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Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer

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Four decades ago, angiogenesis was recognized as a therapeutic target for blocking cancer growth. Because of its importance, VEGF has been at the center stage of antiangiogenic therapy. Now, several years after FDA approval of an anti-VEGF antibody as the first antiangiogenic agent, many patients with cancer and ocular neovascularization have benefited from VEGF-targeted therapy; however, this anticancer strategy is challenged by insufficient efficacy, intrinsic refractoriness, and resistance. Here, we examine recent discoveries of new mechanisms underlying angiogenesis, discuss successes and challenges of current antiangiogenic therapy, and highlight emerging antiangiogenic paradigms.

Introduction

The development of new blood vessels, termed *angiogenesis*, is a hallmark of cancer development that has long been considered an attractive therapeutic target. The signaling molecule VEGF plays a central role in angiogenesis and is frequently highly expressed in cancers. Thus, clinical efforts to develop antiangiogenic therapies have largely focused on inhibiting VEGF. VEGF blockade in neoadjuvant trials show promising benefit, but largely negative results have been obtained in the adjuvant setting. Furthermore, in certain advanced metastatic cancers, blocking VEGF alone is insufficient to prevent progression, induces resistance, and possibly even increases invasion and metastasis, although this matter remains debated. Thus, we need additional angiogenesis targets and predictive preclinical models and biomarkers, and we must better understand the context-dependent angiogenic activity of several targets. Here, we review newly discovered proangiogenic molecules and discuss emerging insights in these outstanding topics. We primarily focus on recent publications and selected angiogenic signals; more historically complete overviews are available elsewhere (1–4).

Vessel sprouting model

Several modes of vascularization exist (see below), but the vessel sprouting (angiogenesis) model has been studied most extensively (5). Key insights have been generated in the mouse retina, where vascularization occurs postnatally, thus representing a physiological angiogenesis model. Upon activation of ECs by proangiogenic molecules, cell-cell junctions (VE-cadherin, ZO-1, and others) and the basement membrane (BM) are remodeled (in part by MMPs) in tandem with pericyte detachment, allowing a tip cell to migrate in response to guidance signals (Figure 1). The sprout then elongates through proliferation of stalk cells, which form a lumen and recruit pericytes for stabilization (Figure 2). Tip cells from neighboring sprouts meet and anastomose to form a perfused branch. Upon perfusion, ECs become quiescent phalanx cells, deposit BM, establish a barrier, and are covered by mature pericytes (Figure 3). From a therapeutic viewpoint, strategies targeting tip and stalk cells would prevent neoangiogenesis, prune immature vessels, and cause vessel regression. Promoting phalanx cell formation would induce tumor vessel normalization (see below).

Tip cell selection: cross-talk between VEGFR2 and Notch

Vessel sprouting requires coordination between migrating tip cells and proliferative stalk cells. ECs at the leading edge extend filopodia and migrate toward angiogenic signals. At the forefront, where VEGF levels are highest, VEGF activates VEGF receptor 2 (VEGFR2) to stimulate tip cell migration (Figure 1). VEGFR2 internalization and activation of ERK1/2 signaling are important for sprouting, likely because rapid receptor turnover and signaling is essential for ECs at the vascular front to respond strongly and quickly to angiogenic signals (6). Such dynamic responses necessitate rapid clearing of activated receptors to finely tune the speed and direction of vessel branching. Signaling through VEGFR2 (and VEGFR3; see below) is enhanced by the coreceptor neuropilin-1 (Nrp1), which promotes tip cell function (7). Although the Nrp1 cytoplasmic domain (and signaling) is dispensable for angiogenesis, it is essential for separation of arteries and veins (8).

