

# NOVEL ANGIOGENIC SIGNALING PATHWAYS AND VASCULAR TARGETS

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■ **Abstract** Intense investigation into the molecular basis of angiogenesis is rapidly revealing novel signaling pathways involved in the generation of new vasculature. These range from elucidation of the mechanism by which hypoxia initiates expression of a proangiogenic gene repertoire via the hypoxia-inducible transcription factors (HIFs) to molecular pathways involved in extra- and intracellular signaling during new vessel formation. Extracellular pathways include those of the Notch/delta, ephrin/Eph receptor and roundabout/slit families, and intracellular pathway members of the hedgehog and sprouty families. The involvement of these pathways in angiogenesis is discussed, together with some comments on recently identified targets in the vasculature that present new therapeutic opportunities.

## INTRODUCTION

Angiogenesis has been a topic of vigorous research for more than a decade, a situation stimulated by the discovery of key angiogenic growth factors vascular endothelial growth factor (VEGF) and basic and acidic fibroblast growth factors. There have been many recent reviews on the processes involved in angiogenesis and its role in cancer and as a therapeutic target [see, for example, (1, 2)]. This review, therefore, focuses on more recently described pathways of the past two to three years, particularly those initially identified in embryonic vascular development and differentiation. These pathways are important in normal and pathological angiogenesis (development of blood vessels from a preexisting vasculature) and also in vasculogenesis (development of blood vessels from progenitor cells).

## Hypoxia Signaling Pathways and Angiogenesis

As tissues or tumors outgrow their blood supply or are deprived of oxygen, a gene response to hypoxia is initiated. Although several transcription factor pathways are involved, most attention has focused on hypoxia-inducible factor 1 (HIF1). This is a heterodimer of two DNA binding proteins, HIF-1 $\alpha$ , and the aryl hydrocarbon nuclear translocator (ARNT or HIF-1 $\beta$ ). In normoxia, HIF-1 $\alpha$  is unstable and rapidly degrades via the proteasome, but as oxygen tension drops below 2%, (air is

20%), HIF-1 $\alpha$  is stabilized, translocates to the nucleus, and interacts with HIF-1 $\beta$  to transcribe a complex gene program via specific hypoxia response elements (HREs). Among the genes upregulated are VEGF, the glycolytic pathway, and pathways involved in invasion (urokinase receptor, PAI1); however, many other genes involved in wound healing and angiogenesis are also induced [see review (3)].

In the past two years, the pathways mediating the hypoxia signal have been elucidated: HIF-1 $\alpha$  is posttranslationally modified on two prolyl residues by prolyl hydroxylases, which require oxygen as a cofactor, as well as ferrous iron, vitamin C, and 2-oxoglutarate (4). There are three well-characterized enzymes that have prolyl hydroxylase domains (PHDs 1, 2, and 3) (5). PHD-2 is hypoxia inducible and most widely expressed. The consequence of hydroxylation is that HIF-1 $\alpha$  is then recognized by the Von Hippel-Lindau protein, an ubiquitin ligase that targets HIF-1 $\alpha$  for destruction in the proteasome (6). Clearly, as oxygen becomes less abundant, there is less modification; therefore, HIF-1 $\alpha$  is stabilized. There are two other HIF $\alpha$ s: HIF-2 $\alpha$  and -3 $\alpha$ . The former is also critical for embryonic development, but its role in adult angiogenesis and cancer is poorly defined compared to HIF-1 $\alpha$ . Although HIF-1 $\alpha$  is commonly upregulated in tumor epithelium, HIF-2 $\alpha$  is much more highly expressed in the stromal cells, such as in macrophages (7), suggesting a different hypoxia response program in this cell type. Other posttranslational modifications, including asparagine hydroxylation (8) and lysine acetylation (9), contribute to inhibition of HIF-1 $\alpha$  function in normoxia.

The angiogenic factor most intensively investigated is VEGF, and it is strongly induced by HIF-1 $\alpha$  via HREs in the 5' and 3' ends of the gene. Many oncogenes also activate the transcription of VEGF and separately enhance function or expression of HIF-1 $\alpha$ . The mechanisms include increased translational efficiency (10), stabilization of the protein (11), or enhanced transactivation (12). Taking into account the hypoxic microenvironment of tumors, it is clear that angiogenic genes downstream of HIF-1 $\alpha$  are likely to be important in tumor angiogenesis and in response to vascular occlusion.

Besides VEGF, endothelins 1 and 2, adrenomedullin, and angiogenin (13) are also induced by hypoxia. The VEGF chaperone, ORP 150, is regulated by hypoxia (14), as are connective tissue growth factor (15), leptin (16), stromal cell-derived factor 1 [CXCL 12] (17), migration inhibitory factor (18), and placenta growth factor (PLGF) (19). The intermediary metabolites produced via glycolysis induced in the absence of oxygen, e.g., pyruvate and lactate, are also angiogenic (20). Several reviews have suggested that hypoxia is a key factor contributing to tumor growth and angiogenesis (21, 22), and it can select for clonal variants, which are more aggressive as they have survived hypoxic stress.

This wide range of angiogenic pathways regulated by hypoxia suggests that drugs targeting the HIF pathways should be considered as antitumor agents (23) and, conversely, drugs activating HIF would be proangiogenic. Experiments modifying HIF in either direction have produced the predicted effects, in general, via a variety of genetic manipulations or with small molecules. Examples include

knockouts of HIF-1 $\alpha$  and use of dominant-negative constructs or antisense (24) versus mutations in HIF-1 $\alpha$  or peptide mimetics (25), which prevent degradation.

