angiogenic molecules stored in the ECM such as VEGF and FGF, and remodel the ECM into an angio-competent milieu. To build a perfused tube and prevent endothelial cells from moving en masse towards the angiogenic signal, one endothelial cell, known as the tip cell, becomes selected to lead the tip in the presence of factors such as VEGF receptors, neuropilins (NRPs) and the NOTCH ligands DLL4 and JAGGED1 (Fig. 2a). The neighbours of the tip cell assume subsidiary positions as stalk cells, which divide to elongate the stalk (stimulated by NOTCH, NOTCH-regulated ankyrin repeat protein (NRARP), WNTs, placental growth factor (PlGF) and FGFs) and establish the lumen (mediated by VE-cadherin, CD34, sialomucins, VEGF and hedgehog) (Fig. 2b). Tip cells are equipped with filopodia to sense environmental guidance cues such as ephrins and semaphorins, whereas stalk cells release molecules such as EGFL7 into the ECM to convey spatial information about the position of their neighbours, so that the stalk elongates. A hypoxia-inducible program, driven by HIF-1α, renders endothelial cells responsive to angiogenic signals. Myeloid bridge cells aid fusion with another vessel branch, allowing the initiation of blood flow. For a vessel to become functional, it must become mature and stable. Endothelial cells resume their quiescent phalanx state (Fig. 2c), and signals such as platelet-derived growth factor B (PDGF-B), ANG-1, transforming growth factor-β (TGF-β), ephrin-B2 and NOTCH cause the cells to become covered by pericytes. Protease inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1) cause the deposition of a basement membrane, and junctions are re-established to ensure optimal flow distribution. Vessels regress if they are unable to become perfused.

The VEGF family

Given the complexity of a process such as angiogenesis, it is remarkable that a single growth factor, VEGF, regulates this process so predominantly. The VEGF family consists of only a few members and distinguishes itself from other angiogenic superfamilies by the largely non-redundant roles of its members. VEGF (also known as VEGF-A) is the main component, and it stimulates angiogenesis in health and disease by signalling through VEGF receptor-2 (VEGFR-2, also known as FLK1)[7,8]. Neuropilins such as NRP1 and NRP2 are VEGF co-receptors, which enhance the activity of VEGFR-2, but also signal independently[9]. Similar to VEGFR-2 deficiency, the loss of VEGF aborts vascular development[2]. In response to a VEGF gradient, established by soluble and matrix-bound isoforms, tip cells upregulate DLL4 expression, which activates NOTCH in stalk cells; this downregulates VEGFR-2 expression, rendering stalk cells less responsive to VEGF, thereby ensuring that the tip cell takes the lead[10]. Soluble VEGF isoforms promote vessel enlargement, whereas matrix-bound isoforms stimulate branching. Paracrine VEGF, released by tumour, myeloid or other stromal cells, increases vessel branching and renders tumour vessels abnormal[11], whereas autocrine VEGF, released by endothelial cells, maintains vascular homeostasis[12]. Emerging evidence indicates that the biological effect of

VEGFR-2 signalling depends on its subcellular localization — for example, for VEGF to induce arterial morphogenesis, VEGFR-2 must signal from intracellular compartments[13]. Activating *VEGFR2* mutations cause vascular tumours, and genetic polymorphisms in *VEGF* and/or its receptors co-determine pathological angiogenesis[14,15], whereas the blockade of VEGF signalling can target angiogenic vessels in malignant and ocular disease in humans. VEGF protein or gene transfer stimulates vessel growth in ischaemic tissues, but often in association with undesired leakage and vessel abnormalities.

VEGF-C, a ligand of the VEGFR-2 and VEGFR-3 receptors, activates blood-vessel tip cells[16]. VEGFR-3 is necessary for the formation of the blood vasculature during early embryogenesis, but later becomes a key regulator of lymphangiogenesis — the formation of new lymphatic vessels from pre-existing ones[17]. In zebrafish, in which the first embryonic vein arises by segregation of venous-fated endothelial cells from a common precursor vessel, the sprouting of venous endothelial cells is restricted by VEGFR-2 but promoted by VEGFR-3 (ref. 18). Venous-derived angiogenesis in the arterial trunk also relies on VEGFR-3 signalling. Anti-VEGFR-3 antibodies that inhibit receptor dimerization or ligand binding slow down tumour growth synergistically, and enhance the inhibition of tumour growth by VEGFR-2 blockade, making VEGFR-3 another anti-angiogenic candidate[16].

Originally discovered as a VEGF homologue, PlGF was also expected to be an angiogenic factor. However, unlike VEGF, PlGF is dispensable for development and is relevant only in disease[19,20]. PlGF is a multitasking cytokine that stimulates angiogenesis by direct or indirect mechanisms, and also activates bone-marrow-derived endothelial progenitor and myeloid cells, as well as stromal cells, to create a nurturing ‘soil’ for tumour cells, in addition to activating tumour cells[19]. By skewing the polarization of tumour-associated macrophages (TAMs), the loss of PlGF improves vessel perfusion and maturation, and enhances responses to chemotherapy[21]. PlGF blockade by neutralizing anti-PlGF antibodies phenocopies the anti-angiogenic effects of genetic *Plgf* (also known as *Pgf*) deficiency in spontaneous mouse tumour models and diseases such as ocular neovascularization[22]. Yet other PlGF-blocking strategies fail to inhibit the growth of tumours in transplantable tumour models[23]. The therapeutic potential of PlGF blockade in patients with cancer thus remains to be established. In preclinical models, PlGF protein or gene delivery increases the revascularization of ischaemic tissues.

Deficiency of the VEGF family member VEGF-B in mice does not impair angiogenesis in normal development, and cannot compensate for VEGF blockade after birth[19]. VEGF-B has only restricted angiogenic activity in certain tissues such as the heart, yet it promotes neuronal survival and induces metabolic effects[19,24]. Divergent effects of VEGF-B on pathological angiogenesis have been reported, and it has been shown to promote the growth of cardiac vessels, without inducing adverse effects such as increased permeability or leakage[25].

The precise role of the VEGFR-1 receptor (also known as FLT-1) in angiogenesis remains elusive[19,26]. VEGFR-1 exists both as a membrane-anchored signalling-competent form and as a soluble secreted form (also known as sFLT-1). By trapping its ligands, sFLT-1 can assist the guidance of the emerging branch or inhibit sprouting altogether. Because of its

weak tyrosine kinase activity, VEGFR-1 may act as a decoy for VEGF, moderating the amount of free VEGF available to activate VEGFR-2 and explaining why VEGFR-1 loss results in vessel overgrowth[19]. However, intracellular VEGFR-1 signalling in angiogenic endothelial, stromal and myeloid cells stimulates pathological angiogenesis[26]. VEGFR-1 signalling also promotes the growth of VEGFR-1$^+$ tumour cells in response to autocrine VEGF production in an angiogenesis-independent manner[27], and upregulates MMP9 in endothelial cells at the premetastatic site. There is evidence to suggest that VEGFR-1$^+$ haematopoietic progenitors form a premetastatic niche in distant organs, but this finding is debated[28,29]. Neutralizing anti-PlGF, anti-VEGFR-1 and anti-VEGFR-2 antibodies are in early clinical development.