The specification between tip cells and stalk cells is regulated by Notch (9). ECs with activated VEGFR2 compete for the tip position by increasing expression of the Notch ligand Delta-like 4 (DLL4), which binds to Notch receptors on neighboring ECs and releases the Notch intracellular domain (NICD). NICD acts as a transcriptional regulator, decreasing *Vegfr2* and *Nrp1* expression while increasing the levels of VEGFR1, which traps VEGF (10, 11) and renders stalk cells less responsive to VEGF (Figure 1). Hence, Notch blockade induces vessel hyperbranching, while gain of function causes the opposite effect (9). In addition to regulation by VEGF/VEGFR2 signaling, initial evidence suggests that cellular or matrix components may also ensure DLL4 expression (12). The tip cell position is fluid: EC interchange occurs at the leading edge, with ECs with the highest VEGFR2 and lowest VEGFR1 levels migrating to the tip position (11). Competition and position exchange couple VEGFR levels to leadership, ensuring that the tip cell is optimally equipped to sense the VEGF gradient. Tumor ECs produce elevated DLL4 levels, and pharmacological blockage of DLL4 reduces tumor growth because it leads to supernumerary hypoperfused tumor vessels (13), but also causes hemangiomas (14).
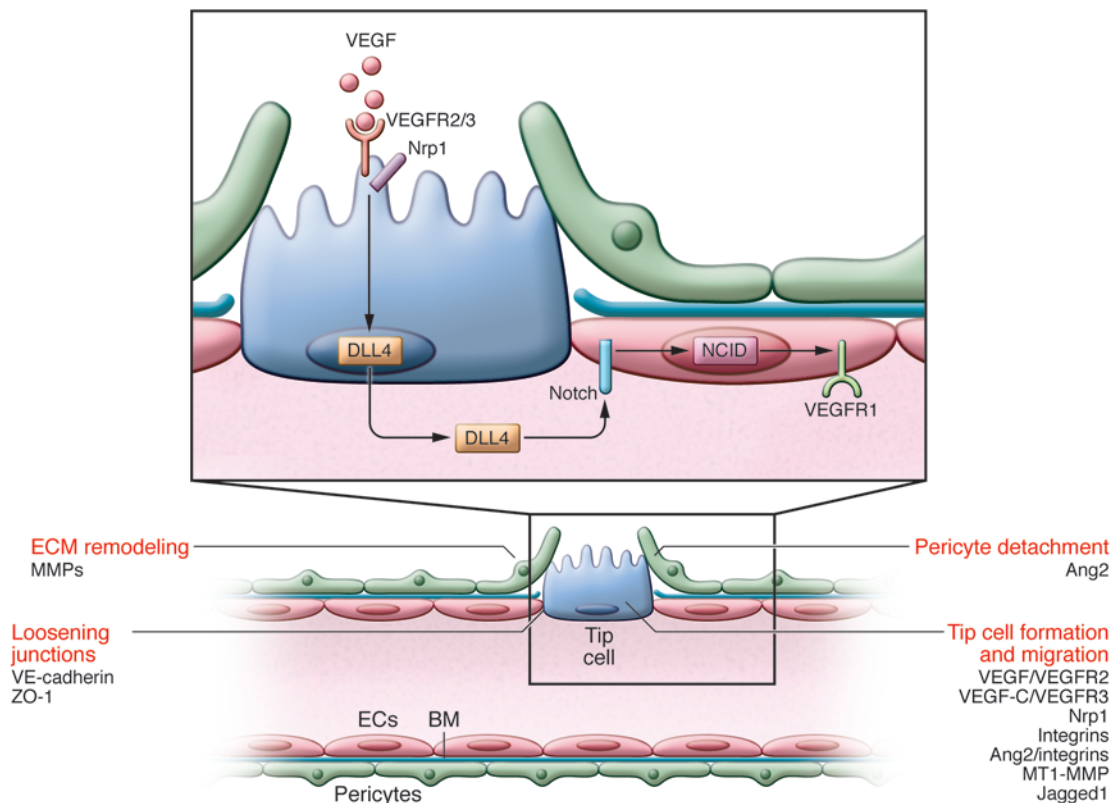
Figure 1
Initial steps of tip cell selection. Vascular sprouting is initiated by proangiogenic factors (e.g., VEGF). ECs at the leading edge of the vascular sprout extend filopodia and migrate toward angiogenic signals. VEGF activates VEGFR2 to stimulate tip cell migration. The coreceptor Nrp1 complexes with and enhances VEGFR2 signaling. ECs become either the migratory vessel-leading tip cell or the proliferating stalk cell, but their phenotype is fluid; Notch regulates this specification. ECs with activated VEGFR2 signaling compete for the tip cell position by increasing their expression of DLL4, which binds to Notch receptors on neighboring ECs, releasing the transcription regulator NICD. NICD transcriptionally downregulates VEGFR2 and Nrp1 expression while increasing VEGFR1, a VEGF trap, thus enhancing the stalk cells' unresponsiveness to VEGF. The tip cell is not a fixed position, and fluidity at the front occurs depending on the VEGFR1/VEGFR2 ratio. Tip cell migration requires BM degradation (in part due to MMP), EC junction loosening (caused by VE-cadherin, ZO-1, and others), and pericyte detachment (regulated by Ang2). VEGF increases the permeability of the vessel, allowing the extravasation of plasma proteins (e.g., fibronectin and fibrinogen) that are deposited as a provisional matrix layer while the preexisting interstitial matrix is remodeled by proteases; these events enable tip cell migration. Key molecular players discussed in this review and elsewhere (5, 132) are indicated.

Role of VEGF-C/VEGFR3 in tip cell formation

VEGF-C binds VEGFR3 (and weakly binds VEGFR2, but not VEGFR1) and induces tip cell activity, though less potently than VEGF (Figure 1). The sprouting activity of VEGF-C/VEGFR3 is more pronounced when VEGFR2 is blocked. Pharmacological VEGFR3 or VEGF-C blockade studies suggest that VEGFR3 activation by VEGF-C promotes tip cell formation (15). However, *Vegfr3* gene deficiency increases tip cell formation (16). These discrepant results are reconciled by a model whereby VEGFR3 has a ligand-dependent ("active") proangiogenic signaling mode and a ligand-independent ("passive") signaling branch that activates Notch, which explains why VEGFR3 deficiency causes hyperbranching. The passive signaling operates by phosphorylation of the intracellular VEGFR3 domain via matrix-dependent activation of Src kinase (16). VEGF-C–producing macrophages that localize to vessel branch points activate Notch target genes, independently of Notch ligands, thereby decreasing the sensitivity to VEGF and facilitating vascular loop assembly. Hence, VEGFR3 regulates the conversion of tip cells to stalk cells at points of sprout fusion, where tip cells

of opposing branches anastomose (16). Furthermore, Benedito et al. (12) reported that Notch downregulates expression of VEGFR3, but not of VEGFR2 (in contrast to ref. 9), and that low Notch signaling induces VEGFR3-driven angiogenesis independent of VEGFR2 signaling (12). Inhibition of VEGFR3's kinase activity, but not ligand binding, suppressed EC sprouting, which suggests that VEGFR3 has ligand-independent activity in low-Notch conditions (12). Future work is required to reconcile these divergent findings on the roles of VEGFR2, VEGFR3, and Notch in a unifying model. Regardless of the mechanisms, VEGFR3 levels are upregulated in tumor vessels, and inhibitors blocking VEGFR3 homodimerization, VEGFR3/VEGFR2 heterodimerization, or VEGF-C binding inhibit tumor angiogenesis in culture and in mice (17).