## NEW ASPECTS OF VASCULAR ENDOTHELIAL GROWTH FACTOR SIGNALING

### The Vascular Endothelial Growth Factor Receptor Family and Ligands

Because this pathway is so critical in angiogenesis and vasculogenesis, a brief summary is provided here, but it has been the subject of many recent reviews (26–30).

There are three VEGF receptors (Figure 1). VEGF receptor 2 (VEGFR2, also known as KDR) is considered to be the main receptor mediating proliferation of endothelial cells after stimulation by VEGF. VEGF receptor 1 (FLT-1) is a high-affinity VEGF receptor and a naturally occurring splice variant, encodes the extracellular domain that is secreted as a soluble protein (sflt1), and is a potent antagonist of VEGF by binding VEGF with high affinity and reducing its interaction with its receptors. FLT-4 is VEGF receptor 3 (VEGFR3) and, in embryonic development, is present in the vasculature but becomes lymphatic specific in the adult. They are tyrosine kinases, forming dimers and signaling via several secondary pathways, e.g., MAP kinases and akt. The kinase domain is interrupted and split into two functional domains, hence, the name kinase insert domain or KDR.

Each of these three receptors has a family of VEGF ligands of different specificities. VEGF-A binds to VEGFR-1 and -2 and sflt1. VEGF-C binds to VEGFR2 and VEGFR3 and has a major involvement in lymphatic growth and development. PLGF1 and PLGF2 bind to FLT-1 soluble and receptor forms only, as does VEGF-B. VEGF-E is a viral VEGF molecule that binds only to VEGFR2. There are multiple splice variants of VEGF-A that affect its ability to bind heparin; the splice variants are 145, 165, and 189 amino acids and all show increased affinity for heparin and increased local effects on angiogenesis. There are also splicing and proteolytic processes for VEGF-C and -D and the other members of the family.

This, therefore, provides an extremely complex environment for local variation and control of angiogenesis by microenvironmental factors, splicing, extracellular matrix interactions, as well as receptor expression and competition between ligands and receptors. Until recently, however, it has been thought that VEGF-A and VEGFR2 were the main angiogenic pathways with relevance to tumor vasculature and revascularization of occluded vessels.

Recent findings of interest have been the demonstration that the HRE in the VEGF promoter (31) provides VEGF with a critical normal role in maintaining CNS function, and in its absence, mice develop a syndrome resembling motor neuron disease. It is likely this is related to VEGF effects via neuropilin receptors on neuronal maintenance.

VEGFR3 in adult vasculature is associated only with lymphatics and is necessary for embryonic blood vessel development. However, in pathological conditions, including inflammation and malignancy, it is again expressed in the abnormal blood vessels (32).

Mutations and functional polymorphisms in the VEGF pathway are also emerging. In juvenile hemangioma, there is spontaneous growth of the lesions in the first year of life, usually followed by spontaneous regression. Missense mutations in the kinase or kinase-insert domain of VEGFR2 and -3 were reported (33), but functional studies have not been done. Polymorphisms in the promoter have, however, been analyzed and a -460/+405 polymorphism increased basal activity, but enhanced responsiveness to phorbol ester fivefold (34). Studies of these genetic differences in predisposition to diseases and cancer will be of major interest and could determine responsiveness to antiangiogenic therapy. The results highlight the individual variability of angiogenesis.

## **Cancer Vaccines to Vascular Endothelial Growth Factor Receptors**

Expression of VEGF receptors is known to be upregulated in tumors compared to normal vasculature. Recently, two studies appeared where, in mouse models, active immunization against the flk1 receptor (KDR in man) (35) or use of cytotoxic T lymphocytes engineered to express VEGF (and so target VEGF receptor-expressing cells) (36) were shown to elicit significant antitumor activities. In the first of these studies, an immune response to flk1 was elicited by immunization with dendritic cells pulsed with a soluble flk1 protein. The immunization generated flk1-specific neutralizing antibodies and CD8+ cytotoxic T cell responses, breaking tolerance to self-flk1 antigen. Both tumor-induced angiogenesis in an alginate bead assay and pulmonary metastases from B16 or Lewis lung carcinomas were strongly suppressed in the immunized mice. In the second approach, recombinant retroviral vectors were generated that encoded a chimeric T cell receptor comprised of VEGF sequences linked to intracellular signaling sequences derived from the zeta chain of the T cell receptor. Transduced murine CD8 cells effectively killed flk1-expressing cells in vitro. Adoptive transfer of the CD8 cells into tumor-bearing mice strongly inhibited the growth of a range of syngeneic murine tumors and human tumor xenografts.

## **Endocrine Gland-Derived Vascular Endothelial Growth Factor**

This factor was reported as an angiogenic molecule with specific activity on endothelial cells derived from the thyroid gland, and was the first evidence for an endocrine or organ-specific angiogenic pathway (37). Endocrine gland-derived VEGF is actually a member of the prokineticin family, of which there are two so far (prokineticin 1 and 2) that were initially discovered because they are potent smooth muscle contractants. Their receptors are two orphan G-protein coupled receptors, ZAQ and I5E respectively (38, 39). They have a role in testicular

angiogenesis (40) but have not been shown to be involved in nonendocrine cancer (41).

