CHAPTER TWO

Vascular Development–Genetic Mechanisms and Links to Vascular Disease

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Contents

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Abstract

Vertebrate development depends on the formation of intricate vascular networks at numerous sites and in precise patterns; these vascular networks supply oxygen and nutrients to the rapidly expanding tissues of the embryo. Embryonic blood vessels are composed of endothelial cells and pericytes that organize and expand into highly branched conduits. Proper development of the vasculature requires heterogeneity in the response of endothelial cells to angiogenic cues provided by other tissues and organs. The pathogenesis of vascular diseases results from genetic mutations in pathways that provide these cues and in signals that coordinate endothelial heterogeneity during blood vessel formation. Here we provide a brief overview of different aspects of blood vessel formation and then discuss three essential signaling pathways that help establish vessel networks and maintain endothelial phenotypic heterogeneity during vascular development: the vascular endothelial growth factor (VEGF), bone morphogenetic protein (BMP), and the Notch/Delta/Jagged pathways. The VEGF pathway is critical for the initiation and spatial coordination of angiogenic sprouting and endothelial proliferation, BMP signaling appears to act in a context-dependent manner to promote angiogenic expansion and remodeling, and the Notch pathway is a critical integrator of endothelial cell phenotypes and heterogeneity. We also discuss human genetic mutations that affect these pathways and the resulting pathological conditions.

1. Introduction

Blood vessels are essential to the development and viability of vertebrate embryos. The vascular system facilitates oxygen and nutrient delivery to cells and removal of metabolic waste from cells, processes that require transport as embryos grow beyond a size that allows for passive diffusion. Thus, blood vessels and the heart are the first organs to function during mammalian development, yet vessel networks continue to form and remodel dynamically even as they function. All stages of vascular development require complex interactions among genetic programs, molecular cues, and cellular behaviors, and these inputs are tightly regulated both spatially and temporally (Adams and Alitalo, 2007; Carmeliet, 2005; Jain, 2003; Risau, 1997). Mutations in numerous vascular genes lead to pathologies characterized by aberrant vessel formation or function (Fig. 2.1).

Insight into the mechanisms underlying vascular development has led to clinically relevant therapies for diseases with vascular components. For example, the prominence of the vascular endothelial growth factor (VEGF) pathway in development and in tumor angiogenesis has instigated the development of several antiangiogenesis drugs that specifically target this pathway (Ferrara, 2005; Heath and Bicknell, 2009; Jain, 2005). However, therapeutic approaches have been less effective than hoped, and we now

