Annu. Rev. Cell Dev. Biol. 2002. 18:541–73
doi: 10.1146/annurev.cellbio.18.012502.105825
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First published online as a Review in Advance on July 18, 2002

SIGNALING PATHWAYS IN VASCULAR DEVELOPMENT

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Key Words vasculogenesis, angiogenesis, artery, vein, signaling

■ **Abstract** The vasculature is one of the most important and complex organs in the mammalian body. The first functional organ to form during embryonic development, the intricately branched network of endothelial and supporting periendothelial cells is essential for the transportation of oxygen and nutrients to and the removal of waste products from the tissues. Serious disruptions in the formation of the vascular network are lethal early in post-implantation development, while the maintenance of vessel integrity and the control of vessel physiology and hemodynamics have important consequences throughout embryonic and adult life. A full understanding of the signaling pathways of vascular development is important not just for understanding normal development but because of the importance of reactivation of angiogenic pathways in disease states. Clinically there is a need to develop therapies to promote new blood vessel formation in situations of severe tissue ischemia, such as coronary heart disease. In addition, there is considerable interest in developing angiogenic inhibitors to block the new vessel growth that solid tumors promote in host tissue to enhance their own growth. Already studies on the signaling pathways of normal vascular development have provided new targets for therapeutic intervention in both situations. Further understanding of the complexities of the pathways should help refine such strategies.

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INTRODUCTION

The formation of the vascular system begins early in development in mammals, with the formation and aggregation of angioblast precursors in the embryo proper and formation of the blood islands in the visceral yolk sac. In the yolk sac, there is a close association between the primitive hematopoietic cells and the developing endothelium, suggestive of a common precursor, the so-called hemangioblast (Robb & Elefanty 1998). Within the embryo itself, the first blood vessels develop in the absence of hematopoiesis, suggesting that these precursors are solely endothelial precursors or angioblasts (Pardanaud et al. 1996). The early blood vessels of the embryo or the yolk sac develop by aggregation of de novo–forming angioblasts into a primitive network of simple endothelial tubes—a process known as vasculogenesis (Risau & Flamme 1995). Later development of the mature vessel system involves a complex process of remodeling and refining the initial pattern, with proliferation and sprouting of new vessels from existing ones—the process of angiogenesis (Risau 1997).

In fact, the full elaboration of the vascular system is much more complex than suggested by this frequently used division into the two phases. As vessels begin to be remodeled into a functioning circulation, they need to undergo localized proliferation and regression, as well as programmed branching and migration into different regions of the body. They need to be specified into different calibre and types of vessels, including division into arteries, veins, and lymphatics, with their separate subdivisions into large vessels, venules, arterioles, capillaries, etc. In addition, they need to recruit supporting cells, smooth muscle cells, and pericytes to ensure the stability of the vessels formed. We still know very little about the total genetic program required to pattern the developing vasculature. By analogy with the growing knowledge about the genetic control of other systems of branching morphogenesis, such as the tracheal system in flies and the lung in vertebrates (Metzger & Krasnow 1999), one would predict that a number of different signaling pathways would be involved in different phases of the development of the system. Some would act to signal the specification of cell types, others to signal proliferation and to provide directional cues for vessel formation and branching morphogenesis. Additional signals would act to recruit associated cells and promote vessel stability.

In recent years, a number of different signaling pathways has been shown to be involved in vascular development. Two pathways, the vascular endothelial growth factor (VEGF) and the angiopoietin/Tie receptor pathways, are almost totally

endothelial-specific and are critical players in vascular development. However, there is also evidence for specific vascular roles for more widely active signaling pathways, including the Ephrin/Eph receptor, transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), and Delta/Notch pathways. In this review we attempt to place the different signaling pathways in the context of their putative roles in the different phases of blood vessel development. The roles of the VEGF/VEGFR and the angiopoietin/Tie pathways have been extensively reviewed previously (Carmeliet 2000, Conway et al. 2001, Yancopoulos et al. 2000) and are only summarized here. We focus on newer information about the roles of the TGF-β, Ephrin, and Notch pathways during the processes of vascular development in the embryo.

ESTABLISHING THE EMBRYONIC VASCULATURE: THE ROLE OF VEGF SIGNALING

Vasculogenesis begins very early after the initiation of gastrulation in the mammalian embryo, with the formation of the blood islands in the yolk sac and angioblast precursors in the head mesenchyme. The signals leading to the early specification of these cells are still not altogether clear, although experiments in chick have suggested that FGF signaling is important for initiation of angioblast specification (Cox & Poole 2000). Recent experiments in the mouse implicate Indian Hedgehog signaling from the primitive endoderm in induction of both vascular and hematopoietic cell fate (Byrd et al. 2002, Dyer et al. 2001). Whatever the upstream signals are that initiate vascular specification in different systems, the immediate downstream response appears to be the induction of expression of the receptors for VEGF, *Flk1* (Kdr, VEGFR2) and *Flt1* (VEGFR1). Expression of *Flk1* represents the earliest marker of the developing endothelial lineage during vasculogenesis (Millauer et al. 1993, Yamaguchi et al. 1993). Indeed, in the yolk sac, *Flk1* expression appears to mark cells prior to the distinction between the outer endothelial cells and the enclosed hematopoietic cells (Yamaguchi et al. 1993), which led to the suggestion that it might also mark the putative common progenitor of the hematopoietic and endothelial system, the hemangioblast (Eichmann et al. 1997, Kabrun et al. 1997). Confirmation of this concept came from studies in which *Flk1*-positive cells were sorted from early differentiating ES cells in vitro and single-marked cells were shown to be capable of producing both endothelial and hematopoietic cells (Choi et al. 1998). Despite its early association with both lineages, *Flk1* is later confined to the endothelial lineage as it develops in both the embryonic and extraembryonic regions and clearly marks endothelial progenitors (Yamashita et al. 2000).

In the yolk sac, the initial endothelial cells associated with the blood islands fuse together to form the primitive capillary plexus, a honeycomb network of developing vessels. Similarly, within the embryo itself, disperse angioblasts start to join together to form a capillary network in the head mesenchyme and posterior lateral plate mesoderm. In the developing cardiac crescent, endothelial progenitors

form the primitive endocardial lining of the developing cardiac tube. These initial phases of vascular development are known as vasculogenesis and seem to be largely dependent on VEGF signaling. Vascular endothelial growth factor (VEGF-A) was the first growth factor shown to have effects specific to the endothelial lineage (Keck et al. 1989, Leung et al. 1989). This specificity stems from the endothelia-restricted expression of its chief receptor, VEGFR2, encoded by the *Flk1* gene. *Flk1* expression is down-regulated in stable, mature blood vessels but is up-regulated again during situations of induced angiogenesis in the adult (Goede et al. 1998, Shweiki et al. 1993) such as the invading blood vessels induced by tumors (Hanahan & Folkman 1996).

The VEGF family of growth factors is a group of homodimeric glycoproteins whose carefully regulated activity is essential for multiple aspects of the formation and modeling of the vasculature. Four *VEGF* genes (A–D), and a related growth factor, placenta growth factor gene, *Plgf*, have been identified in humans and mice (Neufeld et al. 1999, Petrova et al. 1999). Five isoforms of the *VEGF-A* gene are produced by alternate splicing in humans; the mouse *Vegf* gene has been shown to produce three isoforms of 120, 164, and 188 amino acids (a.a.) (Ferrara & Davis-Smyth 1997, Shima et al. 1996). Of these the 120- and 164-a.a. proteins are the most abundant, with the 164-a.a. isoform acting as the strongest mitogen. The VEGF isoforms differ in their ability to interact with heparan sulfate proteoglycans; the larger VEGF isoforms can bind heparin, and associate with the extracellular matrix, whereas the smallest isoform has been shown to diffuse freely (Robinson & Stringer 2001). VEGF ligands have been shown to bind to three major receptors, VEGFR-1/Flt1, VEGFR-2/KDR/Flk1, and VEGFR-3/Flt4 (Neufeld et al. 1999), in addition to accessory receptors, the neuropilins (Neufeld et al. 2002, Soker et al. 1998).

The VEGFR1-3 receptors are characterized by the presence of seven immunoglobulin-like domains used for binding to the VEGF ligand and an intracellular kinase domain (Figure 1). VEGF binding induces homodimerization of the VEGFR-1 and VEGFR-2 receptors, followed by autophosphorylation and activation of the downstream signaling cascade. VEGFR-2(*Flk1*) binds VEGF-A, -C, and -D, whereas VEGFR-1(*Flt1*), which is also specifically expressed in developing vessels (Dumont et al. 1995), binds VEGF-A and -B (Gale & Yancopoulos 1999), as well as specifically binding another related growth factor, PlGF (Clauss et al. 1996, Sawano et al. 1996). VEGFR-3 (*Flt4*) is expressed in all early vessels but then becomes restricted to the developing lymphatics (Kaipainen et al. 1995, Kukk et al. 1996). It binds VEGF-C (Joukov et al. 1996) and -D (Achen et al. 1998). Of the two most-well characterized receptors (VEGFR-1 and -2), VEGFR-1 has a 10-fold higher affinity for VEGF than VEGFR-2 (Petrova et al. 1999). Interestingly, VEGFR-1 undergoes little detectable phosphorylation when bound to VEGF, whereas VEGF binding to VEGFR-2 results in autophosphorylation at four major sites, followed by the activation of the Ras/MAP kinase pathway (Matsumoto & Claesson-Welsh 2001).