### **PLGF1 and VEGFR1 Signaling**

Although most emphasis has been on the role of VEGFR2 in angiogenesis, several recent studies have highlighted a separate and significant role for VEGFR1. In transgenic mouse models deficient in PLGF1, embryonic vasculature was not impaired, but adult responses to ischemia, wound healing, inflammation, and cancer were (42). This effect was related to poor response by VEGF, not other angiogenic factors. It could not be corrected by another ligand binding to FLT1 only, such as VEGF-B, suggesting a specific interaction between PLGF1 and VEGFR1. The deficit could be recovered by marrow transplantation, and this suggested a role of progenitor cells recruited from the marrow in PLGF angiogenesis (see below). PLGF was shown to reconstitute hematopoietic stem cells via VEGFR1 (43). Conversely, antibodies specific for FLT1-inhibited tumor angiogenesis and inflammatory angiogenesis mainly by inhibiting mobilization of bone marrow-derived myeloid precursors. PLGF was also able to induce arteriogenesis in adult rabbits *in vivo* much more effectively than a ligand specific for VEGFR2 (VEGF-E), (44, 45). In tumor cell lines transfected with PLGF and grown *in vivo*, it was shown that there was an antiapoptotic effect on macrophages and endothelial cells, which encouraged macrophage infiltration and survival (46).

An added complexity has been the finding that cells coexpressing PLGF and VEGF produce heterodimers that are inactive on VEGFR2, so clearly localization of production is also likely to be important in the overall outcome of PLGF effects (47). Macrophages often have an important role in sustaining angiogenesis partly by producing VEGF (48). These results can be considered together as an endocrine effect of secreted PLGF recruiting endothelial, myeloid, or monocyte precursors from the marrow to sites of angiogenesis and providing several cell types that contribute to different types of angiogenesis, e.g., inflammatory and tumor associated. This endocrine effect is mediated via VEGFR1 and suggests that combined blockade of both this receptor and VEGFR2 is likely to be much better therapeutically. Many anti-VEGFR drugs being studied were developed against VEGFR2 specifically.

### **Neuropilins 1 and 2, Vascular Endothelial Growth Factor, and Semaphorin Receptors**

Neuropilins are extracellular receptors for two different secreted protein families, VEGF and semaphorin (49, 50). They were initially discovered because of their role in axon guidance in response to semaphorins. VEGF and semaphorin 3A (Sema 3A) have opposing effects in the growth and survival of neurons and endothelial cells. Sema 3A decreases axonal and endothelial growth and survival, whereas VEGF promotes both in these cell types. Specific heparin-binding splice variants of VEGF bind, but VEGF121 does not bind (51). Binding to neuropilin

enhances signaling several-fold through VEGFR2. A naturally occurring extracellular form of neuropilin 1 (NP1) is an antagonist of VEGF signaling, and Semaphorin 3A antagonizes VEGF signaling via the interaction with NP1 (52). Although NP1 has only a short intracellular domain, tumor cells overexpressing NP1 have enhanced *in vivo* growth with increased angiogenesis (53). Cancer cell lines expressing NP1 have increased motility owing to VEGF upregulating CXCR4, and VEGF also enhances survival in these cells (54,55). Studies have focused on increased signaling via VEGFR2, but NP1 also interacts with VEGFR1 (56).

A second neuropilin, NP2, is less well defined, but both are important for yolk sac and embryonic angiogenesis (57). VEGF 165 binds to both NP1 and NP2, but VEGF 145 only to NP1. Additionally, VEGF-B and PLGF2 bind to NP1, and PLGF2 and VEGF C to NP2 (58). However, additional modulating effects on VEGFR signaling are currently unknown, although this highlights the complexity of developing therapeutics.

The striking concordance of vascular and neuronal branching (59) is also due to the neuropilin pathway, with similar responses of the two systems to morphogen gradients of VEGF and Semaphorin 3A, rather than direct influences of one system on the other (60).

## Notch and Delta

Notch signaling is a highly conserved pathway, initially discovered in *Drosophila* development (61). It is a widely used mechanism for regulating cell fate in virtually every cell type studied to date. Its importance in vascular development is highlighted by the genetic defects that arise in humans with mutations in the pathway, *i.e.*, the Alagille syndrome with loss of function mutations in a ligand for Notch, Jagged 1, and CADASIL (cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (62). The latter has mutations in the receptor Notch 3, and both syndromes have a component vascular phenotype or pattern of lesions that is correlated with normal expression patterns of the pathways in vessels.

There are four Notch receptors (Notch 1–4) and five ligands (Jagged-1 and -2, Delta-1, -3, -4) (63). The classical description of the function of this pathway is lateral inhibition, whereby signaling from one cell expressing the ligand interacts with a nonautonomous cell expressing the receptor. The activation of Notch results in cleavage at an intramembrane site that releases the intracellular domain, which translocates to the nucleus to activate transcription. On arrival at the nucleus, the intracellular domain induces induction of a series of basic helix-loop-helix proteins that inhibit transcription of factors involved in differentiation. Thus, Notch-on blocks differentiation and allows cells to proliferate and respond to a later developmental ligand.

There are actually three distinct cleavage sites in Notch: One occurs during its intracellular processing before membrane localization, with the cleaved site ending

up on the extracellular surface. The second and third are involved in signaling, with further extracellular cleavage resulting in loss of its external domain, mediated via a metalloprotease. A final intracellular cleavage via presenilin proteases releases the intracellular signaling domain.