The PDGF family

For vessels to function properly, they must be mature and covered by mural cells. Several growth-factor families, such as PDGFs, angiopoietins and TGF-β, contribute to this process[30]. To stabilize endothelial cell channels, angiogenic endothelial cells release PDGF-B to chemoattract PDGF receptor-β (PDGFR-β)$^+$ pericytes[31,32]. Hence, pericyte deficiency after PDGF-B ablation causes vessel leakage, tortuosity, microaneurysm formation and bleeding. Knockout of the genes encoding the PDGF-B protein retention motif (necessary for pericyte adhesion) in mice results in tumour vessel fragility and hyperdilation, whereas PDGFR-β-hypomorph mice have insufficient pericytes around brain vessels, leading to blood–brain barrier (BBB) defects and neurodegenerative damage owing to the leakage of toxic substances[33]. Tumour-derived PDGF-B also recruits pericytes indirectly by upregulating stromal-cell-derived factor-1α (SDF-1α; encoded by *CXCL12*). Besides a local origin, pericytes can also arise from perivascular PDGFR-β$^+$ pericyte progenitors, recruited from the bone marrow[34]. By inhibiting PDGFR-β signalling in mural cells, VEGF reduces pericyte coverage and renders tumour vessels abnormal.

PDGFR inhibition diminishes tumour growth by causing pericyte detachment, leading to immature vessels that are prone to regression[35]. Other pericyte-deficient mouse strains that lack the proteoglycan NG2 (also known as CSPG4) also form abnormal tumour vessels and smaller tumours. Paradoxically, the overexpression of PDGF-B in mice inhibits tumour growth by promoting pericyte recruitment and inducing endothelial cell growth arrest[36]. Because the survival of endothelial cells depends on pericyte VEGF production, pericytes protect endothelial cells from VEGF withdrawal and confer resistance to VEGF blockade. This protection requires a close endothelial-cell–pericyte interaction, as PDGF-B blockade reduces pericyte coverage and vessel number only when VEGF is produced by pericytes and not by more distant tumour cells[37]. Initial studies using multi-target receptor tyrosine kinase inhibitors (TKIs) showed that blocking PDGF-B renders mature vessels more sensitive to VEGF blockade by depleting the vessels of pericytes[31]. Recent studies with more specific inhibitors have shown that combination therapy is no more efficient than anti-VEGF monotherapy[38].

PDGFR-β$^+$ pericytes have a dual role in metastasis. In primary tumours, pericytes limit tumour cell intravasation, because the more loosely assembled vessel wall is no longer a barrier for disseminating tumour cells after depletion of pericytes[39]. The absence of

pericytes around vessels also correlates with metastasis in patients, and a trial evaluating PDGF-B blockade was aborted because of excessive leakage. These studies indicate that blocking vessel maturation can promote malignancy. However, other reports have shown that pericytes, co-opted by tumour cells at micrometastatic sites, allow tumour colonization by releasing angiogenic factors. Overall, future studies are needed to explore the benefits and risks of PDGF blockade for the treatment of cancer.

PDGF-B blockade may be used therapeutically for non-malignant vascular diseases such as pulmonary hypertension, whereas PDGF-B activation may offer therapeutic opportunities for stabilizing vascular malformations[40]. PDGF-CC, another family member released by cancer-associated fibroblasts in VEGF-inhibitor-resistant tumours, stimulates vessel growth and maturation, and attenuates the response to anti-VEGF treatment[41,42]. By preventing the activation of perivascular PDGFR-α^+ astrocytes, which together with pericytes constitute the BBB, the blockade of PDGF-CC preserves the integrity of the BBB during stroke. Inhibition of PDGF-DD suppresses ocular neovascularization, whereas PDGF-DD overexpression normalizes tumour vessels and improves drug delivery.

TGF-β signalling

Human hereditary haemorrhagic teleangiectasia is characterized by vascular malformations. Human genetic studies have shown that this disorder is due to mutations in the genes that encode endoglin (*ENG*) or activin receptor-like kinase (*ALK1*, also known as *ACVRL1)* — receptors of the TGF-β family. Mouse studies have confirmed that the loss of the TGF-β receptors ALK-1, TGFR-1 (also known as ALK-5), TGFR-2 or ENG results in arteriovenous malformations, reminiscent of those seen in patients with hereditary haemorrhagic teleangiectasia[43]. However, understanding the molecular basis of this pathway has been challenging owing to inconsistent results. This is partly due to the context-dependent pro- and anti-angiogenic effects of TGF-β family members. Furthermore, although TGF-β promotes VSMC differentiation, and deficiency of ENG or ALK-1 impairs mural cell development, it remains unclear whether other TGF-β components mediate their vascular effects *in vivo* by means of endothelial cells or VSMCs[43]. Preclinical studies have shown that antibodies against ENG or ALK-1 can inhibit tumour angiogenesis and growth. Several TGF-β blockers are now in early-phase clinical trials.

The FGF superfamily

The superfamily of FGFs and their receptors controls a wide range of biological functions[44]. bFGF was among the first discovered angiogenic factors and, like FGF1, has angiogenic and arteriogenic properties; FGF9 stimulates angiogenesis in bone repair. FGFs activate receptors (FGFRs) on endothelial cells or indirectly stimulate angiogenesis by inducing the release of angiogenic factors from other cell types[44]. For instance, in the heart, FGF-mediated signalling fuels vessel growth by stimulating the release of hedgehog, ANG-2 and VEGF-B. Low levels of FGF are required for the maintenance of vascular integrity, as inhibition of FGFR signalling in quiescent endothelial cells causes vessel disintegration[45]. Aberrant FGF signalling promotes tumour angiogenesis and mediates the escape of tumour vascularization from VEGF- or epidermal growth factor receptor (EGFR)-inhibitor

treatment[46]. Development of specific FGF or FGFR inhibitors for blocking angiogenesis is lagging behind, partly because *Fgf1* or *Fgf2* deficiency in mice did not produce vascular defects and the FGF superfamily shows substantial redundancy[44]. FGF protein or gene transfer has been tested for therapeutic angiogenesis, but without sustained success in the clinic.

The ANG and TIE signalling system

Healthy vessels must be equipped with mechanisms to maintain quiescence, while remaining able to respond to angiogenic stimuli. The ANG and TIE family is a binary system to offer such a switch. The human ANG family consists of two receptors, TIE-1 and TIE-2, and three ligands, ANG-1, ANG-2 and ANG-4. ANG-1 functions as a TIE-2 agonist, and ANG-2 functions as a competitive ANG-1 antagonist in a context-dependent manner (ANG-4 has not been as well studied, but is thought to act like ANG-1). Because no ligand for TIE-1 has been identified, this orphan receptor may act as a negative regulator of TIE-2, but its precise role remains elusive[47]. ANG-1 is expressed by mural and tumour cells, whereas ANG-2 is released from angiogenic tip cells. In confluent endothelium, ANG-1 induces TIE-2 clustering *in trans* at cell–cell junctions to maintain endothelial cell quiescence[48]. ANG-1 also stimulates mural coverage and basement membrane deposition, thereby promoting vessel tightness. In the presence of angiogenic stimulators, sprouting endothelial cells release ANG-2, which antagonizes ANG-1 and TIE-2 signalling to enhance mural cell detachment, vascular perm eability and endothelial cell sprouting[47]. In accordance, *Tie2* (also known as *Tek*) deficiency in mice causes vascular defects, and activating germline and somatic *TIE2* (*TEK*) mutations in humans result in venous malformations. Tumour-derived ANG-2 also promotes angiogenesis by recruiting pro-angiogenic TIE-2-expressing monocytes (TEMs)[49].