The three VEGF receptors are largely restricted in their expression to the endothelial and lymphatic lineages, although there are reports of VEGFR-2

expression and perhaps function in other tissues, including the nervous system (Meirer et al. 2001, Ogunshola et al. 2002, Yang & Cepko 1996). Interestingly the co-receptor, neuropilin, was first shown to have a role in axon guidance in the nervous system and later shown to have additional roles in blood vessel development. The two mammalian neuropilin genes, *Np1* and *Np2*, encode transmembrane proteins that act as receptors for the axon repellant factors of the class III semaphorin subfamily (Chen et al. 1997, Giger et al. 1998, He & Tessier-Lavigne 1997, Kolodkin et al. 1997). Their additional roles in endothelial signaling stem from their ability to bind specific splicing isoforms of VEGF. Np1 binds $VEGF_{164}$ but not $VEGF_{120}$ in a heparin-dependent manner, and such binding enhances the VEGF-VEGFR-2 dependent migration of endothelial cells in culture (Soker et al. 1998). This effect likely stems from complex formation between neuropilin and VEGF-2 (Whitaker et al. 2001). More complex interactions are also possible. Np2 can bind $VEGF_{165}$ and $_{145}$ but not $_{121}$ (Gluzman-Poltorak et al. 2000), and both neuropilins have recently been shown to be able to complex with other VEGF ligands (Gluzman-Poltorak et al. 2000, Migdal et al. 1998) and the VEGFR-1 receptor (Fuh et al. 2000, Gluzman-Poltorak et al. 2001).

Targeted mutations in mice have given further insight into the normal developmental roles of these various components of vascular signaling. Embryos lacking VEGFR-2 die around 9 days of development and show no development of any blood vessels or hematopoietic cells (Shalaby et al. 1995). The loss of both lineages could suggest that VEGFR2 is required in the putative common precursor, the hemangioblast. However, chimera studies suggest that the failure to form the hematopoietic lineage is a result of the incorrect migration and location of the progenitors of the endothelium and the blood system in the mutant yolk sac (Shalaby et al. 1997). This is consistent with studies showing that *Flk1* mutant ES cells are capable of some hematopoiesis in vitro (Hidaka et al. 1999, Schuh et al. 1999) where cell migration is not required. Studies in other systems also suggest a chemotactic role for early VEGF signaling. For example, in *Xenopus*, the hypochord is known to produce a low-weight isoform of VEGF. Angioblast precursors arise in the lateral plate mesoderm and migrate toward the hypochord to form the dorsal aorta. Implantation of tissue expressing VEGF near to the hypochord results in migration of the angioblasts to the site of ectopic expression (Cleaver & Krieg 1998). Early defects in *Flk1* mutant embryos cannot all be ascribed to migration defects, however. In the embryo itself, loss of VEGFR-2 signaling leads to a total block in the development of the angioblasts and the failure to develop even a primary capillary plexus (Shalaby et al. 1995). VEGF signaling is thus absolutely essential for the specification, proliferation and migration of early vascular progenitors.

The neuropilin co-receptors for VEGFR-2 also appear to be critical for normal vasculogenesis. Embryos double-mutant for *Np1* and *Np2* die in utero around 8.5 days of development and are totally avascular (Takashima et al. 2002). The similarity of the *Flk1* and the *neuropilin* mutant phenotypes suggests an obligate relationship between the VEGF receptor and the neuropilins for normal signaling in early embryogenesis. A null mutation of the ligand VEGF-A also leads to early

lethality. Interestingly, VEGF-A is haploinsufficient; heterozygotes die early in gestation with severe reduction in the size and caliber of their developing blood vessels (Carmeliet et al. 1996, Ferrara et al. 1996). Homozygous embryos were produced by aggregation of homozygous mutant ES cells with tetraploid wild-type embryos (Nagy et al. 1993). These embryos showed a more severe phenotype, similar to but not quite as severe as the *Flk1* mutants (Carmeliet et al. 1996). In such embryos, the primitive endoderm of the yolk sac is wild-type, derived from the tetraploid cells. Because yolk sac endoderm is a known source of VEGF, this may explain the reduced severity of the phenotype.

VEGFR-1/*Flt1* mutants also die early in development but have disorganized and malformed primary blood vessels (Fong et al. 1995). The phenotype is more suggestive of an overgrowth of endothelial cells than a loss of cells, as confirmed by chimera studies (Fong et al. 1999). This suggests that *Flt1* could be a negative regulator of VEGF signaling in the early embryo, acting to modulate VEGF levels by sequestering ligand. A targeted mutation in *Flt1* that disrupts the kinase domain but leaves the extracellular and transmembrane regions intact is viable (Hiratsuka et al. 1998), consistent with this hypothesis. VEGFR-1 may have a later signaling role in the monocyte lineage through its binding of PlGF (Clauss et al. 1996), but it is likely to act largely as a modulator of VEGF action in the endothelial lineage. Again consistent with this, mutation of *Plgf* has no effect on the embryonic development of the vasculature (Carmeliet et al. 2001).

Although *Flt4* was proposed to be a major player in development of the lymphatic system, mutants die earlier than the beginning of lymphatic development with major disorganization of their blood vessels (Dumont et al. 1998) suggesting that this receptor also has a function in development of the entire vascular system. Although the full complexity of the roles of the different ligands and receptors in the early development of the vasculature is not yet understood, it is clear that the VEGF-A/VEGFR-2 interaction, modulated by the accessory receptors neuropilin and VEGFR-1, is fundamental.

VEGF signaling is not only important during the initial phases of vasculogenesis, when, in fact, no other signaling pathway has been shown to be essential, but continues to play key roles throughout development and into the adult, in both normal and pathological situations. There is an extensive literature on the ability of VEGF to promote angiogenesis in vivo and in vitro, making the VEGF signaling pathway a key target for clinical intervention in situations such as preventing tumor angiogenesis and promoting revascularization after tissue ischemia (Ferrara & Alitalo 1999). However, the relative importance of VEGF signaling in the later stages of embryonic angiogenesis and vascular remodeling, when other signaling pathways come into play, is less clear. Inducible knockouts of the genes in the pathway at different stages of development will be needed. However, several pieces of genetic evidence point to a critical continuing role for VEGF throughout development. Mutation of *neuropilin1*, a co-receptor for VEGF, produced a variety of vascular defects, all less severe than loss of either VEGF or its receptor (Kawasaki et al. 1999). Mutation of *Np2* produced no obvious vascular defects, but embryos homozygous for a mutation in one of the neuropilins and heterozygous for the

other showed vascular defects intermediate between avascular double mutants and the single *Np1* mutants (Takashima et al. 2002). These phenotypes suggest a continued requirement for VEGF signaling to promote vascularization of developing organs, such as the brain and heart, as well as expansion and growth of the major blood vessels. Overexpression of neuropilin in transgenic embryos led to excess blood vessels in the developing brain (Kitsukawa et al. 1995), consistent with Np1 acting as a co-activator of the VEGF receptor.

A conditional knockout of the VEGF gene that led to partial loss of function produced neonatal lethality, with reduced vascularity in multiple tissues (Gerber et al. 1999), whereas a hypermorphic allele of VEGF also led to late-acting vascular defects (Miquerol et al. 2000), demonstrating the extreme dosage sensitivity of VEGF action. In addition, an isoform-specific knockout, which only allowed production of the $VEGF_{120}$ isoform, led to impaired myocardial angiogenesis and milder loss of vascular density in other organs (Carmeliet et al. 1999). All these studies point to ongoing roles for VEGF signaling during all phases of later vascular development.

BRANCHING, REMODELING AND VESSEL STABILIZATION—THE ANGIOPOIETIN/ TIE/PDGF INTERACTION

Once the primary capillary plexus is formed, the next phases of formation of the complex vasculature involve induction of new vessel branches, as well as pruning of excess branches and enlargement and fusion of endothelial tubes to form major vessels. The complexities of this process are still not well understood. Also important in these phases is the recruitment of supporting cells, pericytes and smooth muscle cells, that stabilize the developing vessels and are critical to the integrity of the developing vasculature. The process of vascular remodeling and vessel stabilization has been shown to depend on multiple signaling pathways, but the angiopoietin/Tie receptor pathway is key.