The intracellular signaling domain binds to the transcription factor RBP-Jk [also called CBF1/Su (H)/Lag-1 (CSL)] and then binds to a specific DNA sequence, the RBP-Jk binding site. This process upregulates HES 1, 5, and 7 and also another recently described series of transcription factors HERP 1, 2, and 3 with endothelial specificity.

The receptors are modified by specific glycosylases that regulate their function and can change the preference for ligands. These enzymes are in the fringe family (manic and lunatic fringe) (64). Proteolysis has been found to be necessary for signaling via other steps in the Notch pathway (65).

All the receptors and ligands have been expressed in at least one vascular compartment, e.g., arteries, veins, capillaries, vascular smooth muscle cells, or pericytes. However, Notch-4 is specifically expressed in arterial vessels (66), whereas the other receptors are widely expressed in many cell types and tissues. Similarly, the ligand Delta-like 4 is endothelial specific (67,68). It has been reported that vascular expression of the Notch pathway receptors and ligands is restricted to arterial vessels (69). More extensive studies have shown that they are expressed in venous vasculature also (63). Receptors do appear, however, to have an important role in arterial venous differentiation during embryonic vascular development, as Notch signaling seems to repress the venous state (70). In studies in the Zebra fish, mutations in the transcription factor grid-lock, regulated by Notch, caused disruption in the assembly of the aorta. The human equivalent gene HERP-1 does not show a similar venous type in mice homozygous for an inactivating mutation (the gene is also called HEY-2). The reasons for this species difference are currently unknown, but it may be due to compensatory mechanisms of the pathways or other family members being able to substitute for the mutation. The HERP family are basic helix-loop-helix transcriptional repressors and are Notch effectors that negatively regulate downstream genes with endothelial specificity (71). They may also form heterodimers with HES proteins.

In vitro studies of endothelial function have shown an important negative role of Notch signaling in angiogenesis. In human umbilical vein endothelial cells, an antisense oligonucleotide to Jagged-1 increased invasion and tube formation during fibroblast growth factor-induced angiogenesis, suggesting a negative role of Notch signaling (72). A secreted form of Jagged-1, which could act as a dominant-negative factor, was found to increase tube growth and angiogenesis in vivo. In concordance with this, activation of Notch in endothelial cells increased adhesion via  $\beta$ -1 integrin, increasing adhesion to collagen through changing the integrin to a high-affinity state rather than through increased protein expression (73). This  $\beta$ -1 integrin pathway is also necessary for VEGF-induced endothelial sprouting in vitro. It is interesting that Notch-4 activation can inhibit angiogenesis by blocking  $\beta$ -integrated adhesion and sprouting. Furthermore, activation of Notch-1 or

Notch-4 in endothelial cells increased expression of HER-1 and downregulated VEGFR2 with a concomitant decrease in proliferation of endothelial cells in response to VEGF but not basic fibroblast growth factor (FGF) (74).

Further links with VEGF are provided by the observation that VEGF, but not basic FGF, induce expression of Notch-1 and Delta-4 in human arterial endothelial cells by VEGFR1 and -2. Activation of Notch signaling stabilizes network formation on matrigel, whereas blocking Notch signaling inhibits network formation (75). This highlights the complexity of the interactions in these pathways whereby a potent angiogenic factor VEGF also contributes to activating a pathway involved in differentiation and downregulation of its receptor. Recent studies in Zebra fish have indicated that sonic hedgehog may regulate VEGF expression; VEGF is then able to regulate downstream signaling to determine arterial fate (see Hedgehog Signaling in Angiogenesis, below).

In recent years, there has been further emphasis placed on the potential signaling in the opposite direction to Notch via its ligands Delta or Jagged-1. The intracellular domain of the ligands interacts with PDZ proteins and can signal in the same cell presenting Jagged-1 (76). Mutations in the PDZ ligands did not affect the ability of Jagged-1 to signal through Notch-expressing cells but did reduce expression of Jagged-1 target genes and activation of reporter constructs. In fact, downregulation of the ligands by proteolysis is essential for efficient activation of Notch. Mind bomb is a gene encoded coding a ubiquitin ligase (77). It interacts with the intracellular domain of Delta to promote its ubiquitination on internalization. In the absence of this pathway, Notch activation in adjacent cells is suppressed. The observations have suggested a model of activation whereby after Delta and Notch interact, there is endocytosis of Delta with the Notch extracellular domain, allowing cleavage of the Notch receptor intracellular fragment and activation of the target genes in the Notch-expressing cells. So, surprisingly, when Delta-4 is low on the surface of the presenting cell, this is an indication of active signaling, whereas when expression is high, it would imply that the proteolytic processing is not occurring and signaling is reduced.

In the context of this new observation, it is understandable why there are conflicting results of the effects of soluble Delta-like domains as to whether they activate or inhibit Notch signaling. Indeed, one study has shown that the concentration and the amount of dimerization of the soluble domain induced affects whether it is an activator or inhibitor of Notch signaling.