The overall effects of the ANG–TIE system on tumours are context dependent[47]. ANG-1 stimulates tumour growth by promoting endothelial cell survival and vessel maturation, but it also inhibits tumour cell extravasation and maintains the integrity of healthy vessels outside tumours. These conflicting biological activities warrant caution when considering ANG-1 as an anticancer target. Instead, ANG-2 may be a more appealing therapeutic target because it stimulates tumour angiogenesis and recruits pro-angiogenic TEMs, and ANG-2 inhibition promotes vessel regression and normalization[50]. Given that ANG-2 and VEGF cooperatively increase angiogenesis, co-blockade of VEGF and ANGs is superior in inhibiting tumour angiogenesis, metastasis and leakage[51]. Various agents that block either TIE-2 or ANG-2 are being evaluated in early-phase clinical trials.

The NOTCH and WNT signalling pathway

The vessel-branching model postulates that, in general, tip cells migrate and stalk cells proliferate. Recent studies have implicated NOTCH signalling in this model[10]. In response to VEGF, activation of VEGFR-2 upregulates DLL4 expression in tip cells. In neighbouring stalk cells, DLL4 then activates NOTCH, which downregulates VEGFR-2 but upregulates VEGFR-1; thus, the stalk cells become less responsive to the sprouting activity of VEGF but more sensitive to molecules such as PlGF. Overall, DLL4 and NOTCH signalling restricts

branching but generates perfused vessels[10]. By upregulating PDGFR-β in NOTCH$^+$ mural cells, DLL4 in endothelial cells also stimulates vessel maturation. JAGGED1, another NOTCH ligand expressed by stalk cells, promotes tip-cell selection by interfering with the reciprocal DLL4 and NOTCH signalling from the stalk cell to the tip cell[52]. NOTCH signalling in stalk cells is dynamic over time, because it upregulates its own inhibitor, NRARP[53].

An unanticipated complexity is that endothelial cells continuously compete for the tip-cell position by fine-tuning their expression of VEGFR-2 versus VEGFR-1, indicating that this signalling circuit is constantly re-evaluated as cells meet new neighbours[54]. In accordance, the inhibition of DLL4 and NOTCH signalling induces the formation of more numerous but hypoperfused vessels, resulting in tumour hypoxia and growth inhibition[55]. However, chronic DLL4 blockage in healthy animals results in vascular neoplasms[56], and endothelial cell inactivation of RBP-J, a transcription factor downstream of NOTCH, also leads to uncontrolled angiogenesis. Although these data indicate that quiescent phalanx cells need low-level NOTCH signalling, they also warrant caution against the indiscriminate use of DLL4 and NOTCH inhibitors for the treatment of cancer. Signalling by the hedgehog family members also participates in embryonic vasculogenesis, vascular morphogenesis and tube formation, as well as in arterial specification, by regulating NOTCH expression[3].

Endothelial cells express various types of WNT ligand and their frizzled (FZD) receptors, of which several stimulate endothelial cell proliferation. NOTCH activates WNT signalling in proliferating stalk cells during vessel branching[53], explaining why NOTCH, which usually suppresses proliferation and promotes quiescence, stimulates proliferation of stalk cells *in vivo*. WNT also activates NOTCH in a reciprocal-feedback system, because WNT signals in endothelial cells induce a NOTCH-like phenotype, characterized by branching defects, loss of venous identity and aberrant vascular remodelling[57]. Gene-inactivation of some of the WNT and FZD members in mice (*Wnt2, Wnt5a, Fzd4* and *Fzd5*) causes vascular defects, whereas the combined loss of *Wnt7a* and *Wnt7b* impairs brain angiogenesis and BBB formation[58]. Because some WNT members inhibit angiogenesis, specific blockers of these proteins will be required.

Integrins and proteases

The ECM provides a physical link between vascular cells and their surrounding tissues. Endothelial cells possess mechanisms to interact with and alter the matrix. Integrins are heterodimeric receptors that mediate adhesion to ECM and immunoglobulin superfamily molecules[59,60]. Upregulation of the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ permits growing endothelial cells to bind to provisional matrix proteins in the tumour milieu; these proteins include vitronectin, fibrinogen and fibronectin, both in native and degraded forms. These adhesive interactions provide survival cues and traction for invading endothelial cells. Other integrins involved in angiogenesis include $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_9\beta_1$ and $\alpha_6\beta_4$ (refs 59, 60).

In addition to signalling induced by ligating ECM components, integrins regulate angiogenesis by other mechanisms. Given their ability to interact with several extracellular molecules and transmit signals in a bidirectional manner, integrins function as ‘hubs’,

orchestrating endothelial cell and VSMC behaviour during angiogenesis[59,61]. Hence, the binding of integrins to growth factors (such as VEGF, FGFs and ANG-1) or their receptors (VEGFR-2 and FGFRs) stimulates vessel growth. Integrins also upregulate and activate zymogen proteases in invading tip cells, and promote vessel maturation by regulating interactions between endothelial cells, pericytes and the basement membrane. Other integrins promote the adhesion of angiogenic BMDCs to tumour endothelial cells. Recent studies have highlighted the complexities in understanding the role of $\alpha_v\beta_3$ in pathological angiogenesis, as tumour angiogenesis was stimulated by gene deficiency in mice but inhibited by pharmacological blockade[59,60]. Nonetheless, integrin blockers are now being evaluated in the clinic.

Quiescent endothelial cells and pericytes share a common basement membrane, which not only physically restrains these cells but also keeps them quiescent owing to the antiproliferative properties of the ECM components. During branching, proteolytic remodelling of the ECM liberates these cells for unrestricted movement and converts the characteristics of the basement membrane into a pro-angiogenic environment. Distinct proteases such as MMPs modulate angiogenesis by several mechanisms[62]. They promote endothelial cell migration and tube formation by proteolytically remodelling the basement membrane, by executing directional matrix proteolysis (membrane type 1-MMP) or by exposing chemotactic cryptic motifs sites in the ECM. MMPs and plasmin also liberate angiogenic factors such as VEGF and FGF from immobilized matrix stores[63]. VEGF isoforms that are cleaved by MMPs (and therefore soluble) preferentially enlarge vessels, whereas MMP-resistant matrix-bound VEGF supports vessel branching[64]. Macrophages, neutrophils and mast cells initiate angiogenesis by MMP9-mediated activation of VEGF[65,66]. Proteases such as MMP9 also participate in the mobilization of progenitors from the bone marrow by shedding soluble forms of membrane-bound cytokines (such as KIT ligand; also known as stem-cell factor or SCF)[67]. MMPs establish a premetastatic niche by allowing the recruitment of marrow progenitors[29]. Given their destructive potential, the activity of proteases must be tightly controlled. For instance, loss of the inhibitor PAI-1 prevents vessel branching because excessive ECM breakdown leaves no matrix support for the sprout[68]. In addition, basement membrane deposition during vessel maturation requires the activity of MMP inhibitors such as TIMPs. Because degradation of ECM components can also generate anti-angiogenic fragments such as tumstatin and angiostatin[1], protease inhibitors must be judiciously evaluated for biological effects.