The two Tie receptors, Tie1 and Tie2/Tek comprise a separate class of endothelial-specific receptor tyrosine kinases (Dumont et al. 1992, Korhonen et al. 1994, Sato et al. 1993, Schnurch & Risau 1993). These receptors have a complex extracellular domain, with immunoglobulin, fibronectin-like, and EGF-like repeats (Figure 1). Both are expressed throughout the vascular system from the earliest stages and remain endothelial-specific throughout development and into adult life (Dumont et al. 1995). The Tie2 receptor forms homodimers on binding ligand, becomes autophosphorylated, and interacts with a docking protein (Dok-R) (Jones et al. 1999). Several other proteins have been shown to interact with Tie2 including Grb7, Grb14, p85, Grb2, and Shp2 (Jones & Dumont 2000). Recent work performed in human, porcine, and bovine cell culture systems suggests that activated Tie2 might activate Akt through PI3 kinase (Papapetropoulos et al. 2000). Much less is known about signaling through the Tie1 receptor, and the identity of the Tie1 ligand(s) remains unknown (Gale & Yancopoulos 1999, Partanen & Dumont 1999).

The ligands for the Tie2 receptor are the angiopoietins. Ang1-4 were found to be secreted glycoproteins containing a novel N terminus, a coiled-coiled domain, and a fibrinogen-like motif (Davis et al. 1996, Maisonpierre et al. 1997, Valenzuela et al. 1999). Studies of the interactions between Ang1-4 and the Tie2 receptor have shown that Ang1 and Ang4 can act as agonists, stimulating Tie2 autophosphorylation and activity of downstream pathways (Davis et al. 1996, Valenzuela et al. 1999). Ang2 and Ang3 mediate only weak receptor activation in endothelial cells and can antagonize Ang1-mediated Tie2 activation (Maisonpierre et al. 1997). Dissection of the functional domains of Ang1 and Ang2 indicate that Ang1 may act as a homotrimer, and Ang2 as a homodimer; in both cases, interactions are mediated by the coiled-coil and N-terminal domains (Procopio et al. 1999). Studies of chimeric proteins have demonstrated that agonist/antagonist activity is conferred by the receptor binding fibrinogen-like domain (Procopio et al. 1999).

Mutation of *Tie2* does not affect initial formation of blood vessels, but embryos died in midgestation with major defects in vascular remodeling and stability (Dumont et al. 1994, Sato et al. 1995). Blood vessels were enlarged, with fewer branches, and endothelial cells tended to round up, dissociate from the underlying support cells and extracellular matrix, and undergo apoptosis. Ang1 is the positive activator of the Tie2 receptor, and a mutation in the gene leads to a phenotype similar to the loss of *Tie2* (Suri et al. 1996). Ang2 can bind to the Tie2 receptor but does not activate it; rather it seems to act as an antagonist, counteracting the effects of Ang1. Consistent with this, overexpression of *Ang2* in the embryo produces a phenotype similar to loss of function of *Ang1* or *Tie2* (Maisonpierre et al. 1997). It has been proposed that Ang1 acts to stabilize vessels induced by the action of the VEGF pathway (Suri et al. 1996), a process involving the recruitment of support cells such as smooth muscle cells and pericytes (Folkman & D'Amore 1996). Overexpression of *Ang1* in the adult leads to hypervascularization, with the production of many compact stable vessels (Suri et al. 1998). This contrasts with the effects of overexpressing VEGF, which also leads to hypervascularization, but with formation of large, leaky, simple endothelial tubes, unprotected by supporting cells (Drake & Little 1995). If Ang1 normally acts to stabilize vessels, then there has to be a mechanism to modulate its action to allow vascular remodeling and new vessel formation. Ang2 is expressed at sites of vascular remodeling and acts to locally block Ang1 action and destabilize vessels (Maisonpierre et al. 1997). If VEGF is also around, then local angiogenesis can occur. In the absence of VEGF, and the presence of Ang2, vessels destabilize, and endothelial cells apoptose, resulting in vessel regression. Through in vitro and in vivo assays, the Tie-Ang pathway has been shown to mediate endothelial cell chemotaxis, cell survival/apoptosis protection, endothelial-ECM interactions, and stimulation of vessel organization and sprouting, which makes it a key player in the complex processes of vascular remodeling (Loughna & Sato 2001a). Little is yet known about the role of the other angiopoietins in angiogenesis, nor is the role of the Tie1 receptor well understood. No ligand has yet been found for this receptor, nor is its role in the processes of tumor angiogenesis understood. Mutation of *Tie1*

leads to death late in gestation (Puri et al. 1995) or close to term (Sato et al. 1995). Defects were seen in the microvasculature. Chimera studies showed that the role of *Tie1* was specific to the late stages of capillary formation (Partanen et al. 1996), but it is not yet clear whether this role is in regulating proliferation or stabilization of the vessels. *Tie1/Tie2* double mutants show a similar phenotype to *Tie2* mutants alone (Puri et al. 1999), indicating that the Ang/Tie pathway plays no critical role in establishing the vasculature, despite the early expression of both receptors.

Platelet-derived growth factor (PDGF) signaling has been shown to be critical for the proliferation and migration of smooth muscle cells and pericytes to the developing vessel wall (Lindahl et al. 1998a), a process involving reciprocal interactions between endothelium and support cells (Drake et al. 1998). Arterial and capillary endothelial cells secrete PDGF-B, whereas developing smooth muscle cells and pericytes (SMC/PC) express the receptor, PDGFR-β (Betsholtz et al. 2001). Mutation of *PdgfB* caused failure of recruitment of pericytes to brain capillaries and to the glomeruli of the kidney (Lindahl et al. 1997, 1998b). A detailed analysis of the vessel development in both *PdgfB* and *Pdgfrb* mutant embryos showed that smooth muscle cells and pericytes initially form around vessels but, as vessels sprout and enlarge, PDGF signaling is required for co-migration and proliferation of supporting cells (Hellstrom et al. 1999). Angiopoietin's effect on vessel maturation may be partly brought about by signaling the endothelial cell to secrete PDGF-B.

OTHER ASPECTS OF VASCULAR REMODELING: THE INVOLVEMENT OF TGFβ SIGNALING

The TGF-β superfamily is a group of growth factors that include transforming growth factors, activins, and bone morphogenetic proteins (Miyazono et al. 2001, Mummery 2001). Three isoforms of the TGF-β ligand exist (TGF-β1, 2, and 3), of which TGF-β1 appears to be critical for vascular development (Pepper 1997). TGF binds to the type II receptor (TβRII), which then recruits and phosphorylates one of two type I receptors (Alk1, or Alk5) (Hoodless & Wrana 1998). In most tissues, including endothelial cells, the activated type II receptor transphosphorylates the Alk5 TβRI receptor (Franzen et al. 1993), which then interacts with and phosphorylates the Smad-2 and Smad-3 signal transduction molecules. Smad-2 or Smad-3 then complexes with the common Smad, Smad-4, and is translocated to the nucleus, where it activates the transcription of target genes (Chen et al. 1998, Hoodless & Wrana 1998). A second signaling cascade can also occur in vascular endothelium, in which the activated TβRII receptor interacts with and phosphorylates Alk1, a type I receptor specifically expressed in endothelial cells (Panchenko et al. 1996, Roelen et al. 1997). The phosphorylated Alk1 receptor works through a different group of signaling Smads (Smad-1and 5) and the co-Smad (Smad-4) to activate transcription (Oh et al. 2000). Endothelial cells also express a membrane-spanning co-receptor, or type III receptor, called endoglin,

which can reduce the activation of the TGF signal response (Barbara et al. 1999, Letamendia et al. 1998).

Studies of TGF-β activity in vascular development have provided a number of interesting and occasionally conflicting observations in vitro and in vivo. In the late 1980s and early 1990s, several groups examined the effects of adding TGF-β ligand to cultured endothelial cells and consistently observed a reduction in their proliferation (Baird & Durkin 1986, Frater-Schroder et al. 1986). Although a growing body of literature was assembled supporting an inhibitory or quiescence-inducing role for TGF-β, there were conflicting reports implying a mitogenic and proliferative role of TGF-β (Iruela-Arispe & Sage 1993, RayChaudhury & D'Amore 1991, Sutton et al. 1991).

Advances in cell culture technique and the use of three-dimensional collagen- or extracellular matrix-based gels did little to clarify the role of TGF-β in vascular development. Although TGF-β often appeared to slow endothelial cell growth, and had been associated with a change in cell morphology and death of one population of quiescent cells (Sutton et al. 1991), other research demonstrated that TGF-β could stimulate tube formation (Gajdusek et al. 1993, Kuzuya & Kinsella 1994). Furthermore, in co-culture experiments involving endothelial cells with pericytes or fibroblasts, TGF-β was found to be chemotactic for the pericytes and fibroblast cells (Antonelli-Orlidge et al. 1989, Joseph-Silverstein & Rifkin 1987).

Early studies of TGF-β activity in vivo provided still more paradoxical findings. Whereas TGF-β appeared to inhibit angiogenesis in vitro, Roberts et al. demonstrated that subcutaneous injection of newborn mice with TGF-β was sufficient to stimulate fibrotic and angiogenic responses in vivo (Roberts et al. 1986). Studies of tumor development showed that TGF-β could act to inhibit angiogenesis in early stages of oncogenesis but that TGF-β overexpression at later stages stimulated angiogenesis in breast cancer (Li et al. 2001) (Schwarte-Waldhoff et al. 2000). Furthermore, other studies demonstrated that a disc soaked in TGF-β, but not saline, could produce a strong angiogenic response when applied to the chick chorioallantoic membrane (Yang & Moses 1990). TGF-β seemed to be angiogenic in vivo, despite repeated observations that it acted to inhibit vessel formation in vitro. In general, it appears that TGF-β acts to inhibit endothelial proliferation and migration and promotes the production of extracellular matrix and proteinase inhibitors. However, under certain conditions, it can act to enhance endothelial invasion and proliferation, suggesting a possible biphasic response.