Delta-4 has been found to be upregulated in tumor vasculature and in areas of angiogenesis. After trauma, there is high Jagged-1 expression in regenerating endothelial cells, and blocking the effects of Jagged-1 decreases cell matrix adhesion can cause cell migration defects. Thus regulation of cell matrix interactions may contribute to the control of cell migration and tissue-modeling vessels via the Notch pathway. Notch and Delta are extracellular proteins, both with arterial specificity and upregulation in many pathological conditions; therefore, they are potentially viable targets for antiangiogenic therapy. Because of the wide distribution of the Notch signaling pathway, biochemical inhibitors of the fringe enzymes are not



as likely to be specific, but specificity could be obtained with soluble domains or antibodies against Delta-4 or Notch-4 and used for delivery of toxins or drugs to block endothelial proliferation. With regard to the direct effects of the Notch pathway, activation of the pathway could help stop proliferation of tumor-induced vasculature, and because of the internalization of both Notch and Delta, toxins attached to antibodies could have highly specific delivery (see below).

## **Ephrins, the Eph Tyrosine Kinase Receptors, and Angiogenesis**

The ephrin ligands and Eph receptors constitute a large family of signaling molecules that are widely expressed in many embryonic and adult tissues. The ephrin ligands and their Eph receptors are membrane-bound molecules, similar to members of the Notch/delta family described above. The ephrin ligands are divided into A and B type molecules that are distinguished by the way in which they are anchored in the plasma membrane. Thus, the ephrin A ligands are tethered to the outer plasma membrane via a glycosylphosphatidylinositol (GPI) anchor, whereas the ephrin B ligands are inserted into the plasma membrane via a transmembrane region followed by a conserved cytoplasmic domain. The ephrins bind to two families of transmembrane EphA and EphB tyrosine kinase receptors. Although the A type ephrins preferentially bind to the EphA receptors and the B type ephrins to the EphB receptors, considerable promiscuity of receptor binding has been demonstrated within each subclass.

The involvement of ephrin ligands and ephrin receptors in vascular development is considerable [see, for example, (78,79)]. Thus, several mouse gene knockouts have been shown to exhibit a vascular phenotype, including the complete ephrinB2 knockout or knockout of the ephrinB2 cytoplasmic domain (80). Interestingly, knockout of the ephrinB2 cytoplasmic domain affects vascular morphogenesis but not cranial neural crest migration (80). Other knockouts showing a vascular phenotype similar to that of the ephrin B2 knockout are the EphB4 and the double EphB2/EphB3 knockout. Neither the single EphB2 or EphB3 knockouts show a vascular phenotype, confirming functional compensation by the receptors.

Several ephrins and Eph receptors have been shown directly to be present on vascular endothelium. These include EphrinA1, which plays a role in the inflammatory angiogenesis induced by tumor necrosis factor- $\alpha$  (81), and ephrinB1, which promotes endothelial capillary-like assembly and attachment in vivo (82). EphrinB2 and the Eph receptors EphB3 and EphB4 are also present in vascular endothelium.

## **Ephrin Ligands and Eph Receptors in Tumor Angiogenesis**

The ephrin A1 ligand and its EphA2 receptor are expressed in tumor angiogenesis (83). Thus, double immunostaining of endothelial cells for CD34 showed ephrin A1 and its EphA2 receptor to be expressed throughout the endothelium in mouse xenografts of human MDA435 and K1767 Kaposi sarcoma cells and in

the vasculature of human cancers. A dominant-negative EphA2 receptor blocked formation of capillary endothelial tubes in vitro. Further studies have shown that soluble EphA2-Fc and EphA3-Fc receptor constructs inhibit tumor angiogenesis and growth in vivo (84), providing the first functional evidence for EphA receptor regulation of tumor angiogenesis. Recent mechanistic studies have shown that blockade of the EphA receptor specifically inhibits VEGF-induced angiogenesis (85). This activity is not restricted to members of the A subclass as Martiny-Baron et al. (85a) have reported similar effects with the soluble extracellular domains of the ephrin B2 and ephB4 receptors. There is clearly much work to do, but it appears that abrogation of the function of both the A and B class ephrins may provide novel antiangiogenic and antitumor activities.

## Hedgehog Signaling in Angiogenesis

Hedgehogs are a class of 19-kDa proteins that interact with heparin on the cell surface through an N-terminal basic domain and are tethered to the surface through cholesterol and fatty acyl modification. Hedgehog signaling is crucial throughout development. There are three human homologues of the *Drosophila* hedgehog gene: sonic hedgehog (Shh), desert hedgehog (Dhh), and Indian hedgehog (Ihh). Of these, Shh is the most widely expressed during development, and lack of Shh is embryonic lethal with multiple defects in early to mid gestation. Ihh is less widely expressed, and mice deficient in Ihh are able to survive until late gestation but die owing to skeletal and gut defects. Dhh-deficient mice are viable but display peripheral nerve and male fertility defects.

Signaling by all three Hedgehog proteins occurs through interaction with the Patched1 receptor, which then activates the transcription factors Gli1, Gli2, and Gli3. The downstream targets of the Gli gene products include both patched and Gli themselves; thus, patched and Gli are both components and targets of the Hh signaling pathway.