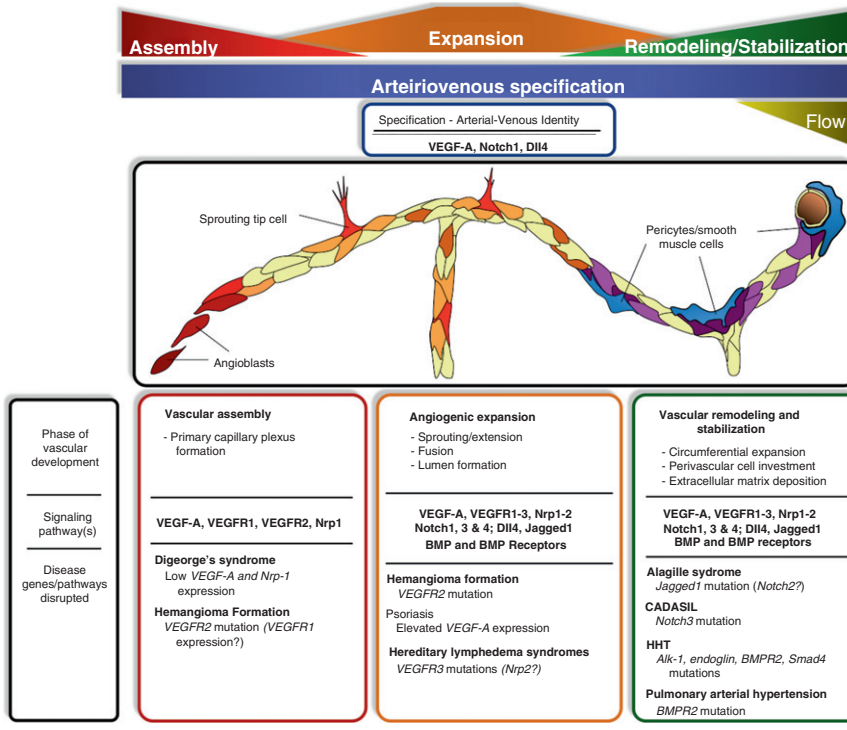

Figure 2.1 Overview of blood vessel formation. The morphological events constituting vessel assembly, expansion, and remodeling intensify in activity and then attenuate during phase transitions, while arteriovenous specification events likely occur throughout the vascular development program. The different stages of vascular development utilize overlapping molecular pathways for these events, and endothelial phenotypic heterogeneity (represented schematically by color variation, i.e., shades of red, brown, and violet) is an essential aspect of each stage. Genetic lesions in human genes that disrupt these pathways are associated with specific vascular diseases. (See Color Insert.)

realize that aspects of blood vessel formation need better elucidation. One such aspect is the concept that endothelial cells in developing vessel networks exhibit phenotypic heterogeneity. This heterogeneity is found at several levels in normal vessels and is essential for vascular development. For example, tip cells migrate to form new sprouts and stalk cells follow behind and proliferate, lateral base cells provide local guidance to emerging sprouts, and endothelial cells behind the sprouting front are said to form a phalanx and become quiescent (Bautch, 2009; Chappell *et al.*, 2009; Gerhardt *et al.*, 2003; Hellstrom *et al.*, 2007; Mazzone *et al.*, 2009). Perturbation of any of these relationships compromises vessel development and function. The endothelial cell heterogeneity that drives blood vessel formation will be highlighted throughout this review.

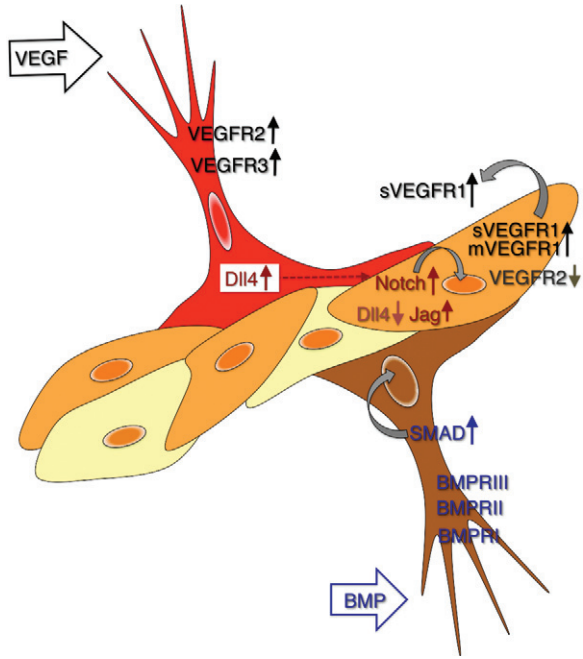

Figure 2.2 Molecular signaling and endothelial heterogeneity in blood vessel formation. As vessels undergo sprouting angiogenesis, tip cells (dark grey) upregulate VEGFR2 and VEGFR3 signaling in response to VEGF-A, and this leads to Dll4 upregulation. Dll4 binds Notch on a neighboring stalk/lateral base cell (medium grey) and activates Notch signaling, which leads to upregulation of VEGFR1, secretion of soluble VEGFR1, and downregulation of VEGFR2. Jagged antagonizes Dll4 in stalk cells. An alternative mode of sprouting (lower cell) involves activation of BMP receptors on some endothelial cells.

We first present a brief overview of vascular development and then focus on how three critical signaling pathways function and interact to promote endothelial heterogeneity: VEGF, bone morphogenetic protein (BMP), and Notch/Delta/Jagged (Fig. 2.2). These pathways were chosen from among many molecular inputs because they both contribute to and respond to endothelial heterogeneity in important ways. We also describe genetic mutations that disrupt these pathways and affect vascular processes in human development and disease.

1.1. Vessel assembly

Blood vessels are one of the first embryonic organ systems to develop and function during vertebrate development. Mesoderm cells give rise to endothelial

precursor cells in several ways. Most angioblasts likely derive from progressive restriction of mesoderm to the endothelial lineage in response to signals such as IHH (Indian Hedgehog), FGF2 (fibroblast growth factor), BMP, and VEGF (Goldie *et al.*, 2008). Some endothelial cells may derive from hemangioblasts, bipotential cells that give rise to both hematopoietic and endothelial cells (Goldie *et al.*, 2008; Vogeli *et al.*, 2006), and some endothelial cells retain the ability to produce hematopoietic cells and are called hemogenic endothelium (Goldie *et al.*, 2008; Yoshimoto and Yoder, 2009).