Recent studies on targeted knockout mice have provided clear evidence of an essential role for TGF-β signaling in vascular development, and suggest that this biphasic response may result from the balance between Alk1 and Alk5 signaling in endothelial cells. Mutations in multiple components of the TGF-β signaling pathway cause early embryonic lethality owing to vascular defects (Dunker & Krieglstein 2000). The first of these genes to be targeted was the TGF-β1 ligand; mice carrying mutations in the *TGFβ1* locus showed one of two phenotypes (Letterio et al. 1994). Half of the predicted homozygous mutant offspring were born alive and survived a few weeks before dying of an inflammatory response

(Letterio et al. 1994). The other half of the homozygous mutant embryos died by embryonic day 10.5 (E10.5) with abnormal development of the yolk sac vasculature (Dickson et al. 1995). Further examination of these mutants showed a range of abnormalities in the extraembryonic vasculature, including delayed vasculogenesis, the development of weak vessels, or even areas in which vessels were entirely absent. Mutant yolk sacs were pale, and defects were found in both endothelial and hematopoietic differentiation. Interestingly, no obvious vascular developmental abnormalities were reported in E9.5 *TGFβ1*$^{-/-}$ embryos. Dickson et al. concluded from this work that deficiency in TGF-β1 resulted in inadequate differentiation of endothelial and hematopoietic cells. The differences between the pre-natal and post-natal lethality groups were attributed to partial rescue by maternally supplied TGF-β1 (Letterio et al. 1994).

Targeted mutation of the TβRII receptor largely phenocopied the embryonic lethal phenotype associated with mutation of the ligand TGF-β1. Homozygous mutant embryos died between E10.5–E11.5 owing to defects in the yolk sac vasculature (Oshima et al. 1996). Like the *TGFβ1*$^{-/-}$ homozygotes, *TβRII* mutant embryos had pale yolk sacs, with layers of endothelial cells detached from the surrounding mesenchyme. Embryos were growth retarded, although no obvious morphological defects were found at E9.5. Mice lacking endoglin also die by day 11.5 with major embryonic and extraembryonic vascular defects, including pockets of blood, and a disorganized yolk sac vasculature, along with defects in endocardial cushion formation (endothelially derived structures) and heart development (Arthur et al. 2000, Li et al. 1999). The similarity of these phenotypes is consistent with the evidence that these genes represent common components of all TGF signaling in endothelial cells. However, biochemical evidence suggested two possible signaling pathways downstream of the common type II receptor; the Alk1/Smad5 and the Alk5/Smad2/3 pathways. If these pathways were redundant, one might expect no vascular defects in individual mutants of *Alk1* or *Alk5*. This is not the case, however. Mutations in each receptor cause embryonic lethality because of vascular failure. Detailed analysis of the phenotypes provides some clues as to the different effects of the two signaling routes.

Embryos deficient for *Alk5* (*TβRI*) die around day 10.5–11.5, with vascular defects similar to those observed in *TGFβ1* and *TβRII* mutants (Larsson et al. 2001). In these mice, blood islands form, and occasionally yolk sac vessels may be seen, but these were fragile and lacked an organized network of branches. The embryonic vasculature was initially normal. Cultured *Alk5*$^{-/-}$endothelial cells produced reduced amounts of fibronectin, were unable to migrate normally, and were not responsive to TGF-β1-mediated growth inhibition (Larsson et al. 2001). When the *Alk1* gene was knocked out in mice, homozygotes died by day 11.5 with vascular defects (Oh et al. 2000, Urness et al. 2000). Defects in yolk sac remodeling and reduced smooth muscle cell differentiation/recruitment were observed, similar to those seen in other TGF-β signaling mutants. However, differences were seen in the embryonic vasculature, where *Alk1*$^{-/-}$ embryos failed to form a capillary network and major vessels were fused and dilated by day 9.5. The phenotype of embryos

lacking *Smad5* is similar to loss of *Alk1*; i.e., embryonic vessels are enlarged and disorganized, with prominent mesenchymal dissociation and apoptosis (Yang et al. 1999).

The differences between the phenotypes in the *Alk1/Smad5* embryos versus the other TGF pathway mutants has been interpreted in terms of a balance model for the role of TGF-β in vessel remodeling and angiogenesis (Oh et al. 2000). During angiogenesis, endothelial cells initially actively proliferate, invade, and migrate to establish new branching vessels and capillaries—the activation phase. However, once vessels are formed, endothelial cells must stop proliferating and migrating and stabilize the vessels by secretion of extracellular matrix and recruitment of supporting cells—the resolution phase. It is proposed that TGF-β, acting through the Alk5 pathway, works together with other angiogenic factors to promote the activation phase, whereas Alk1 signaling promotes transition to the resolution phase. Thus loss of Alk1 signaling would lead to extended activation phase, perhaps explaining the enlarged vessels observed.

Although this general concept of a balance between the two signaling pathways is intriguing, it is not clear that all the genetic or in vitro experiments fit with this simple model of a switch between TGF-β signaling pathways. A different, but not mutually exclusive, explanation for the different roles of the Alk1 and Alk5 receptor pathways is suggested by further observations on the *Alk1* mutant embryos. Major arteriovenous malformations develop in the embryo, and the distinction between arteries and veins fails to be established (Urness et al. 2000). This suggests a specific role for Alk1 but not Alk5 signaling in establishing artery-vein identity, which is discussed further in the next section.

Further evidence implicating TGF-β signaling in arteriovenous development comes from human studies. Mutations in *Alk1* and *endoglin* have been shown to cause hereditary hemorrhagic telangiectasia (HHT) in humans (Azuma 2000). HHT is a dominant disorder of the vasculature, which results in arterio-venous shunting (AVMs), external bleeding, and telangiectases in patients. In mice, no heterozygous phenotype has been reported so far for the *Alk1* mutation, but the *endoglin* $+/-$ mouse develops some of the symptoms characteristic of HHT patients: vascular malformations, external and gastrointestinal bleeding, and telangiectases (Bourdeau et al. 1999). Examination of representative organs in symptomatic mice show a major increase in vascularization, with strongly affected individuals having massively dilated vessels and hemorrhage.

FORMATION OF ARTERIES VERSUS VEINS

It has become apparent in recent years that the distinction between artery and vein is an early event in the development of the vascular system, rather than a later effect resulting from the differential effects of hemodynamic pressure on the developing vessels. The discovery that members of the Eph/ephrin family are differentially expressed in artery and veins from very early stages of development, prior to development of a functional circulation, was one of the first indications that artery-vein identity is intrinsically programmed.

Ephrin Signaling

The Eph receptors constitute a large family of receptor tyrosine kinases with membrane-bound ligands, the ephrins (Flanagan & Vanderhaeghen 1998, Xu & Wilkinson 1997). The ephrins and their receptors are divided into two subgroups, A and B. Ephrin-As are attached to the membrane by a GPI anchor; ephrin-Bs have a conserved intracellular domain (Davis et al. 1994). EphA receptors bind to ephrin-As and EphB receptors to ephrinBs, although at least one EphA receptor (EphA4) can bind to both A and B ligands (Gale et al. 1996). EphrinBs may also signal through their intracellular tails (Henkemeyer et al. 1996, Holland et al. 1996), setting up the possibility of bidirectional signaling when receptor and ligand interact. Roles for the ephrins were first found in axon guidance and boundary maintenance in the central nervous system (Gale & Yancopoulos 1997, Orioli & Klein 1997), as well as in guiding neural crest migration (Holder & Klein 1999). More recently, their involvement in vascular development has begun to be recognized (Cheng et al. 2002). Strikingly, ephrinB2 is specifically expressed in the endothelium of developing arteries, whereas its cognate receptor, EphB4 is expressed in venous endothelial cells from very early stages of development (Wang et al. 1998).

EphrinB2 is also expressed in some mesenchymal cells, and other members of the Eph/ephrin family show vascular expression (Cheng et al. 2002). However, it does appear that the ephrinB2-EphB4 interaction is critical for vascular development, as shown by the similarities in the phenotype of the knockout mice. Loss of either *EphrinB2* (Wang et al. 1998) or *EphB4* (Gerety et al. 1999) led to embryonic lethality around E10.5 with major defects in vascular remodeling in the yolk sac and the embryo. Double mutants in two other Ephs, *EphB2* and *EphB3*, also show similar defects (Adams et al. 1999), at a low penetrance, suggesting that there may be multiple ephrin pathways active in the developing vasculature. Some of these defects could be the result of failure of interaction between developing arteries and veins. However, the defects are complex and seem similar to the defects in *Ang1/Tie2* mutants. This suggests a more general role for ephrins in vascular remodeling, perhaps involving endothelial-mesenchymal interaction rather than a role restricted to the endothelial cell. An endothelial-specific knockout of *ephrinB2*, however, produces very similar vascular defects to the complete null, indicating that the major role for ephrinB2 signaling is endothelial-specific (Gerety & Anderson 2002).