There is increasing evidence of a role for Hh signaling in angiogenesis. For example, hypervascularization of the neuroectoderm is seen following transgenic overexpression of Shh in the dorsal neural tube of zebra fish. As with Notch signaling, it appears that both up- and downregulation of Hh proteins result in vascular defects. These observations clearly suggest a role for Hh signaling in angiogenesis but do not actually prove a direct role for the Hh protein. Although Shh has been shown to have an indirect role in angiogenesis by acting upstream of angiogenic factors (86), it has also been shown to be a potent angiogenic agent in vivo. Thus, when Shh was administered to aged mice, it induced new vessel growth in ischaemic hind limbs. The Shh-induced vessels showed a characteristically large diameter. Despite this, Shh had no effect in vitro on endothelial-cell migration or proliferation but did induce expression of proangiogenic VEGF and angiopoietins-1 and -2 from interstitial mesenchymal cells. The indirect nature of hedgehog signaling has been confirmed in zebra fish (87). Thus, it was shown that zebra fish embryos lacking Shh activity fail to undergo arterial differentiation, as defined by

the expression of artery-specific markers, such as *ephrin-B2a*. However, injection of mRNA encoding Shh into the zebra fish could induce ectopic vascular expression of *ephrin-B2a*, as did the injection of *veg*f mRNA (87). The loss of arterial marker gene expression and ectopic *flt4* transcripts in the dorsal aorta is similar to that of embryos with defective Notch signaling, suggesting that the *veg*f and the Notch pathway act in a common signaling system to induce arterial differentiation. Notch in the absence of *veg*f is able to rescue *ephrin-B2a* expression, providing evidence for such a common cascade. It seems that Shh may have a role in the spatial-temporal production of angiogenic growth factors during embryonic and postnatal angiogenesis, working upstream of *veg*f, which, in turn, operates upstream of Notch.

## Sprouty and Angiogenesis

Sprouty (Spry) was first identified in *Drosophila* as an inhibitor of FGF, signaling during tracheal development. Thus, in *Drosophila*, Spry is expressed at the tips of growing primary branches of the tracheal system, in the eye imaginal disc, the embryonic chordotonal organ precursors, and in the midline glia. Unlike members of the Notch/Delta and ephrin/Eph families, Spry is an intracellular protein localized to the inner leaflet of the plasma membrane by a cysteine-rich domain. There are four known isoforms of Spry in mammals, each having a highly conserved C terminus but a variable N terminus. All four mammalian Sprys exhibit a restricted expression pattern in the embryo during early development, showing a dose correlation with sites of FGF signaling, suggesting that Spry proteins may function as negative regulators of FGF signaling during vertebrate development as well as in *Drosophila*. Thus, a decrease in Spry2 expression in the mouse results in increased lung branching morphogenesis (88). In addition, FGF, VEGF, platelet-derived growth factor, ephrinB2, and Tie-2 are all components of tracheal and blood vessel development. In view of these many similarities between *Drosophila* tracheal development and mammalian angiogenesis in terms of gene function, it was anticipated that Spry would also have a role in angiogenesis.

Direct evidence of a role for Spry in angiogenesis comes from a study in which the mouse Spry4 (mSpry4) was overexpressed in the developing endothelium of a mouse embryo using an adenoviral vector (89). It was found that embryos expressing mSpry4 had decreased sprouting of smaller vessels from the larger ones. By embryo whole-mount staining with an anti-PECAM (platelet-endothelial cell adhesion molecule) antibody it was found that mSpry4 injection resulted in the development of a primitive vasculature with poor branching and minimal sprouting of vessels. Furthermore, 24 h after injection, the hearts of the mSpry4-expressing embryos were beating but incompletely developed. When human umbilical vein endothelial cells (HUVEC) in vitro were transfected with mSpry4 there was a decrease in cell migration and cell cycle arrest at the G1/S phase with no apoptosis (89). The action of Spry4 appears to be via receptor tyrosine kinase pathways because there was a reduction in both basal and bFGF or VEGF-induced MAPK

phosphorylation in Spry4-expressing cells. MAPK signaling is involved in the regulation of proliferation, migration, and differentiation during angiogenesis and the inhibition of tyrosine kinase–stimulated MAP kinase activation probably accounts for the observed cell cycle arrest (89, 90).

## Roundabouts and Slits in Angiogenesis

Roundabouts and slits comprise a family of signaling molecules thought to be restricted to cells of neuronal lineage. Roundabout was so named because of the neuronal phenotype arising from its deletion in *Drosophila* (91). The slits and roundabouts are involved in axon guidance where they mediate a repulsive signal (92, 93). In light of the neuronal expression of roundabouts, the identification of magic roundabout (*Robo4*), a novel roundabout receptor restricted to endothelial cells, was unexpected (94). In situ analysis has shown magic roundabout to be absent from adult tissues but strongly expressed on the vasculature of tumors, including those of brain, bladder, and colon metastatic to the liver (95). Thus *Robo4*, like *Delta4*, is a promising therapeutic target (see below). Neuronal roundabouts contain a PPPPVPPPAI motif in their cytoplasmic tails that couples to c-abl through which they signal intracellularly (96). In contrast, the motif is absent from human and mouse magic roundabouts, and they presumably signal intracellularly by some other pathway.