Junctional molecules

Cell–cell communication is fundamental for vessels to act as a synchronized unit along their longitudinal axis. Such coordination is accomplished by cell–cell communication through gap junctions, established by connexins, which inform upstream feeding vessels about the perfusion status of downstream tissues to prevent shunting, a well-known defect in tumour vessels[69]. Apart from these long-range communication junctions, endothelial cells and pericytes have junctions for short-range communication.

Quiescent endothelial cells form a monolayer of interconnected cells, whereas angiogenic endothelial cells dissociate their junctions to migrate. The tight junctional molecules

claudins, occludins and junctional adhesion molecules maintain barriers, such as the BBB, whereas adherens junctions establish cell–cell adhesion, cytoskeleton remodelling and intracellular signalling[70]. Loss of VE-cadherin does not prevent vessel development, but induces defects in vascular remodelling and integrity[2]. VE-cadherin is also required for localizing CD34 and its sialomucin receptor to cell–cell contacts for lumen formation[71]. In quiescent phalanx endothelial cells, VE-cadherin promotes vessel stabilization by inhibiting VEGFR-2 signalling while activating TGFR pathways. Notably, oxygen sensors control VE-cadherin expression in a feedback loop, so vessel perfusion can be optimized when the oxygen supply is insufficient[72]. N-cadherin stabilizes contacts between endo thelial cells and pericytes. During sprouting, the adhesive function of VE-cadherin between adjacent cells is reduced by endocytosis in response to VEGF and angiogenic factors[70]. At the same time, the localization of VE-cadherin at filopodia allows tip cells to establish new contacts with cells on outreaching sprouts. Antibodies, recognizing neoepitopes of VE-cadherin that are exposed after dissociation of adherens junctions during sprouting, offer opportunities for selective blockage of endothelial cell growth without affecting endothelial cell maintenance.

Chemokines and G-protein-coupled receptors

Chemokines regulate angiogenesis by recruiting pro-angiogenic immune cells and endothelial progenitor cells, or through the direct activation of endothelial G-protein-coupled chemokine receptors (GPCRs). A well-known chemokine is SDF-1α, which binds to its receptor CXCR4 on tip cells[73]. SDF-1α is upregulated by HIF-1α in hypoxia, and supports mobilization and retention of pro-angiogenic $CXCR4^+$ BMDCs to promote revascularization of ischaemic organs. Cancer-associated fibroblasts also release SDF-1α. Another chemokine is the biologically active lipid sphingosine-1-phosphate (S1P), which binds to the S1P family of G-protein-coupled receptors (S1PRs) and regulates endothelial cell barrier function, vessel stability and angiogenesis, in part by crosstalking to PDGF and VEGF receptors — a process known as GPCR-jacking[74]. Inhibitors of SDF-1α, CXCR4 and S1P are being developed for cancer treatment[73]. Another recently identified GPCR is GPR124, which regulates BBB differentiation[75].

Other pathways and challenges in translation

Other pathways also regulate angiogenesis, some of which provide guidance signals to navigating tip cells (Box 2). Given the ancestral function of vessels to supply oxygen, vessel formation is under the control of oxygen sensors (Box 3). As already mentioned, various anti-angiogenic avenues in addition to VEGF blockade are under development[14]. A challenge for the future will be to identify optimal treatment regimens for these agents, either as monotherapy or in combination with VEGF blockade.

A major hurdle in translating the above-mentioned insights into clinically successful treatments stems from the fact that various anti-angiogenic approaches have different effects or are more effective in preclinical than clinical settings. This divergence may be due to several factors. First, most preclinical studies examine the effect of anti-angiogenic agents on transplantable, rapidly growing primary tumours, whereas most anti-angiogenic drugs have been approved for spontaneously arising, slowly evolving cancers in metastatic settings

or for advanced disease in patients. The spontaneous tumour models in genetically engineered mice also do not recapitulate various aspects of the human disease. Differences in malignancy, vascularization and the stromal microenvironment between humans and mice lead to different responses. Second, only a few preclinical studies have analysed the effects of anti-angiogenic agents in residual disease after cytoablative therapy or in the adjuvant setting, and often without chemotherapy. Third, the doses used in preclinical mouse studies are often higher than those given to patients, resulting in more pronounced antivascular and antitumour effects in mice. Fourth, most genetic studies use mice in which the relevant angiogenic gene has been deleted before tumours become established, which is different from pharmacological intervention in patients after the cancer has become detectable. Finally, the dose and schedule of anti-angiogenic and chemotherapeutic drugs in the clinic have not been optimized, owing to cost and other considerations[14].

Clinical anti-angiogenesis with VEGF blockers

Several VEGF blockers have been approved for clinical use in cancer and eye diseases[6,7]. So far, the US Food and Drug Administration has approved the use of the VEGF-neutralizing antibody bevacizumab (Avastin) for metastatic colorectal cancer, metastatic non-squamous non-small-cell lung cancer, metastatic breast cancer, recurrent glioblastoma multiforme (GBM) and metastatic renal cell carcinoma (RCC) (Table 1). In addition, several multi-targeted TKIs, which block the signalling of pathways such as VEGF, have been approved, including sorafenib (Nexavar) for metastatic RCC and unresectable hepatocellular carcinoma, and sunitinib (Sutent) and pazopanib (Votrient) for metastatic RCC (Table 1). Recently, vandetanib (Zactima) has been approved for unresectable or metastatic medullary thyroid cancer and sunitinib has been recommended for approval for advanced pancreatic neuroendocrine tumours, but the clinical data have not yet been published. Treatment with VEGF inhibitors generally prolongs the survival of responsive patients with cancer of the order of months (Table 1). Two anti-VEGF compounds — intravitreous injection of the VEGF aptamer pegaptanib (Macugen) and the anti-VEGF Fab antibody ranibizumab (Lucentis) — have been approved for treatment of the wet (neovascular) form of age-related macular degeneration, which causes blindness owing to the formation of leaky neovessels. Bevacizumab is also used off-label for this condition.

Notwithstanding these successes, the clinical use of VEGF blockers in patients with cancer has shown that anti-angiogenic therapy is more challenging than anticipated. For example, VEGF receptor TKIs are effective as monotherapy in certain cancers, but fail in others or are toxic when combined with chemotherapy[6,14]. The use of bevacizumab is approved only when combined with cytotoxic or cytokine therapy (with the exception of patients with GBM). Many patients with metastatic disease are refractory or acquire resistance to VEGF inhibitors[46], and biomarkers to identify responders are missing[14]. In a recent trial, bevacizumab prolonged disease-free progression but not overall survival in patients with metastatic RCC[76], and failed to show benefit in the adjuvant setting[77]. Moreover, questions have begun to arise about whether anti-angiogenic therapy causes cancer cells to become more malignant[78,79]. What are the reasons for these problems, and what can be done to move forward? The discussion in the next sections does not offer an answer to the daily

challenges in oncological practice, but provides some avenues for developing future strategies.

Refractoriness to VEGF blockade in advanced cancer

A fraction of patients with cancer are refractory to VEGF-inhibitor treatment[46]. The extent of refractoriness varies from one cancer to another, differs between micro- and macrometastatic disease, and differs for various types of VEGF blocker. Patients can be intrinsically refractory and never show any response to treatment, or develop evasive resistance during the course of treatment. Several mechanisms have been proposed to explain these phenomena, which are related to changes in the tumour cells, endothelial cells or other stromal cells[6,14,46,80] (Fig. 3). It is important to note that these mechanisms have been identified for advanced, late-stage, macrometastatic disease only.