Although mutations of the reciprocally expressed *ephrinB2* and *EphB4* do not produce phenotypes that can be considered to be a simple result of altered artery-vein identity, there are effects on both arterial and venous vessels in both mutants. This is consistent with the possibility that bidirectional signaling of both ligand and receptor is involved in ephrin action in the vasculature. A targeted mutation in *ephrinB2* that lacks the cytoplasmic tail required for reverse signaling reproduces the vascular remodeling defects, but not the neural crest defects, seen in the original *ephrinB2* mutants (Adams et al. 2001), supporting this concept. Bidirectional ephrin signaling may act to help separate venous and arterial endothelial cells and ensure the correct development of arterioles and venules and major arteries and

veins. It may also help ensure that "like" vessels fuse only with "like" as the vasculature is remodeled. This would suggest that ephrin signaling is downstream of the pathways that initially establish the identity of arterial and venous endothelial progenitors.

The Notch Pathway

Recent work from studying human disease mutations, as well as experimental analysis in mice and zebrafish, has suggested that Notch signaling may be key to the establishment of artery versus vein fate upstream of ephrin signaling. The Notch signaling pathway has been implicated in many aspects of cell-fate specification during development (Artavanis-Tsakonas et al. 1999, Kojika & Griffin 2001). The *Notch* gene, first identified in *Drosophila*, encodes a 300-kDa single-pass transmembrane protein, characterized by extracellular EGF repeats and a characteristic RAM motif in the intracellular domain (Artavanis-Tsakonas et al. 1999). Four different Notch receptors (Notch 1–4) have been identified in mammals, with homologues in other vertebrates (Kojika & Griffin 2001). Notch receptors are known to interact with five ligands in mammals (Nye & Kopan 1995). Delta-like (Dll) 1, 3, 4, Jagged1, and Jagged2, are single-pass membrane-bound ligands that contain EGF repeats and interact with the Notch receptors through a common DSL (Delta, Serrate, Lay) motif, located in the extracellular domain of the protein. These molecules are homologues of the Delta and Serrate ligands first identified in flies. Once Notch binds to its ligand, the intracellular domain of the receptor is proteolytically cleaved (Schroeter et al. 1998, Struhl & Adachi 1998); this receptor fragment binds to the transcription factor RBPJ-k, [also known as CBF-1/RJBk in mammals and Su(H) in *Drosophila*] (Fortini & Artavanis-Tsakonas 1994). The complex then enters the nucleus where it can activate specific downstream target genes (Artavanis-Tsakonas et al. 1999). Direct targets of Notch activation include the *Hairy* genes in *Drosophila* and the related bHLH transcription factor genes, the *Hes* genes, in vertebrates (Davis & Turner 2001, Kageyama & Ohtsuka 1999).

Expression analysis has revealed that multiple Notch ligands and receptors are expressed in and around the developing vasculature. In mice, Notch 1, 3, 4, Delta-4, Jagged1, and Jagged-2 are all expressed in association with the vasculature in y E10-E17 embryos (Villa et al. 2001). Of these, Notch1, Notch4, Delta4, Jagged1, and Jagged2 were specifically expressed in the arterial endothelium and were found at low levels (if at all) in veins. Notch3 was localized to the smooth muscle cells surrounding arteries but not veins, whereas Jagged1 was expressed in both the endothelial and smooth muscle cells associated with the arterial vessels. Notch4 and Delta4 are the only members of the Notch pathway whose expression appears to be entirely restricted to the endothelium, including the developing capillaries (Shirayoshi et al. 1997, Shutter et al. 2000, Uyttendaele et al. 1996).

Clues as to the function of the Notch signaling pathway in vascular development came first from human disease analysis (Joutel & Tournier-Lasserve 1998). The Notch pathway has been shown to be involved in three human diseases: mutations in

the human *Notch1* gene result in T cell acute lymphoblastic leukemias/lymphomas (Ellisen et al. 1991), mutations in the *Jagged1* ligand cause Alagille syndrome (Li et al. 1997, Oda et al. 1997), and a deficiency in *Notch3* leads to CADASIL (Joutel et al. 1996). The *Jagged1* and *Notch3* disease phenotypes are of particular interest to this review because they both have significant vascular components. Alagille syndrome is a cause of liver disease and liver failure in about 1/70,000 live births (Piccoli & Spinner 2001). Patients suffering from this syndrome have hepatic lesions, a reduction in the number of bile ducts, and skeletal defects. Importantly, Alagille patients also have abnormally formed blood vessels, arterial stenosis, and heart disease. CADASIL, for Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy, is a syndrome in which patients experience a series of strokes, migraines, and progressive dementia, beginning at age 40–45 (Ruchoux & Maurage 1997). Physiologically, CADASIL patients exhibit a degeneration of the smooth muscle layer surrounding the cerebral arteries and skin arterioles. These human syndromes clearly demonstrate a requirement for Notch signaling in vascular development and adult vessel maintenance.

Studies of targeted mutation in mice have provided further evidence for a role of Notch signaling in vascular development. In examining the phenotypes of mice carrying homozygous mutations, it is clear that four genes—*Notch1*, *Notch4*, *Delta Like-1* (*Dll1*) and *Jagged1*—are associated with a vascular phenotype (Conlon et al. 1995, Hrabe de Angelis et al. 1997, Krebs et al. 2000, Xue et al. 1999). Embryos homozygous for a null allele of *Notch1* die by day 11, with growth retardation by day 9, cranial cell death, and defects in somitogenesis (Conlon et al. 1995). Interestingly, further study by Krebs et al. of *Notch1*-deficient embryos showed that homozygotes had pale yolk sacs, with a normal primary capillary plexus, but with severe defects in vessel remodeling and occasional vascular degeneration (Krebs et al. 2000). Placental defects were also observed with vessels failing to migrate into the labyrinthine layer of the placenta. *Notch1*$^{-/-}$ embryos also demonstrated defects in the embryonic vasculature, including abnormalities in the aorta and other major vessels (Krebs et al. 2000). Targeted mutation of the vessel-specific receptor, *Notch4*, showed no defects in homozygous mutant mice, but embryos homozygous for *Notch1* and *Notch4* had more severe vascular defects than in embryos homozygous for the Notch1 mutation alone (Krebs et al. 2000). The *Notch1*$^{-/-}$, *Notch4*$^{-/-}$ double homozygotes showed defects in yolk sac remodeling, appearing unable to form either the large vitelline vessels, or the small capillaries, which resulted in a disorganized vasculature. Furthermore, studies of the embryonic vasculature showed marked defects, with the anterior cardinal vein missing in some of the more severely affected embryos. Significant disorganization was observed in the intersomitic vessels of the double mutants, and some embryos showed a collapse of the dorsal aortae. Clearly the Notch pathway is essential in some manner for the remodeling of the primary vascular plexus.

Further evidence for a vascular role of the Notch signaling pathway comes from the targeted mutation of Notch ligands Dll1 and Jagged1. The Dll1 knockout mouse, characterized by Hrabe de Angelis et al., was described to have major

defects in somite formation (Hrabe de Angelis et al. 1997). The embryos also showed prominent hemorrhaging, although the vascular abnormalities were not examined further. Embryos lacking a functional *Jagged1* gene die by day 10, with vascular defects (Xue et al. 1999). Like the *Notch1* and *Notch4* mutants, *Jagged1*$^{-/-}$ homozygotes are capable of endothelial cell differentiation and the formation of the primary capillary plexus. However, the *Jagged1* mutants are unable to form the large vitelline vessels (Xue et al. 1999). Embryonic vessels are also defective; *Jagged1* mutants show a poorly formed cranial network with reduced branching.

Expression of activated *Notch* in the developing vasculature also disrupts vessel organization in mouse embryos (Uyttendaele et al. 2001). Constitutively active *Notch4* (*int-3*) was expressed under control of the *Flk1* promoter, effectively restricting *int-3* expression to cells of the endothelial and hematopoietic lineages. Although the ectopically expressing embryos were able to form primary capillary plexuses and underwent normal hematopoiesis, the mutant embryos formed fewer small vessels, made disorganized networks, and produced many dilated vessels. Some loss of vessel integrity was also observed in these mutants, and the vessels of the mutant yolk sacs degenerated in a gradient. The fact that both gain and loss of function of Notch signaling can disrupt the same events is typical of pathways dependent on Notch signaling, where often the state of activation of Notch signaling in a given cell determines cell fate choice.