Role of Ang2/Tie2 in tip cell formation

Angiopoietin1 (Ang1) and Ang2 bind Tie2, a tyrosine kinase receptor expressed in stalk and phalanx cells. Perivascular cell expression of Ang1 stabilizes and tightens the EC barrier by recruiting complexes between Tie2 and the phosphotyrosine vascular
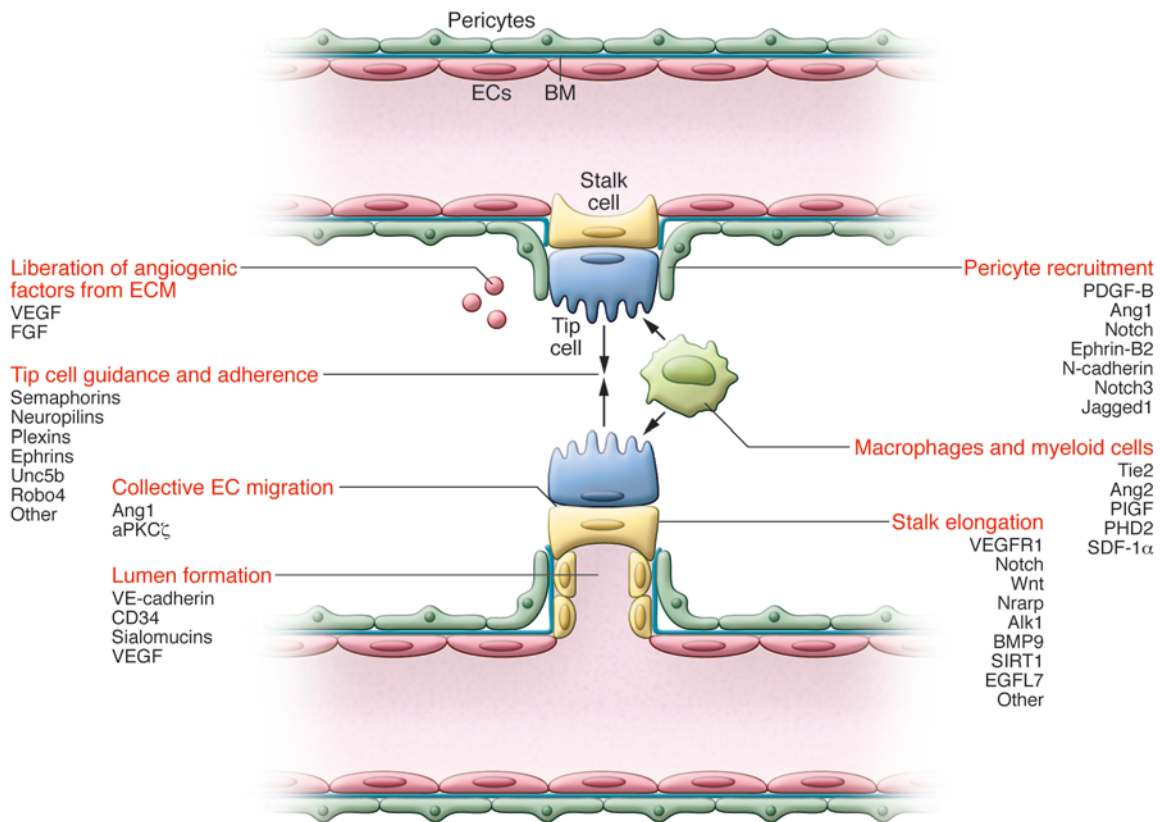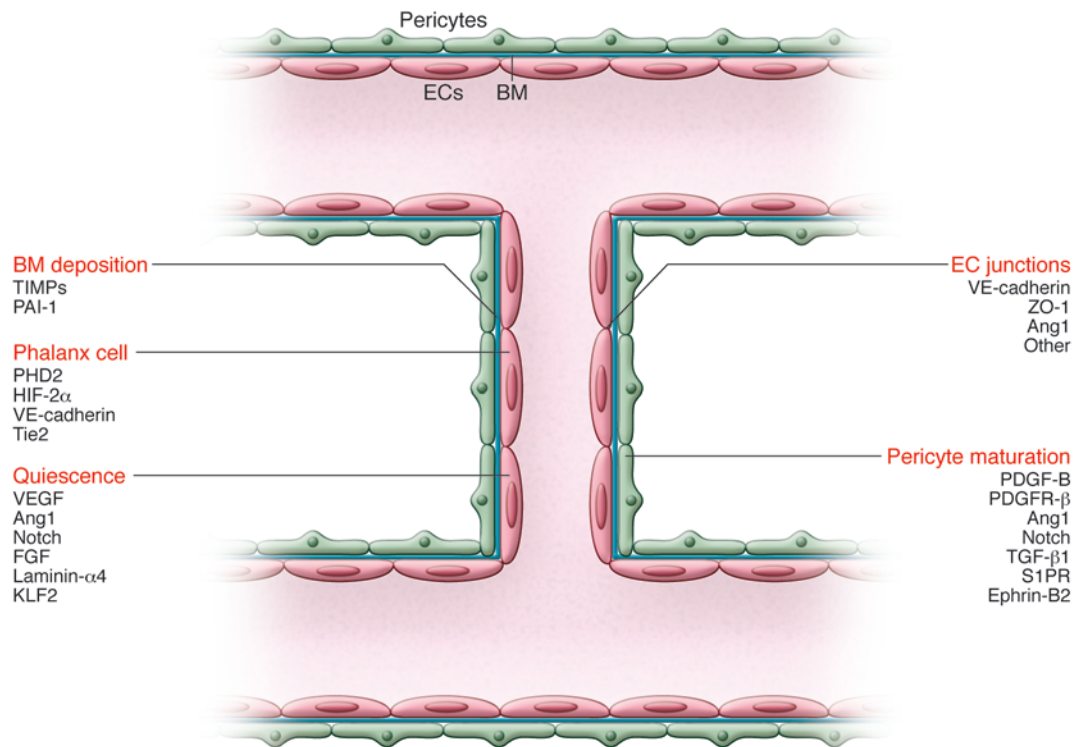
Figure 2
Tip cell guidance and stalk elongation. The growing sprout moves along a VEGF gradient. Tip cells adhere to the ECM, mediated by integrins, and migrate toward guidance signal molecules (e.g., semaphorins and ephrins). Stalk cells trail behind the tip cell and proliferate to allow sprout elongation and lumen formation. While Notch signaling inhibits proliferation, expression of Nrarp at branch points allows Wnt signaling to maintain stalk cell proliferation. This system allows vascular migration/directionality (by tip cells) and elongation of the shaft (by proliferating stalk cells). When two tip cells meet, they fuse (anastomose); this mechanism is assisted by macrophages, which accumulate at sites of vascular anastomosis to act as bridge cells by interacting with the neighboring tip cells' filopodia. Once contact between the tip cells has been established, VE-cadherin–containing junctions further strengthen the connection. Perivascular macrophages further stimulate sprouting by producing angiogenic factors or proteolytically liberating them from the ECM. The stalk cells also deposit BM and recruit pericytes, thus stabilizing the forming vessel. Pericyte precursors are attracted to vessels by EC-expressed PDGF. Once at the vessel, these mesenchymal precursor cells differentiate to pericytes in response to TGF-β and decrease EC migration, proliferation, and vascular leakage, resulting in nascent vessel stabilization. Key molecular players discussed in this Review and elsewhere (5, 132) are indicated.

endothelial protein tyrosine phosphatase (VE-PTP) to cell-cell junctions and by preventing VEGFR2-induced internalization of the junctional molecule VE-cadherin (18). Ang1-Tie2 complexes assemble in *trans* at EC-EC junctions, promoting EC-EC adhesion and EC survival. Ang1 also promotes collective directional migration of ECs by relocating atypical PKCζ to the leading EC edge, where it forms a complex with β-catenin that interacts with polarity proteins at adherens junctions (19). In atypical PKCζ morphant zebrafish, tip cells, after initial sprouting from the aorta, separate from the secondary connector stalk cells and lose polarity cues by extending filopodia more randomly (Figure 2). In ischemic tissues, Ang1 promotes vessel growth and enlargement, but without inducing vessel leakage (as VEGF does), making it a potential target for therapeutic angiogenesis (20).