Tumour angiogenesis can become VEGF independent at a more advanced stage because of the production of other pro-angiogenic molecules, and thus respond poorly to VEGF blockade. Hypoxia induced by vessel regression after VEGF blockade can also switch on a more invasive and metastatic program, whereas in other cases, cancer (stem) cells can become hypoxia-tolerant when acquiring extra mutations and survive in poorly oxygenated niches. VEGF blockade inhibits sprouting angiogenesis, but may not be as efficient in suppressing other modes of tumour vascularization, relying on the recruitment of BMDCs, vessel co-option, vasculogenic mimicry or vessel splitting. Certain tumours, such as pancreatic carcinoma, contain a hypovascular stroma and are therefore less sensitive to anti-angiogenic agents. Vessel pruning by VEGF blockade can aggravate hypoxia, resulting in the upregulation of angiogenic factors such as PlGF, FGFs, chemokines and ephrins, and this may rescue tumour vascularization[46]. Some tumour endothelial cells show signs of cytogenetic abnormalities and transforming stem-cell potential[81], which could alter sensitivity to VEGF inhibition. Furthermore, GBM-like stem cells can differentiate into tumour endothelial cells, and VEGF blockers can only partially inhibit this process[4].

Other stromal cells contribute to the resistance to VEGF blockade. Hypoxia promotes the recruitment of angiocompetent BMDCs, including TEMs, TAMs, neutrophils, mast cells and $CD11b^{+}GR\text{-}1^{+}$ (also known as $ITGAM^{+}Ly6G^{+}$) myeloid-derived suppressor cells, which release angiogenic signals such as VEGF, BV8 (also known as PROK2) and MMPs[82]. VEGF blockade is often combined with chemotherapeutics — by sensitizing endothelial cells to cytotoxic damage, VEGF inhibitors impair endothelial cell survival and regrowth, but recruitment of BMDCs after chemotherapy can revascularize tumours (‘vasculogenic rebound’)[6,83]. The release of angiogenic factors such as PDGF-CC by cancer-associated fibroblasts also contributes to resistance. Furthermore, vessels in most tumours are covered with few pericytes, but microvessels in some cancers acquire a dense pericyte coat with a thick basement membrane; such mature vessels are usually less sensitive to VEGF blockers[31,32]. Understanding the molecular basis of these cancer-type-dependent resistance mechanisms against VEGF blockade offers opportunities to improve anti-angiogenic treatment.

VEGF blockers in the adjuvant setting

On the basis of the clinical experience with VEGF inhibitors in macrometastatic cancer, VEGF blockers were anticipated to be beneficial for micrometastatic disease in the adjuvant setting (that is, after surgical resection of the primary tumour). However, compared with chemotherapy alone, adjuvant treatment of patients with micrometastatic disease in combination with bevacizumab and chemotherapy failed to prolong disease-free survival after three years[77]. The administration of an anti-VEGF antibody initially prolonged disease-free survival in patients, but this benefit was lost after three years. The precise reasons for this remain unclear. It is possible that the micrometastatic tumour cells were less responsive to anti-VEGF treatment because they were in a state of angiogenic dormancy[84]. The recruitment of proangiogenic BMDCs may convert micrometastasis to macrometastasis, but it unclear whether VEGF blockade eliminates this rescue pathway. Another hypothesis is that an angiogenesis rebound occurs after the arrest of anti-VEGF treatment, as documented in animal models[85]. However, such vascular rebound did not occur after long-term treatment with a pan-VEGFR TKI in patients with GBM[86].

Another question is whether the transient disease-free survival benefit of anti-VEGF treatment was attributable to a change in the nature of the disease, and whether VEGF blockade caused the cancer to become more malignant after an initial delay. Some preclinical models show that VEGF blockade aggravates hypoxia and induces a pro-tumorigenic inflammatory state, which promotes invasiveness and metastasis, despite inhibition of primary tumour growth and prolongation of survival[78,79]. However, another preclinical study reported no effect of VEGF blockade on metastasis in the adjuvant setting[87], and clinical trials have not shown an increase in malignancy or tumour-growth rebound after VEGF blockade, at least not in the metastatic setting. Moreover, a recent randomized phase II trial showed that continuous dosing and discontinuous dosing (four weeks on and two weeks off) of sunitinib have the same outcome in RCC patients. Finally, a meta-analysis of advanced cancers shows that VEGF blockade does not aggravate metastatic disease[88]. Recurrent GBM is an exception, in which VEGF blockade increased tumour invasion, but even in these studies, tumours might have become more malignant because the treatment prolonged survival and allowed the cancer to progress further. Overall, there is an urgent need for an improved mechanistic understanding of vessel growth and resistance to anti-angiogenic therapy, particularly in micrometastatic lesions.

Tumour vessel abnormalities as a future target

Another parameter that could determine the overall efficiency of anti-VEGF therapy is the abnormal nature of tumour vessels. Tumour vessels become abnormal in almost all aspects of their structure and function[89]. They are heterogeneous, tortuous, branch chaotically and have an uneven vessel lumen. In addition to abnormal endothelial cells, pericytes and the basement membrane are also abnormal. Owing to the leakiness of tumour vessels, escaping fluid raises the interstitial fluid pressure. As a result, blood flow is heterogeneous, and oxygen, nutrients, immune cells and drugs are distributed unevenly. Because radiation therapy and many chemotherapeutics rely on the formation of oxygen radicals to kill cancer cells, tumour hypoxia reduces their efficacy. These vessel abnormalities create a hostile

milieu, characterized by hypoxia, low pH and high fluid pressure, which can select for more malignant cancer cells and lower barriers to their escape through leaky vessels.

These findings raise questions for the future. Excessive vessel pruning and growth arrest by anti-angiogenic agents could aggravate tumour invasiveness and metastasis by increasing hypoxia and creating a protumorigenic inflammatory state. Vessel normalization could provide new therapeutic opportunities to slow down tumour invasiveness and dissemination, and increase tumour responses to chemotherapeutics and radiotherapy[89]. Another consideration is how vessel normalization should be combined with an anti-angiogenic treatment. Vessel normalization was first recognized in mice xenografted with colon cancer and treated with an anti-VEGF antibody, but it is transient in mice and patients[14,89,90]. Recent genetic studies in mice have shown that sustained vessel normalization can provide benefits. Indeed, haplodeficiency of the oxygen-sensor PHD2 in endothelial cells induces sustained normalization of tumour vessels, without altering vessel density or size[72]. In these vessels, leakage, tortuosity and remodelling are reduced, whereas endothelial cell quiescence, barrier tightening and vessel maturation are increased — changes that boost perfusion and decrease hypoxia[72]. A streamlined monolayer of phalanx endothelial cells is also formed, providing a more impenetrable barrier for intravasating tumour cells[72]. These changes do not affect tumour growth, but reduce tumour cell invasiveness, intravasation and metastasis[72]. Although these genetic studies offer an elegant example, the challenge will be to develop therapeutic strategies that translate these insights into daily practice in the clinic.