Although it is clear that Notch signaling is essential for the proper development of the vascular network, its precise role is not readily apparent from these descriptions, and several different possibilities have been proposed. From study of all the mutants, Notch signaling does not appear to be required for hemangioblast differentiation, initiation of hematopoiesis, or the formation of the primary capillary plexus. One possible role for the Notch pathway is for the induction or regulation of branching morphogenesis in the developing vasculature. Certainly this is seen in the case of the *Jagged1* and *Notch1/Notch4* mutants; in these cases, the vascular networks of the yolk sac and cranium have reduced branching, with disorganized vessels of inappropriate size (Krebs et al. 2000, Xue et al. 1999). The idea that Notch signaling is important for branching is not inconsistent with findings in other systems; Notch has been shown to be important for the branching of the epithelial tubes in mammary gland development (Uyttendaele et al. 1998) and also has a role in patterning tracheal branches in *Drosophila* (Steneberg et al. 1999).

Notch may also be required for vascular maintenance. If one looks at the phenotype of mutant embryos, it is clear that although the embryos have defects in remodeling the vasculature, several mutants also exhibit a degeneration of the vasculature and occasional collapse of major vessels. This connection is tenuous, as there is no evidence for a loss of smooth muscle cell coverage. Much more convincing is the finding that CADASIL patients have defects in maintaining smooth muscle cells, which results in lesions in the vasculature and hemorrhage (Joutel & Tournier-Lasserve 1998). In this case, Notch3 is required to keep the vessels healthy and functional. It is possible that Notch could have a role in maintaining the smooth muscle layer of the adult vasculature, and it is also possible that it

might play an earlier role in determining smooth muscle cell fate. It is intriguing to speculate that the levels of Notch signaling could have an effect on initial differentiation of smooth muscle cells in the embryo. It was recently shown that a common progenitor cell may exist that can give rise to both endothelial cells and smooth muscle cells in vitro (Yamashita et al. 2000). What determines the decision of the progenitor to take either pathway is currently unknown, but levels of Notch activation might affect such a choice. Certainly some of the vascular defects seen in the various Notch signaling mutants could result from an alteration in the endothelial/smooth muscle cell ratio.

However, the most intriguing possiblity is that Notch signaling acts to determine artery versus venous fates, perhaps upstream of ephrin signaling. Hints as to this possibility come from the description of the various mouse embryos mutant in the Notch pathway. For example, dilated vessels and missing major veins have been reported in both gain and loss of function mutations. However, none of the studies specifically looked at whether arteriovenous shunts and altered artery-vein differentiation were produced. Given that *Notch1*, *Notch4*, *Delta4*, *Jagged1*, and *Jagged2* are all found at higher levels in arteries than in veins (Villa et al. 2001), the possibility of a role in this cell choice needs to be considered. The best evidence comes from studies in zebrafish, which strongly suggest that Notch signaling is necessary to suppress venous identity in the developing arteries and thus helps determine cell fate choice. The mouse experiments will need to be reexamined in the light of these studies.

The Notch pathway was inhibited in the developing vasculature of the zebrafish through the injection of a dominant-negative form of Suppressor of Hairless (the common downstream effector of Notch signaling) in two different studies (Lawson et al. 2001, Zhong et al. 2001). Injected embryos showed decreased arterial expression of *ephrinB2* and *Notch3*, two genes known to be markers of arterial cells in zebrafish as in mouse. These embryos also showed ectopic expression of venous markers in the dorsal aorta. Circulatory shunts between the dorsal aorta and the posterior cardinal vein in the trunk were also reported in one study (Lawson et al. 2001), and another study showed progressive loss of sections of the dorsal aorta and expansion of the vein (Zhong et al. 2001). Expression of an activated *Notch* construct, on the other hand, decreased expression of venous markers but did not induce ectopic arterial markers (Lawson et al. 2001). This suggests that during normal development, activation of the Notch signaling pathway in developing artery cells is required to suppress the venous fate and allow arterial differentiation.

The hairy-related transcription factor genes of the *Hes* family are direct downstream targets of Notch signaling in many situations where Notch determines cell fate choice. These genes act as repressors of transcription and can suppress specific cell fates (Castella et al. 2000, Cau et al. 2000). One member of this family is *HRT2*, which has been shown to be a direct transcriptional target of Notch signaling in mammalian cells (Nakagawa et al. 2000). The zebrafish homologue is mutated in the *gridlock* mutation (Zhong et al. 2000), which causes a localized patterning defect in the developing dorsal aorta. *Gridlock* is expressed specifically in the aorta

and not in the developing veins, consistent with it being a Notch target in vivo (Zhong et al. 2000). Graded reduction in *gridlock* expression leads to progressive loss of the artery and expansion of the adjacent vein, in a manner very similar to the effect seen by blocking Notch signaling with a dominant-negative *Su(H)* construct (Zhong et al. 2001). Although it has not been proven that *gridlock* is a direct target of Notch signaling—the two reports on the use of dominant-negative *Su(H)* constructs differ as to whether gridlock expression is downregulated or not—the phenotypic similarities make this an attractive hypothesis. The role of the repressor, gridlock, could then be to repress venous markers like EphB4 in arterial cells.

Other Signaling Pathways

As described in the section on TGF-β signaling, there is evidence for arterio-venous malformations in mouse embryos mutant for *Alk1*, a TGF-β type I receptor (Urness et al. 2000). Embryos lacking *Alk1* show formation of the dorsal aorta and cardinal veins in the correct positions, but shunts develop between the vessels and the artery fails to turn on expression of arterial-specific *ephrinB2*. Ectopic expression of venous markers was not reported, nor is it known whether ectopic expression of activated receptor can cause ectopic arterial differentiation. However, it may be that the TGF-β pathway, acting through Alk1, is part of the arterial specification pathway that activates Notch signaling and represses venous fate. The exact order of action of the two signaling pathways is unclear. Artery-versus vein-specific activity of another supposedly generic vascular signaling pathway, the VEGF pathway, has also been reported. Mice lacking the $VEGF_{164}$ isoform, but expressing the other forms, showed a variety of vascular patterning defects. Notably, arteriole outgrowth in the retina was specifically affected, whereas venules were relatively normal (Stalmans et al. 2002). Although the VEGF receptor *Flk1* is expressed in both arteries and veins, the co-receptors, neuropilins, have recently been shown to be differentially expressed: *Np1* is artery-specific, whereas *Np2* is restricted to the venous system (Herzog et al. 2001). Because Np1 preferentially binds $VEGF_{164}$, this may indicate a special role for the particular combination of $VEGF_{164}$/Np1/VEGFR2 in promoting artery outgrowth. To date, no signaling pathway has been shown to be specifically involved in positive activation of venous differentiation.

A possible model for arterio-venous separation is shown in Figure 2. Initial specification of angioblasts and formation of the capillary plexus requires VEGF signaling. At some time early in this process, either at the angioblast stage or at the early endothelial tube stage (the exact point is not clear), angioblasts make the initial decision to be artery or vein, perhaps involving differential Notch signaling (Zhong et al. 2001). Subsequently, signaling through the Alk1 TGF-β receptor is positively required to specify arterial fate, whereas signaling through the Notch pathway acts to repress venous differentiation. $VEGF_{164}$ signaling via Np1/VEGFR2 complexes on developing arteries specifically promotes arteriole outgrowth. Downstream of these events, the Ephrin/Eph interaction between

developing arteries and veins acts to ensure continued separation of the two and perhaps also to assist in the correct branching and guidance of the developing vasculature. Many parts of this model are still unresolved but are testable in genetic experiments in fish and mice.

PATTERNING THE DEVELOPING VASCULATURE: INTRINSIC VERSUS EXTRINSIC EFFECTS

Although considerable information is now accumulating on the signaling pathways required to initiate vascular development, determine artery versus vein identity, and regulate vascular remodeling and vessel stabilization, the mechanisms by which the exquisitely complex stereotypic patterning of the entire vasculature is brought about are still unclear. Why do vessels form in the same position in all embryos? How is branching and sprouting controlled locally? How do the different properties of the vasculature of different organs develop? An extensive review of the many possible mechanisms is beyond the scope of this review, but a few examples of recent studies highlighting the complex inputs into vascular patterning are described.

Vascular Polarity

Although the final network of capillary branching in many tissues does not appear to follow a clear intrinsic pattern, it is obvious that higher-level structuring of the vasculature is remarkably stereotypical and presumably under genetic control. The vascular network of both arteries and veins is formed by progressively ordered and directed branching. In some situations, such as the formation of the repeated intersomitic arteries and veins, this order is bilaterally symmetric. In other cases, for example, the development of the major vessels coming in and out of the heart, asymmetries develop in a predictable manner between the right and left side. This latter situation is critical for normal function of the circulation. Defects in the patterning of the great vessels are a common congenital cardiovascular anomaly. Intriguingly, it has recently been shown that embryos lacking both *Ang1* and the orphan Tie receptor, *Tie1*, show very specific disruption of the right side of the developing venous system (Loughna & Sato 2001b). *Tie1* is expressed symmetrically, but *Ang1* shows polarized expression in the developing sinus venosus, suggesting that somehow the asymmetrical expression of the ligand disproportionately influences one side of the developing venous system. This example suggests that already known signaling pathways may play a role in the specific patterning of the vasculature, as well as in the generic processes of vasculogenesis and angiogenesis.