During this initiation phase, called vasculogenesis, angioblasts aggregate into cords and differentiate into endothelial cells. For example, dorsal aorta formation in *Xenopus* and zebrafish models involves the recruitment and orientation of angioblasts at the midline by VEGF and other molecular signals (Cleaver *et al.*, 1997; Jin *et al.*, 2005). In other tissue beds, however, it remains unclear exactly how these vessel cords arise and form basic networks. Nevertheless, the rudimentary structures within these networks provide initiation points for the subsequent expansion of vessels. A hierarchy of vessels (i.e., arteries, capillaries, veins) is initially established by the activity of arteriovenous specification molecules during vasculogenesis (Swift and Weinstein, 2009). Sonic hedgehog expression from the zebrafish notochord induces somite VEGF production, which upregulates arterial specification molecules such as ephrinB2 through Notch signaling (Lawson *et al.*, 2001, 2002). Subsequent maintenance of arteriovenous identity requires flow-mediated remodeling, since in the chick and mouse yolk sac alterations in flow dynamics perturb vessel maturation (le Noble *et al.*, 2004; Lucitti *et al.*, 2007).

1.2. Angiogenic expansion of blood vessel networks

Primitive vessels must branch and establish new connections. This angiogenic expansion phase requires phenotypic heterogeneity among endothelial cells in their response to stimulatory cues (Fig. 2.2). Sprouting angiogenesis entails the selection of an individual endothelial cell for outward migration from a parent vessel (Gerhardt *et al.*, 2003; Phng and Gerhardt, 2009). This leading tip cell responds to a VEGF gradient by migrating up the gradient (Ruhrberg *et al.*, 2002). Endothelial cells behind the tip cell in the emerging sprout, termed *stalk cells*, do not migrate independently, but instead proliferate and eventually lumenize. The mechanism by which vessels acquire a patent lumen remains controversial and may actually be region specific (Iruela-Arispe and Davis, 2009). The lumen of the mouse dorsal aorta forms by organized changes in endothelial cell shape (Strilic *et al.*, 2009), while an alternative model for lumenization has been proposed in which intracellular and intercellular vacuoles form and fuse along connected endothelial cells (Blum

et al., 2008; Kamei *et al.*, 2006). Before expanding the vessel lumen, the stalk cells are initially lateral to the emerging tip cell. In these lateral base areas, endothelial cells provide soluble Flt-1/VEGFR1, which neutralizes VEGF to provide local guidance cues for the emerging sprout (Chappell *et al.*, 2009). The tip cell must eventually fuse with its target to establish a new branch, although the mechanism of endothelial cell fusion has yet to be elucidated. Observations from the developing mouse retina suggest that tip cell filopodia engage with those of a nearby tip cell to form a "bridge" and the foundation of a new vessel (Bentley *et al.*, 2009). This connection then develops a patent lumen so that blood can flow through the newly formed branch. During the transition from active sprouting to quiescence, endothelial cells adopt a "phalanx" phenotype that promotes vessel integrity and stabilizes the vasculature (Bautch, 2009; Mazzone *et al.*, 2009).

1.3. Vessel remodeling and stabilization

As the vasculature matures, vessel remodeling also contributes to network patterning. Changes in blood flow, metabolic demands, and growth factor secretion induce pruning of some vessels (Benjamin *et al.*, 1999). In contrast, other vessels become more stable through increased adhesion between endothelial cells and deposition of matrix and basement membrane (Stratman *et al.*, 2009). Hemodynamic stress and local molecular signals in the microenvironment regulate circumferential growth of vessels (Garcia-Cardena *et al.*, 2001; Masumura *et al.*, 2009; Skalak and Price, 1996; Zeng *et al.*, 2007). For example, skeletal muscle vessels exposed to elevated circumferential wall stress have increased diameters (Price and Skalak, 1996). Furthermore, mechanical and molecular factors also direct the recruitment and investment of mural cells such as pericytes and vascular smooth muscle cells (Armulik *et al.*, 2005; Gaengel *et al.*, 2009; Lindahl *et al.*, 1997). Platelet-derived growth factor (PDGF)-BB and transforming growth factor (TGF)-β promote vessel maturation by stimulating mural cell precursor migration and differentiation, respectively (Hirschi *et al.*, 1998). These stimuli work together with arteriovenous specification cues to facilitate proper development and enlargement of arteries and veins (Jones *et al.*, 2006).