EC-expressed Ang2 antagonizes Ang1 activity and thereby stimulates vessel destabilization and sensitizes ECs to proangiogenic signals (Figure 1 and ref. 21). In this case, Tie2 translocates to cell-matrix contacts. However, Ang2 also stimulates angiogenesis by activating Tie2. Indeed, Ang2 attenuates Ang1-Tie2 activation in

the presence of Ang1 (in mature tumor supply vessels), but activates Tie2 signaling when Ang1 is absent (in immature pericyte-deprived tumor vessels), which suggests that Ang2 is a partial agonist (22). Ang2 also stimulates tip cell migration by activating integrins independently of Tie2 (Figure 1 and ref. 23). Tie1, an orphan receptor homologous to Tie2, heterodimerizes with Tie2 and regulates Ang2 activity. In the presence of Tie1, Ang2 is unable to activate Tie2; however, loss of Tie1 reveals agonist capabilities of Ang2.

Tumor ECs express elevated Ang2 levels, and an increased Ang2/Ang1 ratio correlates with tumor angiogenesis and poor prognosis in many cancers, making Ang2 an attractive therapeutic target. Anti-Ang2 antibodies inhibit tumor angiogenesis and growth and improve the antiangiogenic efficacy of VEGF blockers in xenograft models (22), while a combination of angiopoietin blockers and cytotoxic drugs increases the progression-free survival (PFS) of patients with ovarian cancer (24). Simultaneous targeting of angiopoietins and VEGF by the chimeric decoy receptor double antiangiogenic protein (DAAP) also inhibits tumor angiogenesis and growth in preclinical models (25). Nonetheless, the effects of

Figure 3
Maturation through resolution of quiescent phalanx cells. Once fusion has occurred, a connected lumen is formed to allow blood flow through the new vessel. This perfuses the hypoxic tissue, and the resultant oxygen and nutrient delivery leads to decreased levels of angiogenic signals, inactivation of EC oxygen sensors, and increased proquiescent molecules that lead to EC quiescence. Establishment of the blood flow remodels vessel connections, which are regulated by the shear stress–responsive transcription factor KLF2. ECs resume a quiescent phalanx phenotype in a tightly apposed monolayer with a streamlined surface that conducts the blood flow and regulates tissue perfusion. Perfusion induces vascular maturation by reestablishment of cell-cell junctions, pericyte maturation, and BM deposition. Autocrine and paracrine signaling from ECs and surrounding support cells by VEGF, FGF, Ang1, and Notch, among others, maintain a quiescent EC phenotype and protect the vessel from environmental stresses. Reduced growth factor signaling can lead to vessel retraction and EC apoptosis. Once stabilized and matured, the vessel forms a barrier between the blood and surrounding tissue, controlling the exchange of fluids and solutes. Key molecular players discussed in this Review and elsewhere (5, 132) are indicated.

Ang2 on tumor progression upon over- or underexpression are complex and often divergent (26). Because of its pleiotropic effects in angiogenic, lymphatic, and macrophage biology as well as the complexities in receptor use and contextual localization, a better understanding of the tumor-specific expression of the Tie receptors and their ligands is needed to improve Ang/Tie-targeted therapy.

Tip cell guidance
ECs express guidance receptors that probe the environment (Figure 2). Nrp1, alone or complexed to plexin family members, interacts with semaphorins (Sema). Sema6a regulates VEGFR2 expression and its downstream signaling (27), while Sema3E activates PlexinD1 in tip cells and maintains the tip cell/stalk cell balance by regulating VEGF activity and DLL4 expression (28). Another guidance class involves SLIT proteins, which are ligands for roundabout receptors (Robo). Robo4 is expressed in ECs, but its role remains debated, as it has been attributed both chemoattractant and repellant activity (29, 30). However, when binding to Unc5B, a netrin receptor, Robo4 increases vessel integrity and reduces angiogenesis by inhibiting VEGF signaling (31). VEGF also promotes the expression of the transcription factor Hlx1, which increases expression of Unc5, plexin 5, and Sema3G,

suggesting feedback with Robo4 (32). Hlx1 is expressed in sprouting ECs, where it maintains the stalk cell phenotype by regulating repulsive signals (33).

Ephrins activate Eph receptor tyrosine kinases to regulate developmental vessel morphogenesis (34). In zebrafish, angioblasts form a precursor vessel that segregates into discrete arterial and venous vessels. Ephrin-B2–expressing ECs, fated to form arterial vessels, segregate from EphB4-expressing ECs, which become venous vessels due to repulsive cues (35). Ephrin-B2 activates Eph receptors in a positive feedback loop and has its own reverse signaling activity, which is important for EC morphology and motility (36). Deletion of Ephrin-B2's PDZ binding motif results in fewer tip cells and filopodia, an effect due to its regulation of VEGFR2/VEGFR3 internalization and trafficking (37, 38). Furthermore, antibody blockade of Ephrin-B2 inhibits tumor angiogenesis in preclinical studies (39).

Macrophages orchestrate vessel fusion and formation
When tip cells of adjacent vessels meet via filopodia, they connect and anastomoze (Figure 2). Imaging in zebrafish reveals that cell junctions at the site of contact expand into rings, generating an interface of apical membrane compartments (40). Macrophages act as "bridge cells" between anastomosing tip cells by releasing angio-

genic factors (41). In disease, macrophages have contextual effects. In ischemia they promote collateral vessel growth (42), while in tumors M1-polarized macrophages are tumoricidal, but M2-polarized macrophages promote tumor vascularization by producing proangiogenic factors (43). It is unclear whether tumors "educate" macrophages to these phenotypes or whether tumors recruit distinct monocyte populations. Targeting myeloid cells is gaining increasing attention for blocking tumor angiogenesis and growth (44). Possible targets include placental growth factor (PlGF), which promotes M2 polarization (45), or Ang2, which increases macrophage association with tumor blood vessels (46, 47). The oxygen sensor HIF-prolyl hydroxylase domain protein 2 (PHD2) also modulates the macrophage phenotype and regulates collateral vessel growth in ischemia (42).