Intersomitic Vessels

The stereotypic production of the intersomitic arteries and veins is currently under investigation in a number of systems as a model of how vascular patterning

could be regulated. A recent study in zebrafish suggests that the site of sprouting of the intersomitic vessels is genetically constrained (Childs et al. 2002). Intersomitic vessels sprout from the dorsal aorta only between the ventral regions of two adjacent somites and then migrate dorsally between the somites. A mutation, *out-of-bounds*, causes intersomitic vessels to arise at abnormal positions and migrate aberrantly (Childs et al. 2002). Interestingly, there is still only one sprout per somite, but the position of the sprout is altered. This suggests that there may be two kinds of controls, one that determines the number of sprouts per somite and another that determines the position of the sprout. The first process might be intrinsically programmed, whereas the position of the sprouts would be determined by extrinsic signals, perhaps from the somites themselves. Consistent with this, the *out-of-bounds* mutation acts non-cell autonomously in the endothelial cells (Childs et al. 2002).

Once the position of the intersomitic sprout is determined, repulsive cues from the somites act to constrain the direction of intersomitic vessel growth. Because ephrin-Eph interactions have been shown to control migration and guidance in other cell types, they are strong candidates for controlling the correct positioning of the developing intersomitic vessels as well. The developing intersomitic veins express EphB4, and the surrounding somites express several ephrinBs. Experiments in *Xenopus* blocking *EphB4* by overexpression of a dominant-negative construct resulted in aberrant growth of intersomitic veins into adjacent somite tissue (Helbling et al. 2000). Mice mutant for *EphrinB2* show abnormal intersomitic vessel sprouts, which penetrate the surrounding tissues (Adams et al. 1999). Similar intersomitic vessel defects were observed in *EphB2/EphB3* mutant mice (Adams et al. 1999), as well as in mice lacking p120 Ras GTPase-activating protein (*RasGAP*) (Henkemeyer et al. 1995). The latter observation suggests that RasGAP could be a key downstream effector of Eph signaling in the migration of endothelial cells. Ephrin signals from somite tissue would thus interact with Eph receptors on the developing vessels to repel them from entering the somites and thereby ensure correct intersomitic growth (Figure 3).

It is interesting that some of the same pathways that are involved in axon guidance also seem to be involved in vascular patterning (Shima & Mailhos 2000). The role of ephrin signaling in guidance of developing vessels seems analogous to their action in the nervous system. Neuropilins, on the other hand, which act as receptors for the axon repellant semaphorins in the nervous system, have not yet been shown to be directly involved in vessel guidance. Rather, they may act as general VEGF co-receptors, enhancing the stimulatory responses of VEGF on endothelial cells. It is also possible that semaphorins could play a direct role on vascular patterning via binding to neuropilins on developing vessels. In many parts of the embryo, there is a close association of the developing axons and blood vessels, suggesting that there could be an actual interplay of signaling between the two lineages. More analysis of the effects on vascular development of defects in nerve development and vice versa should help elucidate whether the association is obligatory.

Hypoxia and Vascular Patterning

As well as specific guidance cues for directional organization of the vasculature, extrinsic signals also act to promote local vascular invasion and proliferation and to influence the specialization of different endothelial cell types in various tissues. One of the key players in regulating the extent of angiogenesis and vessel growth is oxygen level in a given tissue. Many genes involved in angiogenesis, including the VEGF gene itself, are upregulated by hypoxia in both normal and pathological situations (Semenza 1999). The hypoxia-response mechanism is an evolutionarily conserved pathway involving the regulated expression of a hypoxia-induced transcription factor complex. Hypoxia-inducible factor (HIF) consists of a heterodimer between HIF1α and the aryl hydrocarbon receptor nuclear translocator (ARNT) protein, both of which are members of the basic-helix-loop-helix-PAS domain gene family (Wang et al. 1995). ARNT expression is not regulated by hypoxia, but HIF1α is regulated by increased protein stability in hypoxic conditions. In normal conditions, HIF1α is rapidly degraded by ubiquitination and proteasome degradation. This ubiquitination is mediated by the product of the von-Hippel-Lindau (*vHPL*) tumor suppressor locus, whose action is blocked in low oxygen (Maxwell et al. 1999). This leads to stabilization of HIF1α and translocation of the HIF complex to the nucleus (Kallio et al. 1999), where it binds to specific hypoxia-response elements (HREs) upstream of a large number of hypoxia-responsive genes (Semenza 1999).

The local induction of the *Vegf* gene by the HIF complex (Forsythe et al. 1996) has been considered to be the main driving force for the development of new vessels by angiogenesis in hypoxic situations, such as rapidly growing tumors in the adult (Grunstein et al. 1999, Hanahan & Folkman 1996). Although overexpression of VEGF in the skin induces increased vascularization, the vessels formed are leaky and tortuous (Detmar et al. 1998, Larcher et al. 1998, Thurston et al. 1999). Expression of *HIF1*α under the same skin-specific promoter also induces neovascularization, but the vessels formed are much more like normal vessels and are functional (Elson et al. 2001). This suggests that hypoxia induces a cascade of hypoxia-response genes, not just *Vegf*, that can lead to development of new fully functional vessels. This raises the possibility that local hypoxia may also be a driving force for activation of angiogenesis in the developing embryo, ensuring that a relationship between oxygen needs and tissue vascularization is maintained. Recent evidence in the *Drosophila* tracheal system has also suggested that hypoxia may be an important determinant of the final branching pattern of the developing tracheal system (Jarecki et al. 1999).

Several pieces of evidence suggest a connection between the HIF pathway, as mediator of the hypoxic response, and the embryonic development of the vasculature, most notably the lethal phenotypes of mutations in the major hypoxia-regulators, *HIF1*α and *Arnt*. Targeted mutations in *HIF1*α result in mid-gestational embryonic lethality characterized by prominent neural defects, myocardial hyperplasia, and defects in both the embryonic and extraembryonic vasculature

(Carmeliet et al. 1998, Iyer et al. 1998, Ryan et al. 1998). Sections of *HIF1α*$^{-/-}$ embryos showed massively enlarged neural vessels, with dilated and disorganized capillaries. Embryos deficient in *Arnt* also exhibited lethality coupled with defects in vascular remodeling (Kozak et al. 1997, Maltepe et al. 1997). Like the *HIF-1α*$^{-/-}$ embryos, *Arnt*$^{-/-}$ embryos were able to differentiate endothelial cells and produce a primary plexus but were unable to form a properly branched network. These results clearly suggest that detection of oxygen levels and translation of that detection into vascular growth and differentiation is key, not just for tumor angiogenesis, but also for normal vascular patterning. Local reduced oxygen tension during development may be important in activating the complex pathways leading to new vessel sprouting and maturation. More studies are needed to determine how much of the developing vascular pattern is driven by local hypoxia versus more specific local growth factor cues, but it is clear that this effect is a necessary and critical part of normal vascular patterning.

Endothelial Specialization

A final component of vascular patterning, which is not at all well understood, is the specialization of endothelial cell types that occurs in different tissues. Endothelial cells in different organs show very different morphological and physiological properties dependent on their particular role in a given environment. For example, the microvasculature of several organs, particularly those involved in endocrine secretion, is highly permeable. This feature depends on the development of actual discontinuities in the plasma membrane of the microvasculature, known as fenestrae. VEGF can induce fenestration in vessel walls (Esser et al. 1998, Roberts & Palade 1997) but presumably cannot be the only factor involved in this specialization because it is also expressed strongly around other developing vessels that do not fenestrate. Recently, a novel growth factor, known as EG-VEGF, has been isolated that can act as an angiogenic mitogen and induce fenestration (LeCouter et al. 2001). This molecule is largely restricted in its expression to steroidogenic glands, suggesting that it may act in conjunction with the more general angiogenic factors to promote local specialization of the endocrine endothelium. Its receptor is currently unknown, and it is it not known if there are similar factors specific to other local endothelial situations. However, it opens the possibility that other specialized endothelia may also be regulated by local signaling pathways.

FUTURE DIRECTIONS

Much research in vascular biology has been driven by the clinical importance of understanding how to induce or inhibit neovascularization in the adult. Initial studies on the induction of angiogenesis helped identify key signaling pathways, such as the VEGF and angiopoietin pathways, that are critical to both normal vascular development and neovascularization. However, such studies did not deal with the issues of how the normal vascular system is patterned, how artery-vein

distinctions develop, and how specialized endothelia develop in different organs. Insights into these processes have come from recent genetic and developmental biology studies in mice and fish. Multiple signaling pathways, some endothelial-specific, some co-opted from other processes, have been implicated in vascular patterning, although many questions remain. Future studies will need to elucidate the complex hierarchies of normal patterning of the vasculature and will provide possible new targets for intervention in a clinical setting.

ACKNOWLEDGMENTS

We thank Masanori Hirashima for constant updates. J. R. is a Distinguished Investigator of the Canadian Institutes for Health Research.