Future directions

An important question is how anti-angiogenic medicine can be improved. In the short term, the use of current anti-VEGF agents should be optimized. Given the low response rates, a step forward would be the discovery of predictive biomarkers to identify responders among the large patient group of non-responders. So far, only a few candidates for predictive biomarkers have been identified, but they emerged from small studies and require prospective validation in independent randomized trials[14]. Another consideration is the optimization of the dose and duration of anti-angiogenic drug delivery. Little is understood about the mechanisms of vascularization of micrometastatic lesions, and agents that can block other modes of tumour vascularization (such as co-option, intussusception, vasculogenesis and vasculogenic mimicry) are needed. Furthermore, understanding the mechanistic differences between VEGFR TKIs and anti-VEGF antibodies (for instance, whether the former are effective as monotherapy because they inhibit several targets, whereas the latter require combination chemotherapy in most instances) will help to optimize the design of anticancer treatments.

In the intermediate term, anti-VEGF agents could be combined with agents that target the escape pathways detected in clinical studies (not in mice). Examples are ANG-2, PlGF, SDF-1α and CXCR4 (ref. 14). The challenge will be when to add these second agents — before, during or after anti-VEGF therapy. In the long term, the therapeutic potential of vascular normalization agents based on recently identified targets should be evaluated in preclinical models, but their clinical development will require years. By using combinatorial therapeutic approaches, it will be important to explore the eradication of most tumour

vessels and normalization of residual vessels for longer durations than are now achievable with VEGF blockers alone. The potential of tumour vessel normalization to improve anticancer immune therapy should be explored further. Finally, it is important to test whether the approved anti-VEGF agents and those under development could be used to treat various non-malignant diseases characterized by abnormal vasculature, which afflict millions of people worldwide and in many cases have no effective treatment — such as age-related macular degeneration causing blindness, schwannomas causing loss of hearing, and atherosclerotic plaques causing stroke and myocardial infarction after rupture[14,91]. A tight integration between preclinical and clinical research is crucial to achieve these goals.

Acknowledgments

We would like to acknowledge the late J. Folkman for inspiring us and others to work in the area of tumour angiogenesis. We would like to thank L. Claeson-Welch, H. Augustin, E. Dejana, S. Kozin, D. G. Duda, S. Goel, L. L. Munn, G. Sledge, R. Stupp and H. D. Suit for their comments, and L. Notebaert for help with the illustrations. We apologize to the authors whose work we could not cite because of the limit on the number of references. The work of P.C. is supported by a Federal Government Belgium grant (IUAP06/30), long-term structural Methusalem funding by the Flemish Government, a Concerted Research Activities Belgium grant (GOA2006/11), Leducq Transatlantic Network ARTEMIS and a grant from Flanders Research Foundation (FWO G.0673.08). The research of R.K.J. is supported by US National Institutes of Health grants P01-CA80124, R01-CA85140, R01-CA115767 and R01-CA126642, Federal Share/NCI Proton Beam Program Income, the National Foundation for Cancer Research and a Department of Defense Breast Cancer Research Innovator Award (W81XWH-10-1-0016).

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BOX 1

Perfusion–independent role of endothelial cells

During embryogenesis, the invasion of endothelial cells into nascent organs confers inductive signals to promote organogenesis, even in the absence of blood flow. This suggests that endothelial cells not only form passive conduits for delivering oxygen but also establish organ-specific vascular niches, which stimulate organogenesis by the production of paracrine-tropic 'angiocrine' factors[92]. Endothelial cells show remarkable heterogeneity in different organs. These organ-specific endothelial cells release signals for pancreatic differentiation, reconstitution of haematopoietic stem cells and expansion of neuronal precursors, and give rise to haematopoietic progenitors by endothelial-to-haematopoietic transition. The vascular adventitia — the outer layer of vessels — also hosts vessel-resident stem and progenitor cells. Emerging evidence indicates that such perfusion-independent activities of endothelial cells also promote tumorigenesis[92]. In addition to constituting the building blocks of vessels and delivering nutrients and oxygen, tumour endothelial cells allow the recruitment of pro-angiogenic bone-marrow-derived cells.

BOX 2

Guidance signals in angiogenesis

Tip cells sense guidance cues, similar to how axonal growth cones explore their surroundings. It is therefore not surprising that molecules used by navigating axons are evolutionarily conserved, and molecules such as VEGF also guide neuronal cells. Navigating endothelial cells express receptors for axon-guidance cues, including ephrin receptors (EPH); neuropilins (NRPs) and PLEXIN-D1, which bind semaphorins; ROBO4, which binds slit proteins; and UNC5B, which binds netrin proteins. Given the size and complexity of these families (and the existing controversies[93]), we illustrate this concept with a few examples that have therapeutic potential.

EPH receptors and their ligands, the ephrins, regulate cell-contact-dependent patterning and can generate bidirectional signals. The signalling cascade in ephrin-expressing cells is known as reverse signalling, whereas signalling in EPH-receptor-expressing cells is termed forward signalling. Ephrin-B2 and its receptor EPHB4 regulate vessel morphogenesis by several mechanisms[93,94]. During vasculogenesis, the vascular plexus is marked by ephrin-B2$^+$ arterial and EPHB4$^+$ venous territories. By avoiding repulsive actions, ephrin-B2$^+$ and EPHB4$^+$ cells prevent intermingling and segregate from each other. In zebrafish, the emigration of venous-fated cells from a precursor vessel leads to segregation of ephrin-B2$^+$ arterial endothelial cells in the dorsal aorta and EPHB4$^+$ venous endothelial cells in the cardinal vein[18]. Moreover, reverse signalling by ephrin-B2 in tip cells induces VEGFR-2 internalization, which is necessary for downstream signalling of VEGFR-2 to elicit VEGF-induced tip-cell filopodial extension[95]. Ephrin-B2 also promotes the recruitment of mural cells and bone-marrow-derived endothelial progenitor cells. In tumours, the overall effect of EPHB4 is pro-angiogenic, making it a target for anti-angiogenic therapy. Indeed, upregulation of EPHB4 stimulates tumour angiogenesis, whereas EPHB4 blockade has the opposite effect. Other EPH receptors and ephrin ligands, such as EPHA2 and ephrin-A1, have a role in vessel growth and maturation[94]. Notably, ephrin-A1 levels are upregulated in tumours treated with VEGF blockers, suggesting that it contributes to resistance against VEGF blockade[46]. Various therapeutics that target EPH receptors and ephrin ligands are being developed, but the complexity of this signalling system should be kept in mind.

Semaphorins are secreted or membrane-anchored, and bind to plexin proteins or their NRP co-receptors. The loss of *Plxnd1* in mice induces erroneous navigation of vessels, because endothelial cells cannot recognize the repulsive semaphorin-3E (SEMA3E) signals in their environment. Many semaphorins inhibit tumour angiogenesis, including SEMA3A, SEMA3B, SEMA3D, SEMA3F and SEMA4A, whereas SEMA3C and SEMA4D promote tumour angiogenesis. NRPs bind ligands such as semaphorins and VEGF, but the vascular defects observed in *Nrp1*-deficient embryos are attributable to defective VEGF signalling, rather than defective semaphorin signalling. An antibody that blocks the binding of VEGF, but not of SEMA3A, to NRPs also inhibits tumour angiogenesis. Dual targeting with antibodies that block both VEGF and NRP1 is more effective than single-agent therapy, presumably because the antivascular remodelling effects of anti-NRP1 antibodies keep vessels in a VEGF-dependent state. In addition, a

soluble NRP2B variant with increased VEGF affinity enhances the tumour-growth-inhibitory activity of an antibody that blocks the interactions of VEGF with VEGFR-2 but not with NRPs.