Stalk cell proliferation and maintenance
Stalk cells elongate the sprout shaft (Figure 2). In vitro, Notch inhibits EC proliferation; however, stalk cells must proliferate to elongate the shaft in vivo. To overcome this, stalk cells express the Notch target Notch-regulated ankyrin repeat protein (Nrarp), which limits Notch signaling at branch points while allowing continued Wnt signaling to promote EC proliferation and vessel stability (48). Because of the pro–stalk cell activity of Notch, posttranslational modifications finely tune its activity to prevent excessive signaling. The NICD is acetylated, which stabilizes the protein against ubiquitylation-dependent proteasomal degradation. Sirtuin-1, a NAD$^+$-dependent deacetylase, associates with NICD and reduces its deacetylation levels (49). Interestingly, sirtuin-1 is more active during fuel and energy stress, which suggests that it promotes vessel branching via Notch inactivation to guide ECs to fuel-rich areas (49). How targeting these stalk cell signals can be used therapeutically for cancer remains to be determined.

Other modulators of tip/stalk cell balance
Activin receptor–like kinase 1 (Alk1), an EC-specific member of the TGF-β/BMP9 receptor superfamily, is inactivated in patients with hereditary hemorrhagic telangiectasia (HHT) (50). Smad1/5, effectors of Alk1 signaling, also orchestrate the balance between tip and stalk cells in the mouse retina (Figure 2). Loss of Smad1/5 impairs Notch signaling and increases tip cells at the expense of stalk cells, possibly because the interdependence between Notch and Smad1/5 results in oscillatory fluctuations of tip/stalk cell targets that determine the dynamic shuffling of ECs at the tip (51). In agreement with this model, Alk1 inhibits retinal angiogenesis by cooperating with Notch: combined blockade of Alk1 and Notch exacerbates hypervascularization, while activation of Alk1 by BMP9 rescues hypersprouting by Notch inhibition (52). However, Alk1's activity is context dependent; in preclinical tumor models, Alk1 is proangiogenic in the presence of VEGF, and an anti-Alk1 antibody and BMP9 trap inhibit tumor angiogenesis (53).

Vessel maturation by pericyte recruitment
Mural pericytes reduce EC proliferation, migration, and vessel leakage, thereby stabilizing nascent vessels (Figure 2 and refs. 54, 55). TGF-β1 promotes the differentiation of precursor cells to pericytes (56). PDGFR-β–expressing pericytes migrate in response to PDGF-B from ECs and surround newly formed vessels. Ang1 was previously suggested to promote pericyte coverage of blood vessels (57). However, conditional global *Ang1* gene inactivation studies showed that early Ang1 deficiency causes vascular morphogenesis

defects, which are caused by cardiac defects and secondary flow disturbance, without affecting pericyte recruitment (58). In postnatal angiogenic conditions, Ang1 deficiency accelerated angiogenesis, which suggests that Ang1 is dispensable for quiescent vessels but modulates the vascular response after injury (58).

Pericyte-expressed sphingosine-1-phosphate (S1P) regulates EC barrier properties by upregulating N-cadherin between ECs and pericytes while downregulating Ang2 in ECs (59). S1P receptor (S1PR) signaling acts as a vascular stabilization mechanism by impairing sprouting via inhibition of VEGF signaling and stabilization of VE-cadherin junctions (60, 61). Reduced pericyte coverage is associated with metastasis in patients, and overexpression of PDGF-B increases pericyte coverage that results in tumor growth inhibition. Concerns have been raised regarding pericyte targeting, as this increases epithelial-to-mesenchymal transition and metastasis because of a reduced barrier for tumor cells to intravasate (62).

Resuming quiescence
Phalanx ECs line quiescent vessels (Figure 3). Once the hypoxic tissue is perfused by neovessels, levels of angiogenic signals are reduced, and proquiescent molecules are increased. Autocrine signals, including VEGF, Ang1, FGF, and Notch, maintain ECs in quiescence (63). Ang1 induces DLL4 expression and NICD signaling (64). Furthermore, deposition of a BM around quiescent ECs promotes vessel stabilization, partly because the BM component laminin-α4 in tip cells limits their number by inducing Notch signaling (65, 66). Phalanx cells in a tightly apposed monolayer optimize conduction of blood flow, establish tissue barriers, and form intercellular junctions to tighten the EC barrier. An oxygen-sensing system ensures that ECs normalize abnormalities in structure and function of ECs to readapt oxygen supply to tissue needs. Indeed, via stabilization of HIF-2α, haplodeficiency of the PHD2 oxygen sensor promotes phalanx differentiation, thereby tightening the EC barrier and reducing tumor cell intravasation (67). Accordingly, endothelial HIF-1α increased leakage and tumor cell intravasation and extravasation, while HIF-2α had opposite effects (68).

Pathological angiogenesis: distinct from vascular development?
It has been postulated that the molecular players and vascular branching model in pathological angiogenesis are parallel to developmental angiogenesis, but have dysregulated expression. However, some molecules have different functions during physiological and pathological angiogenesis. For example, VEGFR1 and its ligands, PlGF and VEGF-B, are dispensable for development, yet they regulate angiogenesis in disease (69, 70). In developmental angiogenesis, VEGFR1 has a negative role by trapping VEGF (71), but this model does not explain its disease-restricted proangiogenic activity (72, 73). Stromal cell PlGF production, induced by contact with tumor cells, not only promotes angiogenesis in the leukemic bone marrow or medulloblastoma, but also stimulates tumor cell proliferation via Nrp1 signaling (74, 75). Although it is superfluous for vascular development, VEGF-B promotes contextual enlargement of myocardial capillaries (76) or growth of coronary vessels (77). Another example is ataxia teleangectasia mutated (ATM), which only regulates angiogenesis in disease, not in health (78). These examples (and others) suggest that part of the molecular basis of pathological angiogenesis is different from that in vascular development. Moreover, insights obtained from developmental angiogenesis models may not completely recapitulate the