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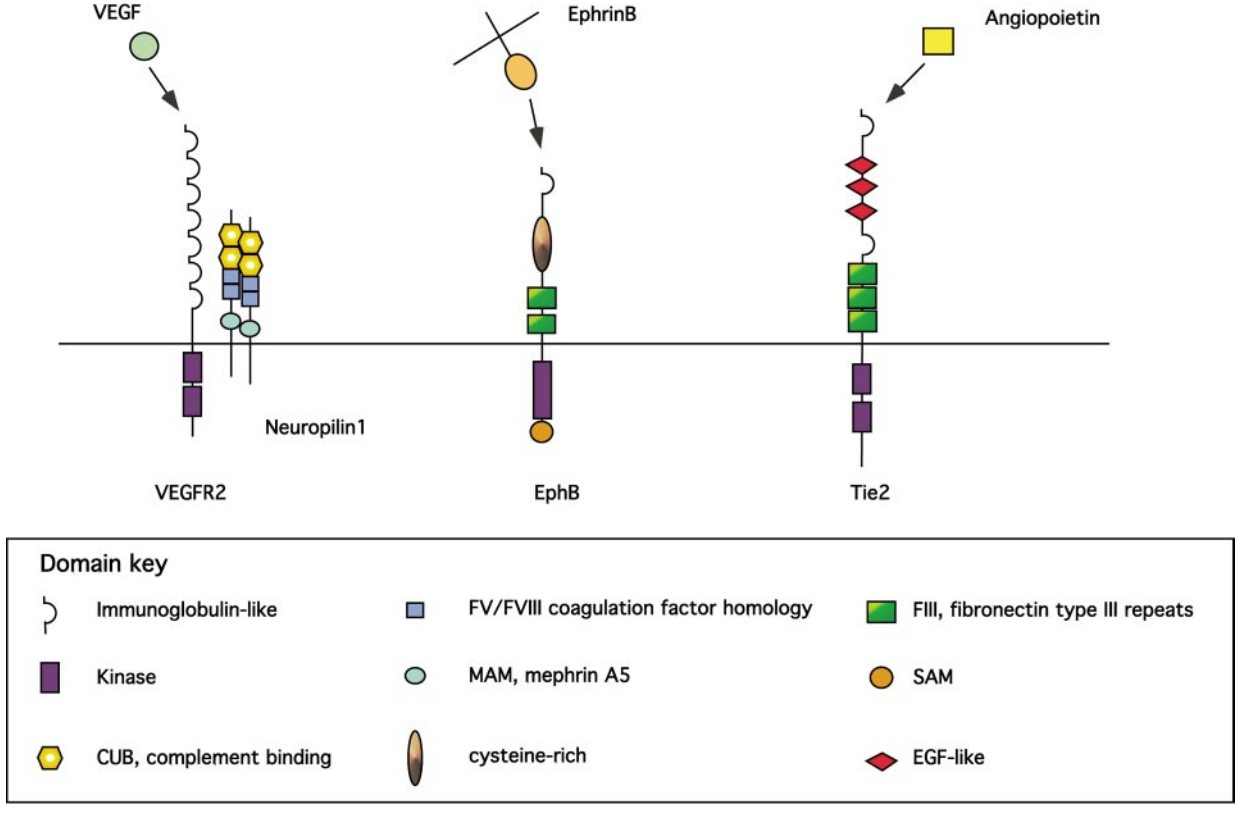

Figure 1 Major receptor tyrosine kinases active in the developing vasculature.

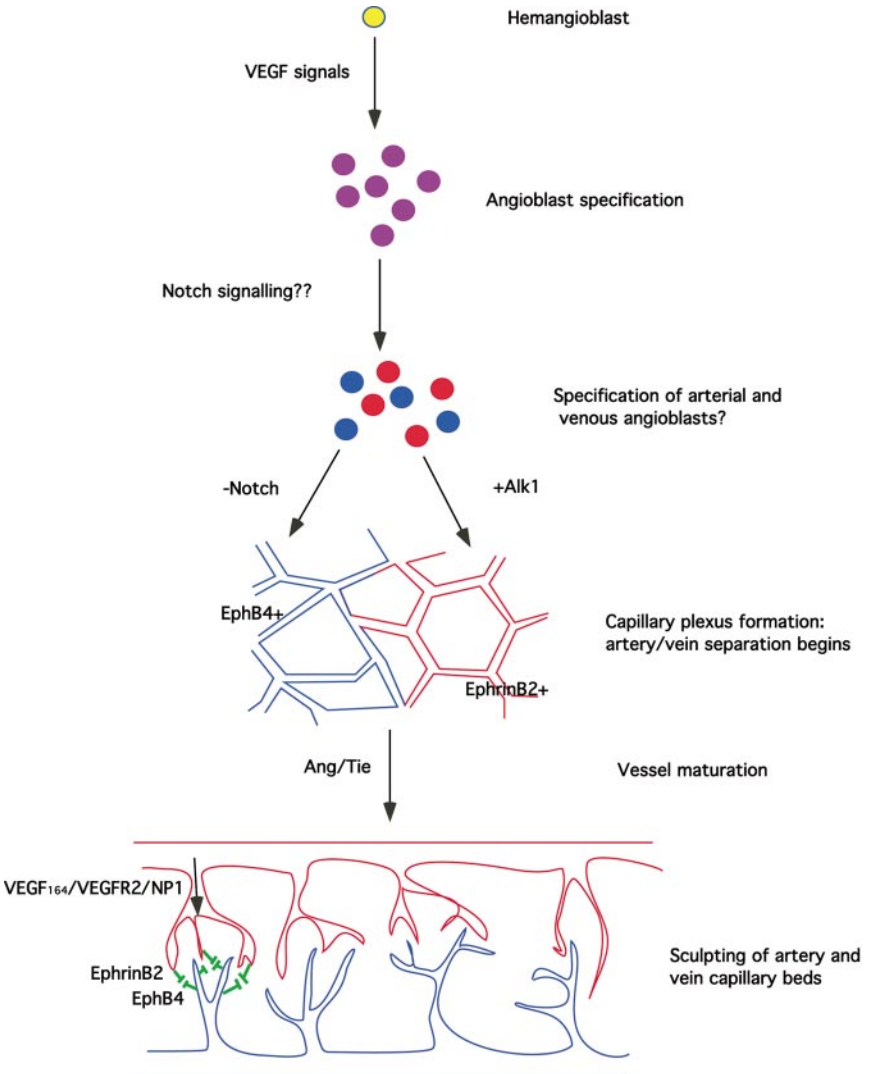

Figure 2 Model for specification of arteries and veins in development. A common hemangioblast progenitor responds to VEGF signaling to become an endothelial precursor. At some point, perhaps before vessel organization, individual angioblasts become committed to artery versus vein fate under the influence of Notch signaling. As the capillary plexus forms, artery vein separation continues, with signaling through Alk1 promoting artery fate and signaling through Notch repressing vein differentiation. As the vasculature matures into major vessels and associated capillary beds, $VEFG_{164}$ signaling via VEGFR2/NP1 complexes promotes arteriole outgrowth, and Ephrin interactions serve to separate arteries and veins.

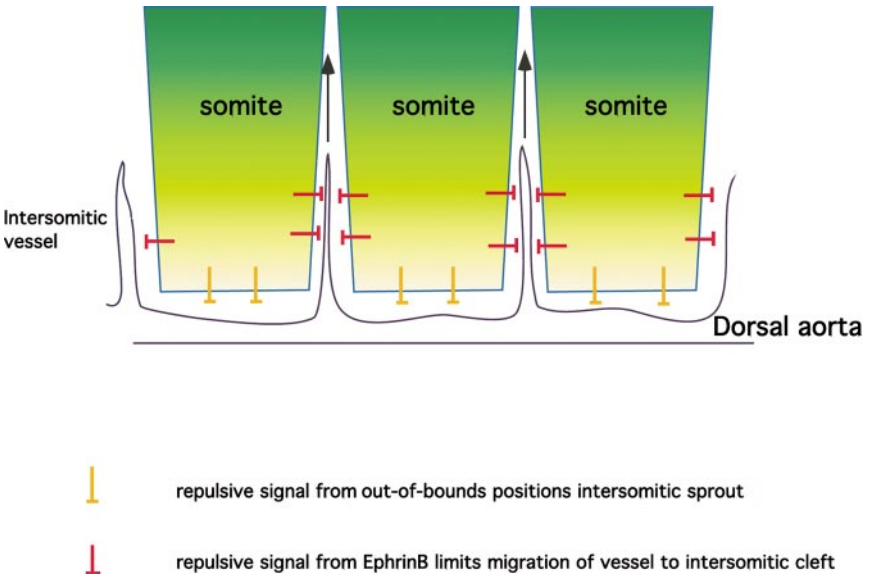

Figure 3 Repulsive cues determine position and migration of intersomitic vessels. The somite produces repulsive cues to limit intersomitic vessels. The out-of-bounds pathway limits vessel sprouting from the dorsal aorta to the intersomitic space, whereas Ephrin signals ensure that the vessel does not encroach into somite territory during its migration.