BOX 3

Hypoxia and epigenetic regulation of angiogenesis

The prolyl hydroxylase domain (PHD) proteins PHD1–3 are oxygen-sensing enzymes that hydroxylate the hypoxia-inducible factor (HIF) proteins HIF-1α and HIF-2α when sufficient oxygen is available. Once hydroxylated, HIFs are targeted for proteasomal degradation[96]. Under hypoxia, PHDs become inactive, and HIFs initiate broad transcriptional responses to increase the oxygen supply by angiogenesis, through the upregulation of angiogenic factors such as VEGF[97]. HIFs are also activated in non-hypoxic conditions by oncogenes and growth factors, allowing tumour cells to stimulate angiogenesis before they become deprived of oxygen. In general, HIF-1α promotes vessel sprouting, whereas HIF-2α mediates vascular maintenance[97]. Reduced HIF-1α levels in mice impair embryonic vascular development, revascularization of ischaemic tissues, and angiogenesis in injured tissues and tumours[97]. The use of HIF-1α inhibitors to block tumour or ocular angiogenesis has therefore received attention. Conversely, *Hif1a* gene transfer in mice or activation of HIF-1α by pharmacological blockade of PHDs promotes ischaemic tissue revascularization.

HIF-1α also regulates tumour angiogenesis indirectly, by releasing chemoattractants such as SDF-1α to recruit pro-angiogenic BMDCs[65]. Gene silencing of *Phd2* in mouse tumour cells enhances vessel growth by similar mechanisms. Hypoxia also regulates the polarization and pro-angiogenic activity of tumour-associated macrophages (TAMs) by means of HIF-1α and HIF-2α with different effects[96]. That hypoxia and inflammation are closely intertwined is illustrated by the finding that signalling by HIF-1α and nuclear factor-κB cross-activate each other. In certain cases, hypoxic upregulation of VEGF occurs independently of HIF-1α, and is mediated by the metabolic regulator PGC-1α in preparation for oxidative metabolism once the ischaemic tissue is revascularized[98]. Because HIF signalling contributes to acquired resistance against anti-VEGF therapy, the combined blockade of VEGF and HIF-1α is being explored as a cancer treatment strategy.

There is increasing evidence for epigenetic control of angiogenesis, particularly by non-coding microRNAs (miRNAs)[15], which induce messenger RNA degradation or block translation. Because miRNAs target multiple genes, they are well positioned to regulate complex processes such as angiogenesis. Endothelial cells express several miRNAs that are induced by hypoxia or VEGF. Most of those stimulate angiogenesis by hijacking pro-angiogenic cascades, while suppressing angiostatic pathways[99]. The expression of miR-126 is induced by the mechanosensitive transcription factor KLF2A and integrates the mechanosensory stimulus of blood flow to shape the vascular system[100]. Endothelial-cell-specific loss of DICER, an exonuclease involved in miRNA biogenesis, impairs pathological angiogenesis. Angiogenic miRNAs seem to offer significant pro- or anti-angiogenic potential.

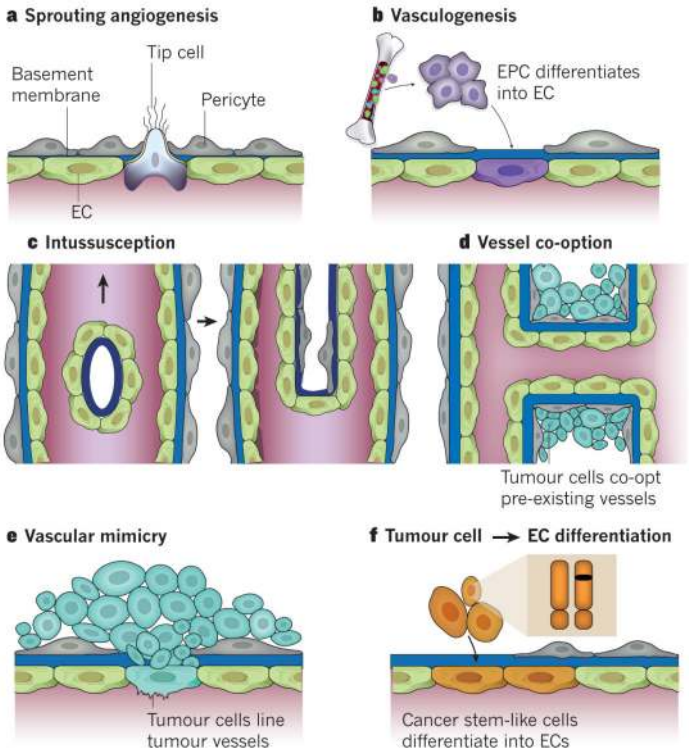

Figure 1. Modes of vessel formation

There are several known methods of blood vessel formation in normal tissues and tumours. **a**–**c**, Vessel formation can occur by sprouting angiogenesis (**a**), by the recruitment of bone-marrow-derived and/or vascular-wall-resident endothelial progenitor cells (EPCs) that differentiate into endothelial cells (ECs; **b**), or by a process of vessel splitting known as intussusception (**c**). **d**–**f**, Tumour cells can co-opt pre-existing vessels (**d**), or tumour vessels can be lined by tumour cells (vascular mimicry; **e**) or by endothelial cells, with cytogenetic abnormalities in their chromosomes, derived from putative cancer stem cells (**f**). Unlike normal tissues, which use sprouting angiogenesis, vasculogenesis and intussusception (**a**–**c**), tumours can use all six modes of vessel formation (**a**–**f**).