Table 1
Clinical results obtained for anti-VEGF agents

Drug	Mode of action	Tumor types treated	Successes	Limitations	Reference
Bevacizumab (Avastin)	Monoclonal anti-VEGF antibody	BRCA, CRCA, NSCLC, RCC, glioblastoma, ovarian cancer, gastric cancer, PACA, PRCA	Improved PFS in the majority of trials	No OS improvement in the majority of trials; no PFS improvement in a trial of PACA and a trial of CRCA	80, 82, 113, 115, 123, 133–145
Aflibercept (Zaltrap)	Chimeric VEGF/PlGF neutralizing receptor	CRCA, PACA, NSCLC	Improved PFS in CRCA and NSCLC; improved OS in metastatic CRCA	Little to no OS improvement; no PFS improvement in PACA	86, 133, 146
Sorafenib (Nexavar)	Small-molecule VEGFR TKI	RCC, HCC, melanoma, NSCLC	Improved PFS in RCC; improved OS in HCC	No OS improvement in RCC; no PFS or OS improvement in metastatic melanoma or NSCLC	133, 147, 148
Sunitinib (Sutent)	Small-molecule VEGFR TKI	RCC, GIST, BRCA, HCC, CRCA, pancreatic NETs, NSCLC, PRCA	Improved PFS in advanced/metastatic RCC, GIST, pancreatic NETs, and PRCA; increased OS in RCC and pancreatic NETs	No OS improvement in the majority of trials; no PFS or OS improvement in metastatic BRCA or CRCA	133, 149–151, NCT00393939, NCT00373113, NCT00457691, NCT00676650
Pazopanib (Votrient)	Small-molecule VEGFR TKI	RCC, NSCLC, STS	Improved PFS in RCC and STS	No OS improvement	152, 153
Vandetanib	Small-molecule VEGFR TKI	NSCLC, MTC	Improved PFS in metastatic MTC	No OS improvement; little to no PFS improvement in metastatic NSCLC	133, 154, 155
Vatalanib/ PTK 787	Small-molecule VEGFR TKI	CRCA	–	No OS improvement; little to no PFS improvement in metastatic CRCA	133
Cediranib	Small-molecule VEGFR TKI	CRCA, glioblastoma	–	No improvement in OS or PFS	156, NCT00777153
Axitinib	Small-molecule VEGFR TKI	RCC, PACA	Improved PFS in RCC	No OS improvement; no PFS improvement in metastatic PACA	157, NCT00471146

BRCA, breast cancer; CRCA, colorectal cancer; HCC, hepatocellular carcinoma; MTC, medullary thyroid cancer; NET, neuroendocrine tumor; PACA, pancreatic cancer; PRCA, prostate cancer; RCC, renal cell carcinoma; STS, soft tissue sarcoma; TKI, tyrosine kinase inhibitor. Only results from phase 3 clinical trials for FDA-approved agents are discussed here; see Supplemental Table 1 for a complete overview.

mechanisms that drive human pathological angiogenesis. To further our understanding of antiangiogenic medicines, it is therefore essential that sufficient feedback about the mechanism of pathological angiogenesis is provided by bedside-to-bench research.

Successes and challenges of antiangiogenic drugs

The field has focused on developing VEGF and VEGFR inhibitors (VEGFIs and VEGFRIs, respectively) (1, 79). While low autocrine VEGF signaling maintains quiescent vessel integrity, increased VEGF/VEGFR2 signaling induces angiogenesis, thereby creating a therapeutic window for antiangiogenic therapy. Current VEGFI/VEGFRI-based therapies prolong PFS and/or overall survival (OS) in a fraction of cancer patients (ref. 1, Table 1, and Supplemental Table 1; available online with this article; doi:10.1172/JCI70212DS1). Bevacizumab also shows efficacy in the neoadjuvant setting in breast cancer (80).

Despite the success of antiangiogenic drugs, several questions warrant further research to improve anticancer treatment. First, some cancers are resistant; even in responsive patients, antiangiogenic drugs generally prolong survival only in the order of months. The FDA revoked the approval of bevacizumab for metastatic breast cancer (81). In general, clinical efficacy is lower than that observed in preclinical cancer models (79). These models often represent rapidly growing ectopic tumors that do not reflect the heterogeneous human cancers developing over years in situ. Even transgenic models do not fully reflect the multistep carcinogenesis that occurs in humans. Another concern is that the majority of preclinical studies were undertaken in the neoadjuvant setting, which is a poor model for human metastatic cancer (79). Moreover, many drug combinations that proved ineffective were not studied preclinically (82).