## Endothelial-Specific Genes as Antiangiogenic and Vascular Targets

With increasing realization that the tumor vasculature is in many ways an ideal anticancer target, there has been intense interest in the identification of molecules that are expressed on endothelial cells lying within the tumor. Vigorous efforts to identify such molecules by researchers have utilized a variety of experimental approaches. Thus, St Croix et al. (97) employed a “brute force” approach involving the isolation of tumor endothelium by fluorescence-activated cell sorting (FACS) followed by the construction of a tumor endothelial serial analysis of gene expression (SAGE) library that could then be used in combination with a normal endothelial SAGE library to identify genes expressed in the tumor but not normal endothelium. New genes called TEMs (tumor endothelial markers) have been further characterized. Although some proved of lesser interest, others, such as TEM1, TEM5, and TEM8, were shown to be abundantly expressed on tumor vessels and the vasculature of the developing embryo but absent from adult vessels (98). There seems little doubt that these are molecules involved in developmental angiogenesis whose expression is reactivated in the tumor environment. Similar molecules have been identified by a bioinformatics technique to identify tissue (in this case endothelial)-specific genes, followed by in vitro and in vivo verification of expression (94). This latter approach has identified the *Robo4* gene, which shows an ideal expression pattern for vascular targeting (95). Hypoxia and endothelial proliferation (for example, in response to VEGF) are very likely

activators of tumor endothelial gene expression. For example, it has been known for several years that even the endothelium of well-perfused vessels in tumors may be profoundly hypoxic (99). A prime example of an endothelial-specific gene induced by hypoxia that is a potential target is Delta4. Researchers have used PCR differential display and hybridization techniques, such as filters or glass chips, to screen for such genes. These studies include the identification of genes differentially expressed when endothelial cells differentiate into tubes in collagen (100); genes induced by hypoxia (101); and proliferative genes induced by exposure of microvascular endothelial genes to VEGF, such as the alpha5beta1 integrin (102). Schnitzer's group has employed the novel approach of raising monoclonal antibodies specific to lung caveolae that specifically target rat lung endothelium following i.v. injection (103). It is conceivable that such an approach could be used to target tumor endothelium in the future. Another widely used technique to identify novel binding motifs is phage library display (for results derived using this technology see Reference 104). Phage display has identified peptides that home to lymphatic (105) as well as organ- and tumor-specific endothelium (reviewed in Reference 106). The recent surge in the identification of new tumor endothelial markers [see Figure 2 (color insert) for examples] will no doubt lead to increased activity in attempts at vascular targeting.

## Endothelial Stem Cells Are Active in Tumor Angiogenesis

Recent studies have led to the realization that endothelial progenitor cells arising in the bone marrow are also significant contributors to the vasculature in tumors. These findings arose from work showing that adult mice that expressed reduced dosages of the transcription factor *Id* were unable to support tumor angiogenesis (107). It was subsequently shown that transplantation of  $\beta$ -galactosidase expressing wild-type bone marrow into lethally irradiated *Id*-mutant mice was sufficient to restore vascularization of implanted tumors (108). If tumors are indeed achieving substantial vascularization by recruitment of circulating endothelial progenitors, this has profound implications for the development of new strategies to block tumor angiogenesis. A key point is what stimulus leads to seeding of the tumor by the endothelial progenitors? An obvious contributor would be hypoxia.

## CONCLUSIONS

Although this review has tried to focus on many of the new pathways described in the past few years, there are several key issues that are poorly understood. These issues include the way the system is coordinated because it appears that blocking any one of many pathways can affect angiogenesis; differentiation and vasculogenesis; and definition of the cascades and feedback loops, which require much more extensive information and modeling. Are there single key pathways that if blocked or activated will be sufficient to restore normal vasculature or completely block tumor vasculature? Can blocking any one pathway ever really completely block

vascular development? Are there several different pathways running in parallel that need to be blocked?

With regard to tumor biology, are there unifying pathways that are common to all tumor vessels or does each tumor tend to select different pathways depending on its genetic background, the polymorphisms within the patient's own stroma, and their ability to respond to environmental hypoxia and stresses? Can profiles be developed to define which pathways are important and individualized?

By applying many of these studies and pathways to human tumor vasculature and relating their expression to the behavior of tumors, we may be able to define the relevant pathways. Currently, patients receive one inhibitor of a single pathway without any assessment of which pathways may be most relevant to their tumor type. The development of a more rational approach remains a major challenge for antiangiogenic therapy.