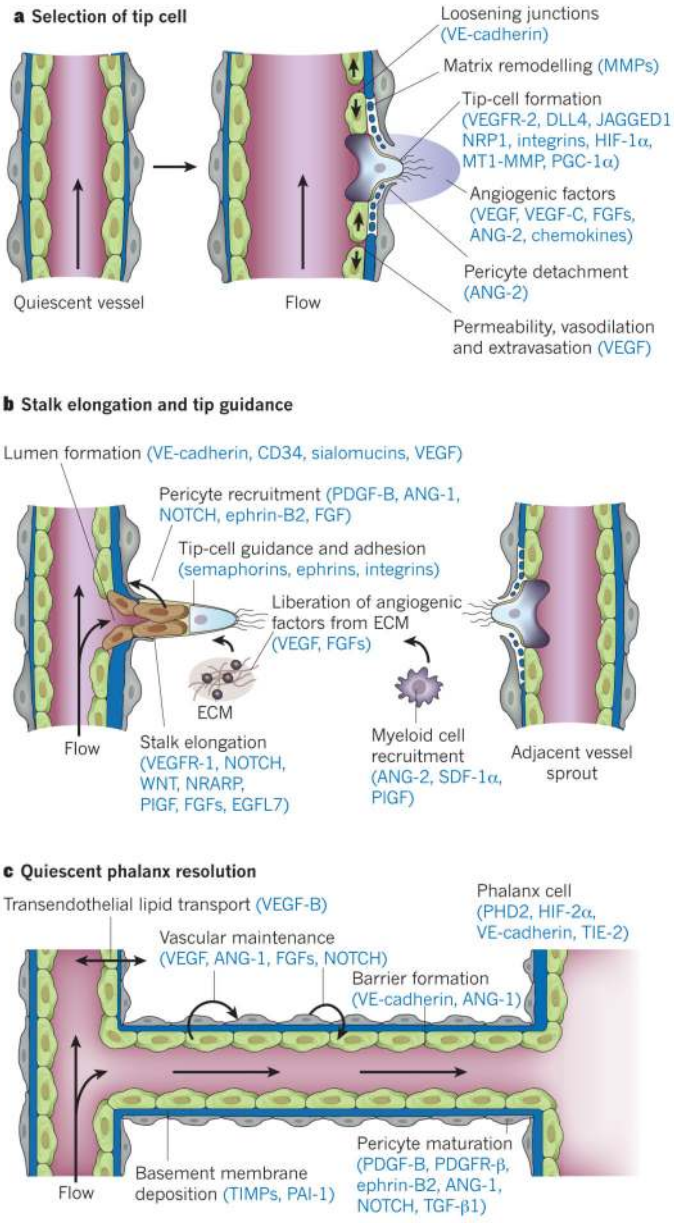

Figure 2. Molecular basis of vessel branching

The consecutive steps of blood vessel branching are shown, with the key molecular players involved denoted in parentheses. **a**, After stimulation with angiogenic factors, the quiescent vessel dilates and an endothelial cell tip cell is selected (DLL4 and JAGGED1) to ensure branch formation. Tip-cell formation requires degradation of the basement membrane, pericyte detachment and loosening of endothelial cell junctions. Increased permeability permits extravasation of plasma proteins (such as fibrinogen and fibronectin) to deposit a provisional matrix layer, and proteases remodel pre-existing interstitial matrix, all enabling cell migration. For simplicity, only the basement membrane between endothelial cells and pericytes is depicted, but in reality, both pericytes and endothelial cells are embedded in this basement membrane. **b,** Tip cells navigate in response to guidance signals (such as

semaphorins and ephrins) and adhere to the extracellular matrix (mediated by integrins) to migrate. Stalk cells behind the tip cell proliferate, elongate and form a lumen, and sprouts fuse to establish a perfused neovessel. Proliferating stalk cells attract pericytes and deposit basement membranes to become stabilized. Recruited myeloid cells such as tumour-associated macrophages (TAMs) and TIE-2-expressing monocytes (TEMs) can produce pro-angiogenic factors or proteolytically liberate angiogenic growth factors from the ECM. **c**, After fusion of neighbouring branches, lumen formation allows perfusion of the neovessel, which resumes quiescence by promoting a phalanx phenotype, re-establishment of junctions, deposition of basement membrane, maturation of pericytes and production of vascular maintenance signals. Other factors promote transendothelial lipid transport.

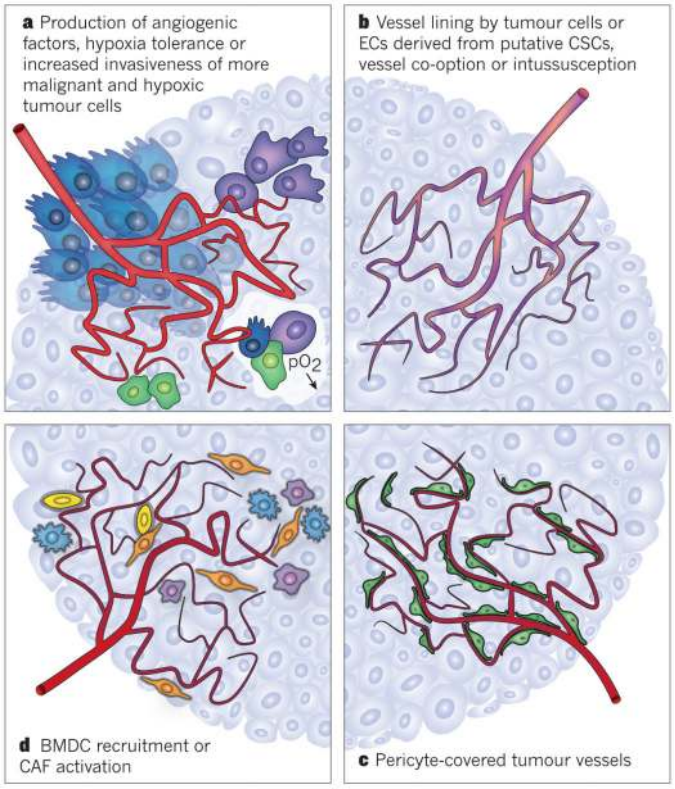

Figure 3. Potential mechanisms of resistance to targeted VEGF therapy in cancer

Different mechanisms underlie the resistance to VEGF blockade seen in some patients with cancer. These mechanisms are not exclusive, and it is likely that several occur simultaneously in a single tumour. **a**, In established tumours, VEGF blockade aggravates hypoxia, which upregulates the production of other angiogenic factors or increases tumour cell invasiveness. Tumour cells that have acquired other mutations can also become hypoxia tolerant. The more malignant tumour cells are shown as dark green, blue and purple cells. **b**, Other modes of tumour vascularization, including intussusception, vasculogenic mimicry, differentiation of putative cancer stem cells (CSCs) into endothelial cells (ECs), vasculogenic vessel growth and vessel co-option (all denoted by the mosaic red–purple vessels), may be less sensitive to VEGF blockade. **c**, Tumour vessels covered by pericytes (green) are less sensitive to VEGF blockade. **d**, Recruited pro-angiogenic BMDCs (yellow), macrophages (blue and purple) or activated cancer-associated fibroblasts (CAFs; orange) can rescue tumour vascularization by the production of pro-angiogenic factors.

Table 1
Overview of anti-angiogenic drugs in cancer

Drug	Approved indication	Improvement in RR (%)	Improvement in PFS (months)	Improvement in OS (months)
Bevacizumab	Metastatic colorectal cancer (with chemotherapy)	10	4.4	4.7*
		0	1.4	1.4*
		7.8	2.8	2.5*
		14.1	2.6	2.1†
	Metastatic non-squamous NSCLC (with chemotherapy)	20	1.7	2.0*
		10.3–14.0	0.4–0.6	NR*
	Metastatic breast cancer (with chemotherapy)‡	15.7	5.9	NS*
		9–18	0.8–1.9	NS*
		11.8–13.4	1.2–2.9	NS*
		9.9	2.1	NS†
	Recurrent GBM (monotherapy)	Currently only phase II data reported		
	Metastatic RCC (with IFN-α)	18	4.8	NS*
		12.4	3.3	NS*
Sunitinib	Metastatic RCC	35	6.0	4.6*
Sorafenib	Metastatic RCC	8	2.7	NS†
	Unresectable HCC	1	NS	2.8*
		2	1.4	2.3*
Pazopanib	Metastatic RCC	27	5.0	NR*†

Anti-angiogenic therapies currently approved by the US Food and Drug Administration (FDA) for treatment of malignancies. Per indication, the results of various trials are shown. The data show the improvement observed after the addition of the anti-VEGF therapy. GBM, glioblastoma multiforme; HCC, hepatocellular carcinoma; IFN, interferon; NR, not reported; NS, not significant; NSCLC, non-small-cell lung carcinoma; OS, overall survival; PFS, progression-free survival; RCC, renal cell carcinoma; RR, response rate. For reference, see http://clinicaltrials.gov.

*First-line therapy.

†Second-line therapy.

‡The FDA recommended the withdrawal of bevacizumab for breast cancer in December 2010; this is under appeal, with a hearing expected in June 2011. However, bevacizumab is approved for metastatic breast cancer in Europe, except in the United Kingdom.