One mechanism underlying resistance is that tumors produce multiple proangiogenic molecules in addition to VEGF, including PlGF, FGFs, interleukin-8, and others. Tumor ECs engineered to overexpress DLL4 develop enlarged mature vessels that are resistant against VEGF blockade, while inhibition of Notch signaling restores the sensitivity to antiangiogenic drugs in a xenograft model (83). Resistance can result from activation of FGF2-FGFR and EphB4–Ephrin-B2 pathways or from decreased levels of VEGFR2 (83). Several proangiogenic molecules become upregulated under selective pressure by VEGFIs/VEGFRIs (79, 84). PlGF and FGF2 plasma lev-
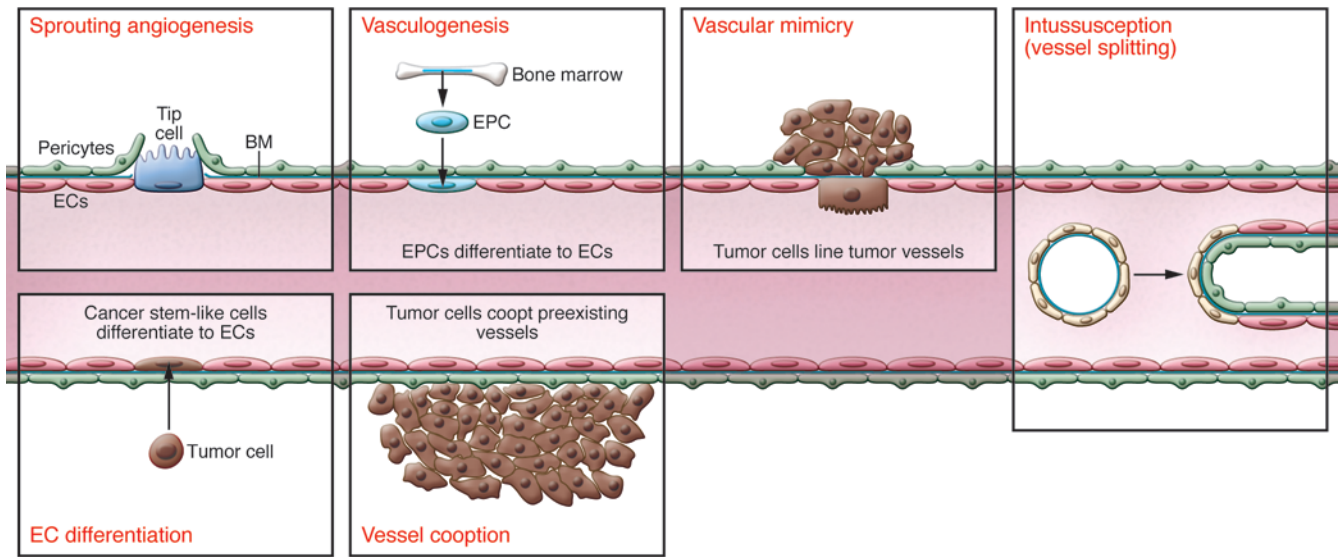
Figure 4
Tumor vascularization modes. After development, the vasculature rarely extends, but does so in tumor formation. Tumor vascularization occurs via a number of potential mechanisms. While angiogenesis is the most investigated, and the focus of this Review, other mechanisms have been observed. Endothelial progenitor cells (EPCs), which can either reside in the vascular wall or migrate from bone marrow in response to chemoattractants from the tumor cell, can differentiate into ECs and contribute to vessel formation. Vascular mimicry can also occur, whereby tumor cells can act as replacement cells for ECs. Another possibility is that chromosomal abnormalities in putative cancer stems cells allows tumor cells to differentiate into ECs. Other mechanisms by which tumor cells can obtain a blood flow include vessel cooption, whereby the tumor cell arises near to (or migrates toward) a preexisting blood vessel, or the process of intussusception, whereby a preformed vessel splits into two daughter vessels by the insertion of a tissue pillar.

els increased prior to progression of colorectal cancer patients treated with bevacizumab and chemotherapy (85). A phase III trial reported the efficacy of aflibercept, which blocks VEGF and PlGF, in patients who progressed on bevacizumab therapy (86).

Cancers also switch between different modes of vascularization, further explaining the resistance to VEGF blockade (Figure 4). Besides sprouting angiogenesis, they use vessel cooption (by growing around preexisting vessels), vascular mimicry (replacement of ECs by tumor cells), and vasculogenesis (vessel growth from bone marrow–derived progenitor cells), although the clinical relevance of these mechanisms remains unclear (87–89). For instance, metastases of melanoma and lung cancer grow in an angiogenesis-independent manner around existing vessels or switch to vessel cooption upon treatment with bevacizumab (90). Furthermore, cancer stem cell–like cells differentiate to ECs that exhibit reduced sensitivity to VEGF blockade (88, 91). Skin cancer stem cells in vascular niches express Nrp1 and produce VEGF, which might promote stemness independently of VEGF's activity (92). Resistance in certain cancers is associated with pericyte-covered vessels, while tortuous uncovered vessels are observed in primary resistance (93).

CD11b+Gr1+ myeloid-derived suppressor cells (MDSCs) confer resistance to initially sensitive tumors (94). G-CSF plays a role in mobilization of MDSCs from the bone marrow (94). Strategies aimed at reducing mobilization and/or tumor infiltration of MDSCs might help to reduce resistance. Antiangiogenic drugs induce a systemic proinflammatory and proangiogenic burst in tumor-bearing healthy mice by upregulating PlGF, G-CSF, and osteopontin (95), which induce mobilization of resistance-conferring MDSCs (96, 97). The tumor microenvironment can also cause refractoriness. For instance, pancreatic adenocarcinomas have high interstitial fluid pressure due

to abundant deposition of hyaluronic acid, which impairs perfusion and drug distribution (98). Notably, disaggregation of hyaluronic acid by enzymatic treatment improved perfusion (98).

Another hypothesis to explain the lower than expected efficacy of VEGF-targeted antiangiogenic drugs is that these treatments increase, rather than reduce, tumor malignancy. Indeed, certain preclinical studies show enhanced metastasis in tumor-bearing mice treated with VEGF-blocking drugs, such as sunitinib (79, 84, 99, 100). However, these findings remain debated because other preclinical studies did not detect increased metastasis (101, 102), and large meta-analyses have not shown more metastatic dissemination in patients (79, 103). Strategies combining antiangiogenesis with inhibition of metastasis might be useful to increase therapeutic efficacy. For instance, VEGF suppresses HGF-dependent c-MET phosphorylation and tumor cell migration, which explains why VEGF blockade promoted invasiveness (104) and combined VEGF/c-MET inhibition reduced metastasis (105, 106). c-MET is upregulated in bevacizumab-resistant glioblastomas (107). VEGF inhibitors also cause class-specific side effects (thromboembolic events, hypertension, gastrointestinal perforation, impaired wound healing, renal toxicity, and congestive heart failure) by depriving quiescent ECs of VEGF's prosurvival effect (108).

Mode of action and schedule of application
The precise mode of action of antiangiogenic drugs in cancer patients remains incompletely understood. Antiangiogenic drugs can block angiogenesis, inhibit recruitment of proangiogenic bone marrow–derived cells (5, 109), induce vessel regression, and promote sensitization to radio- and chemotherapy by depriving ECs of VEGF's prosurvival effect (5, 109). Antiangiogenic drugs also inhib-

Table 2
Challenges with the use of antiangiogenic drugs and possible considerations for overcoming them