Each phase of vascular development requires the integration of multiple signaling pathways to precisely coordinate cell behavior such that multicellular vessel networks form and expand. The following sections will describe how the VEGF, BMP, and Notch pathways provide this critical regulation for vascular development and morphogenesis, and how genetic mutations in components of these pathways contribute to human pathology.

2. VEGF in Vascular Development

The VEGF pathway is a vital regulator of vascular development and has been the subject of several comprehensive reviews (Ferrara *et al.*, 2003; Olsson *et al.*, 2006). We provide a brief characterization of this pathway, describe observations from genetic mutation experiments, and discuss human genetic disorders related to the pathway.

2.1. VEGF-A signaling pathway

VEGF-A is the predominant ligand for VEGF receptor 2 (VEGFR2, Flk-1 in mice), which positively signals for endothelial cell proliferation, migration, and survival (Shibuya and Claesson-Welsh, 2006). VEGF receptor 1 (VEGFR1, Flt-1 in mice), which has transmembrane and soluble isoforms due to alternative splicing, selectively binds VEGF-A, -B, and placental growth factor (Fischer *et al.*, 2008; Kendall and Thomas, 1993; Shibuya, 2006). Deletion of the intracellular signaling domain of Flt-1 is compatible with normal vascular development, indicating that signaling through Flt-1 is not required for its developmental role (Hiratsuka *et al.*, 1998). We showed that Flt-1 acts primarily as a ligand sink during vessel development, and it thus negatively modulates the amount of available VEGF-A that can bind and activate its receptor Flk-1 (Kappas *et al.*, 2008; Roberts *et al.*, 2004). This modulation negatively regulates endothelial proliferation but paradoxically positively regulates branching morphogenesis, and recent work from our laboratory illustrates a critical role for the soluble Flt-1 isoform in providing local sprout guidance cues necessary for proper vessel branching (Chappell *et al.*, 2009; Kearney *et al.*, 2002, 2004; Zeng *et al.*, 2007). The VEGF coreceptor Neuropilin (Nrp)-1 also provides regulation of the VEGF pathway (Larrivee *et al.*, 2009). Specifically, Nrp-1 enhances signaling by increasing Flk-1 affinity for VEGF-A (Whitaker *et al.*, 2001) and facilitating Flk-1 clustering (Soker *et al.*, 2002). This in turn promotes endothelial cell migration and guidance for proper vessel patterning (Gerhardt *et al.*, 2004; Jones *et al.*, 2008).

Spatial regulation of VEGF-A ligand availability is critical for coordinating its effects on vessel network formation. Alternative splicing of VEGF-A mRNA generates three primary isoforms (Tischer *et al.*, 1991), and the absence or presence of heparin-binding domains determines the affinity for the extracellular matrix and thus the spatial distribution of each isoform (Ruhrberg *et al.*, 2002; Stalmans *et al.*, 2002). Recent work suggests that matrix-bound and soluble VEGF-A isoforms provide distinct signaling cues to endothelial cells (Chen *et al.*, 2010). Endothelial cells themselves contribute to the spatial regulation of VEGF by responding differentially

to stimulatory cues to express VEGF receptors in a spatially heterogeneous manner, and Flk-1 receptor activation is also heterogeneous in vessel networks (Jakobsson *et al.*, 2009; Kappas *et al.*, 2008; Sainson *et al.*, 2008). Moreover, heterogeneity of soluble Flt-1 expression from endothelial cells is thought to be required for local sprout guidance (Chappell *et al.*, 2009). Thus, proper VEGF signaling, and in turn blood vessel morphogenesis, requires the appropriate spatial distribution of VEGF ligand and receptors and heterogeneous activation of VEGF-mediated signaling.