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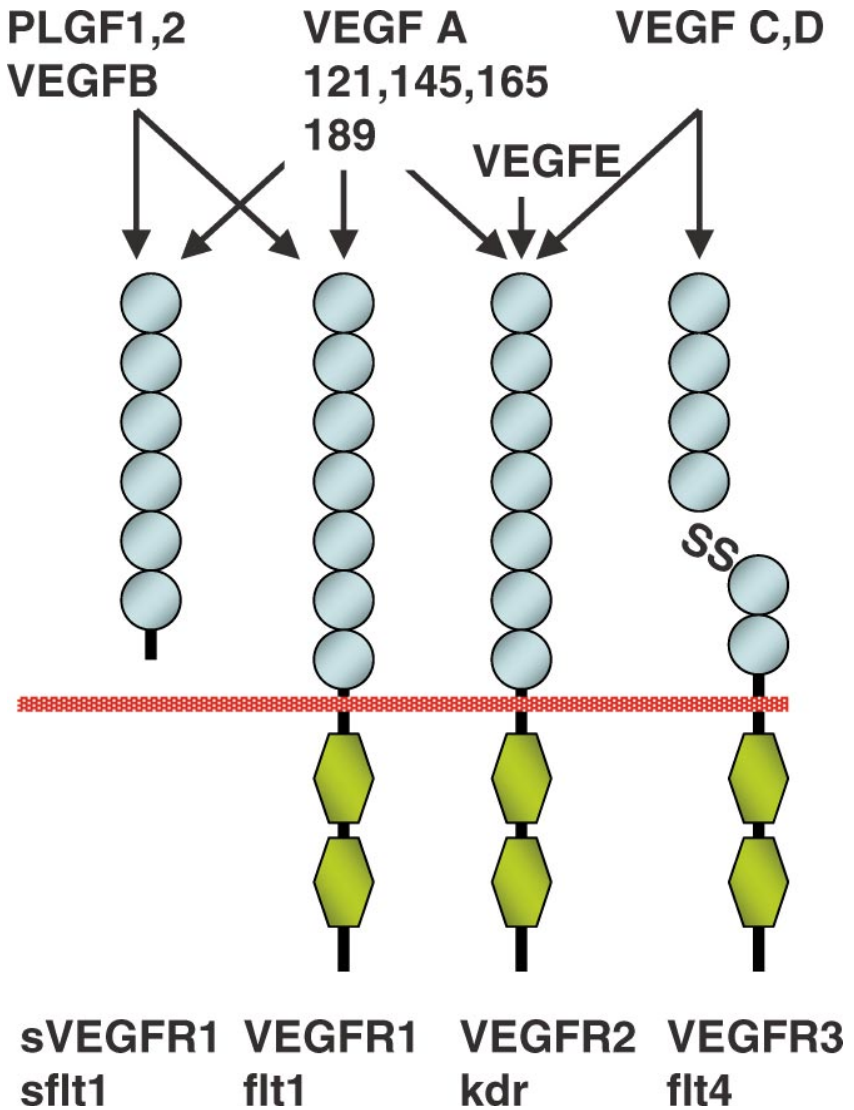


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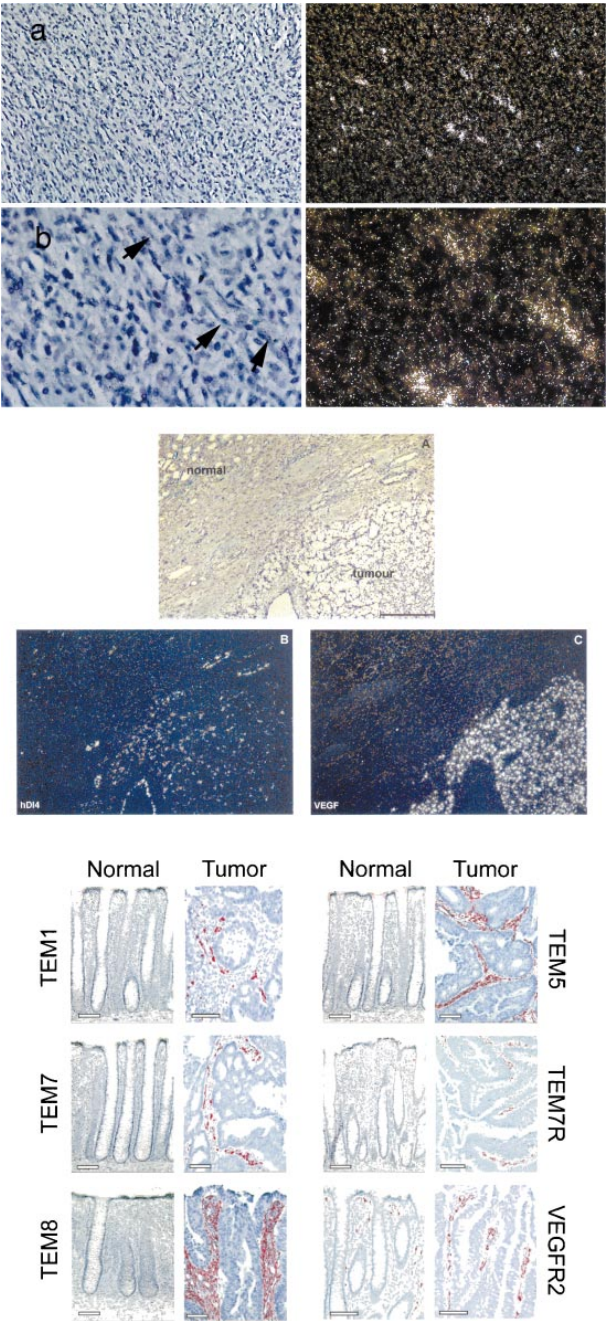
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**Figure 1** Schematic showing the known VEGF receptor family members and their cognate ligands. Blue = IgG domain and green = tyrosine kinase domain.



See legend on next page

**Figure 2** Expression of genes restricted to tumor endothelium in the adult. Sections show in situ hybridization data. (*Top*) Magic roundabout on the endothelium in a ganglioglioma, left light field, right dark field ( $a \times 20$ ,  $b \times 50$ ) (95). Arrows indicate a tumor vessel. (*Middle*) Delta4 expression in the endothelium of a renal clear cell carcinoma (67): (*a*) light field showing normal kidney tissue at top left and tumor bottom right, (*b*) delta4 expression, and (*c*) VEGF expression. Note that the delta4 expression corresponds to that of VEGF in a presumably hypoxic area of the tumor (both genes are induced by hypoxia) but is restricted to tumor vessels (scale bar 100  $\mu\text{M}$ ). (*bottom*) TEM1, TEM5, TEM7, and TEM8 on the vessels in a colorectal carcinoma but absent from endothelium in the normal colon tissue (98).