Challenge	Possible considerations
Lower clinical efficacy than in preclinical studies (79)	Use appropriate endpoints (OS) and preclinical testing of drug combinations used in the clinic (79)
Multiple angiogenic factors besides VEGF promote tumor angiogenesis (79, 83, 158)	Develop inhibitors of alternative proangiogenic targets and test combination therapies (79, 83, 158)
Primary tumor/metastasis are vascularized by mechanisms other than sprouting angiogenesis (88–90, 159)	Preclinical research to identify targets regulating alternative modes of tumor vessel growth (88–90, 159)
Resistance due to myeloid cell infiltration (94, 96)	Develop antimyeloangiogenic therapies (96, 97)
Antiangiogenic drugs induce inflammatory/proangiogenic host response (79, 95–97)	Perform (pre)clinical research to understand host response; inhibit host-derived factors (PlGF, osteopontin, G-CSF, etc.) (79, 96)
Profibrotic host reaction by cancer-associated fibroblasts increases interstitial fluid pressure and reduces perfusion (98)	Develop antifibrotic therapies (enzymatic digestion of matrix) (98)
Enhanced tumor cell motility elicited by antiangiogenic drugs (79, 84, 99, 100)	Concomitant use of antiangiogenic and antimetastatic therapies (pan-VEGFR and Met TKI) (105)
Mode of action is incompletely understood (5, 109, 132)	Preclinical research to understand vessel-normalizing (4, 45, 160, 161) versus vessel-regressing (111) effects; analyze effects on cancer stem cells (92)
Optimal duration of application is unclear (113, 114)	Randomized clinical studies with prolonged application beyond progression, also with TKIs
Reasons for context-specific efficacy (early-stage vs. metastatic) are unclear (113, 121)	Research to better understand differences in vascularization of macro- vs. micrometastases
Toxicity of orthosteric antiangiogenic inhibitors (108)	Develop allosteric antiangiogenic inhibitors (130, 131)

Challenges were identified in (pre)clinical studies. TKI, tyrosine kinase inhibitor.

it VEGFR-expressing tumor cells (5, 109) or deprive cancer stem cells from EC-derived angiocrine signals (110). Moreover, VEGF inhibitors reduce the number and self-renewal capability of cancer stem cells (92). Alternatively, antiangiogenic drugs block excess VEGF levels secreted by tumor cells. This uncoordinated secretion of VEGF induces a chaotic proangiogenic response characterized by hyperbranching and lack of maturation, which renders tumor vessels dysfunctional. Consequently, delivery of chemo- and radiotherapy is impaired. Anti-VEGF normalizes tumor vessels and restores delivery of radio- and/or chemotherapy in tumors, although this subject remains debated (4, 45, 111). Nonetheless, vessel normalization can explain why bevacizumab works better when combined with chemotherapy. The challenge in the clinic is to identify agents that cause permanent tumor vessel normalization.

Another question is the optimal duration and dose of antiangiogenic drug application. Certain preclinical studies indicate rapid revascularization after cessation of treatment (112); the clinical relevance of this observation requires future study. Adjuvant application of bevacizumab for 12 months had a beneficial effect on PFS of early-stage colorectal cancer patients when analyzed after 15 months, but this effect was lost after 36 months (113); additionally, the outcome of patients with metastatic colorectal cancer was better when bevacizumab was given beyond progression (114). A phase III trial in colon cancer showed a modest OS advantage for patients treated with bevacizumab beyond progression (115). Bevacizumab beyond progression is currently being investigated in other cancer types.

Contextual mode of action
The efficacy of antiangiogenic therapies differs among cancer types. Antiangiogenic drugs are more efficient in well-vascularized cancers (e.g., clear cell renal cancer), in which bevacizumab is effective without chemotherapy (116, 117). In contrast, antiangiogenic agents are less effective in less vascularized cancers (e.g., pancreatic adenocarcinoma and gastric cancer) (118, 119). Nonetheless, pharmacological VEGF blockers can have dose- and drug class–dependent effects (120). Indeed, in mouse models of metastasis, an anti-VEGF antibody did not promote metastasis, in contrast to small-molecule receptor tyrosine kinase inhibitors. One of those, sunitinib, enhanced metastasis and lung permeability and promoted tumor cell extravasation by inhibiting tyrosine phosphorylation of proteins, important for EC-EC junctions (120). Another concern is the context-dependent efficacy of antiangiogenic agents in micro- versus macrometastatic disease. Two phase III studies failed to show benefit of bevacizumab combined with chemotherapy in early-stage (stage II and III) colorectal cancer in the adjuvant setting (113, 121). Similarly, in breast and ovarian cancer, bevacizumab lacks efficacy in the adjuvant setting (122, 123). The reasons remain elusive, but may be due to rebound angiogenesis, although this has not been observed when using tyrosine kinase inhibitors (124). Another possibility is that micrometastases grow in an angiogenesis-independent fashion and might survive antiangiogenic treatment in a dormant state, or use other vascularization principles, such as vessel cooption, which is less sensitive to VEGF inhibition (125). In vivo imaging documented that anti-VEGF treatment prolonged the dormancy of micrometastatic tumor cell aggregates by blocking angiogenesis (90). Alternatively, vessels in micrometastases might be less abnormal, because proangiogenic factors are less abundant and the chemosensitizing effect of bevacizumab would therefore be less important. Given that more than 20,000 cancer patients are being enrolled in trials testing anti-VEGF therapy in the adjuvant setting, understanding the modes and mechanisms of vascularization of micrometastatic disease is a center-stage priority.

Conclusion and perspectives

Research in angiogenesis continues to yield new molecular insights about vessel branching. Emerging avenues include studying the role of metabolism in ECs (126), the molecular determinants of cellular shape and size in patterning connectivity, the role of pericytes in neurovascular disorders, the mechanistic basis of EC polarity, the different modes of tumor vascularization, and the genetic basis of tumor vessel normalization. Another intriguing area of research is how ECs feedback on cancer (stem) cells by providing angiocrine signals (110, 127). Still, much needs to be clarified about the context-dependent activity of many angiogenic factors. The challenge is to translate this knowledge into improving therapy (Figure 2). Numerous areas of clinical research are of high priority, including the optimization of drug regimes, the use of predictive biomarkers to identify putative responders versus nonresponders (as illustrated by a *VEGFR1* genetic locus, ref. 128; and short VEGF isoform, ref. 129), the development of antiangiogenic treatment of pediatric tumors (such as anti-PlGF therapy of medulloblastoma, ref. 75), the development of vessel normalization drugs, and the development of VEGF-independent antiangiogenic drugs that can be used in combination with existing antiangiogenic therapies. The development of allosteric antiangiogenic inhibitors, which offer a superior advantage of safety, specificity, and efficacy over current orthosteric antiangiogenic antagonists, is also commendable (130, 131). Finally, more bedside-to-bench studies are needed to provide the necessary feedback needed to further improve the overall efficiency of antiangiogenesis therapy.

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