2.2. VEGF-A mutations

2.2.1. In development

Genetic mutations in the mouse VEGF signaling pathway show critical roles for both ligands and receptors. Heterozygous disruption of the *Vegf-A* gene is lethal at approximately embryonic day (E) 9.5 due to abnormal blood island formation, perturbed angiogenesis, and disruption of vessel organization and lumen formation, suggesting that strong quantitative regulation of the VEGF pathway is important for development (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Vascular-specific deletion of *Vegf-A* leads to endothelial apoptosis and postnatal lethality despite intact paracrine VEGF (Lee *et al.*, 2007), indicating that vessel-derived VEGF is required for vascular maintenance. Genetic deletion of *flt-1* causes overproliferation of endothelial cells but reduced branching that results in embryonic lethality at E9.5 (Fong *et al.*, 1995; Kearney *et al.*, 2002, 2004). The vessel dysmorphogenesis observed in the *flt-1*$^{-/-}$ loss-of-function situation is consistent with the model described above in which an effective gain of function for VEGF signaling results from perturbed *flt-1* function (Kappas *et al.*, 2008; Roberts *et al.*, 2004). Lack of *flk-1 (VEGFR2)*, the primary transducer of VEGF signaling, disrupts both vasculogenesis and angiogenesis, and *flk-1*$^{-/-}$ mice die *in utero* (E8.5–9.5) from lack of blood island and primitive vessel network development (Shalaby *et al.*, 1995). *Nrp-1* knockout mice suffer from developmental defects in both the nervous system and the vascular system, including impaired brain, spinal cord, and yolk sac vascularization, and endothelial specific deletion of *Nrp-1* leads to dilated and poorly branched vessels (Gu *et al.*, 2003; Kawasaki *et al.*, 1999). Additionally, the yolk sacs of mice with mutations in both *Nrp-1* and *-2* are avascular, and these mice exhibit severely disrupted embryonic angiogenesis (Takashima *et al.*, 2002). These co-receptors thus make important contributions to the precise regulation of endothelial responses to VEGF.

2.2.2. In human disease

How genetic defects in VEGF signaling influence human development is unclear. Because VEGF signaling is tightly regulated both quantitatively and

qualitatively in normal vessel development, gross disruptions in pathway components are likely to cause early fetal lethality in humans. Recent data, however, suggest that defective expression of VEGF pathway components contributes to several diseases. DiGeorge syndrome is a common genetic disorder (affecting 1 in 4000 infants) with multiple developmental abnormalities, including defects in blood vessel formation (Lindsay, 2001). Deletion of approximately 3 million base pairs in chromosome 22q11 (del22q11) results in haploinsufficiency of *tbx1*, but its variable expression suggests a role for additional modifiers. Stalmans and colleagues demonstrated interactions between Tbx1 and VEGF-A as well as an association between VEGF promoter haplotypes and cardiovascular birth defects in DiGeorge syndrome patients (Stalmans *et al.*, 2003). In addition, aberrant pharyngeal arch artery formation was exacerbated experimentally by VEGF knockdown in developing zebrafish with a concurrent tbx-1 knockdown, and regression of this artery was preceded by downregulation of Nrp-1, suggesting an increased risk for vascular complications in del22q11 patients with genetic lesions in VEGF pathway components (Stalmans *et al.*, 2003).

Genetic lesions in VEGF-A have also been associated with early-onset psoriasis, a chronic inflammatory disease with abnormal vessel expansion in the skin (Creamer *et al.*, 1997; Young *et al.*, 2004). Dermal microvessels within psoriatic plaques are tortuous, excessively dilated and permeable, and composed of hyperproliferative endothelial cells (Braverman and Sibley, 1982). Genetic analysis of psoriasis patients revealed the presence of single nucleotide polymorphisms in *Vegf-A* (Young *et al.*, 2004), and indeed elevated levels of VEGF-A protein are associated with this disease (Detmar *et al.*, 1994; Young *et al.*, 2004). Interestingly, the levels of circulating VEGFR1 were also higher in affected patients, which may represent hyperactivity of a feedback loop regulating VEGF-A availability or a compounding defect in VEGFR1 gene expression and activity.

Genes encoding the VEGF receptors have also been linked to vascular pathologies. In endothelial cells derived from infantile hemangiomas, a missense mutation in *Vegfr2* causes increased complex formation with tumor endothelial marker-8 (TEM-8) and β1-integrin (Boscolo and Bischoff, 2009; Jinnin *et al.*, 2008). The aberrant association of these molecules leads to decreased nuclear factor of activated T cells (NFAT) transcription of VEGFR1 and ultimately to focal regions of overgrown and disorganized cutaneous vessels. As stated previously, loss of VEGFR1 is essentially a gain of function for VEGF activity and results in elevated VEGF signaling and vessel dysmorphogenesis. Therefore, it follows that genetic mutations disrupting the tight regulation of VEGF signaling would result in vascular malformations. Nevertheless, more work remains to be done to uncover the exact mechanistic role for the VEGF signaling components in hemangioma formation and other disorders.

2.3. VEGF-C/VEGFR3 signaling pathway

Blood and lymphatic endothelial cells express VEGFR3 (Flt-4 in mice), a receptor for VEGF-C and -D (Saharinen *et al.*, 2004). During vascular development, higher *Vegfr3* expression in endothelial tip cells relative to neighboring stalk cells (Tammela *et al.*, 2008) indicates that this receptor also participates in the endothelial phenotypic heterogeneity that is necessary for proper network expansion. Expression of *Vegfr3* gradually becomes limited to the lymphatic endothelial cells, where it mediates VEGF-C signaling for lymphangiogenesis (Karpanen *et al.*, 2006b; Makinen *et al.*, 2001). Neuropilin-2, which interacts with VEGFR3, is also expressed by the lymphatic vessel endothelium and can bind VEGF-C (Karpanen *et al.*, 2006a; Xu *et al.*, 2010; Yuan *et al.*, 2002).

2.4. VEGF-C/VEGFR3 mutations

2.4.1. In development

Loss of *Vegf-C* results in impaired lymphatic vessel formation and abnormal fluid accumulation in various tissues (Karkkainen *et al.*, 2004). Cardiovascular failure occurs around E9.5 in *Vegfr3*$^{-/-}$ embryos, which also have vessel remodeling abnormalities and pericardial edema, highlighting a role for this receptor in vascular development in addition to its role in lymphangiogenesis (Dumont *et al.*, 1998). Genetic loss of *Nrp-2* impairs lymphatic endothelial cell proliferation as well as the development of capillaries and lymphatic microvessels (Yuan *et al.*, 2002). Given the importance of these VEGF pathway components in both angiogenesis and lymphangiogenesis, it is not surprising that developmental defects arise when their expression is disrupted.

2.4.2. In human disease

Genetic mutations in *Vegfr3* are linked to lymphedema in humans. Lymphedema arises from dilated lymphatic capillaries, which prevent adequate removal of lymph fluid from tissues (Ferrell, 2002). Congenital lymphedema in some families is associated with the *Vegfr3* locus on distal chromosome 5q (Cueni and Detmar, 2006). Moreover, missense mutations in the *Vegfr3* gene have been identified in patients with hereditary, early-onset lymphedema (Witte *et al.*, 2001). These genetic abnormalities impair the signaling activity of the VEGFR3 receptor (Alitalo and Carmeliet, 2002), thus leading to defective formation of lymphatic vessels and in turn excessive tissue fluid accumulation. Mutations in *Vegf-C* and *Nrp-2* have yet to be linked to human pathology, but establishing their contribution to lymphatic diseases could lead to improved therapies.

3. BMP in Vascular Development

BMPs are part of the TGF-β superfamily of signaling molecules, and the role of TGF-β family members in angiogenesis has been thoroughly reviewed (Holderfield and Hughes, 2008). The importance of BMP signaling in vascular development is increasingly apparent, although a global model to describe BMP effects on vessels is lacking (David *et al.*, 2009; Moreno-Miralles *et al.*, 2009). One effect of perturbing BMP signaling in vascular development is that distinctions between arteries and veins are often not maintained, leading to arteriovenous shunts, and an emerging theme is that BMP signaling has differential effects on arteries versus veins.

3.1. BMP signaling pathway

There are multiple components of BMP signaling that interact in complex ways during vascular development. Thus, it is quite likely that numerous unique combinations of BMP signaling components occur in different vessels, leading to context-dependent effects of BMP signaling on development and maintenance programs. In general, a Type II BMP receptor (i.e., BmprII or ActRII) and a Type I receptor (i.e., Alk 1/3/6) form heterodimers via ligand binding (i.e., BMP2, BMP4, BMP7) and also utilize a co-receptor (sometimes called a Type III receptor, i.e., endoglin) to initiate signaling. Signaling usually goes through transcription factors called SMADS (i.e., SMAD 1/5/8) that complex with a co-SMAD (SMAD 4) for translocation to the nucleus and modulation of transcription. However, BMP can also signal through MEK/ERK and p38 MAPK. BMP inhibitors such as noggin, chordin, gremlin, and BMP-binding endothelial cell precursor-derived regulator (BMPER) also modulate BMP signaling. In fact, the notochord in amniotes expresses BMP antagonists that promote the formation of an avascular zone at the embryonic midline (Bressan *et al.*, 2009; Reese *et al.*, 2004).

3.2. BMP–VEGF crosstalk

There is evidence that BMP stimulates VEGF expression and secretion in different cell types. Several studies showed that BMPs stimulate VEGF expression in osteoblast cells, and BMP-dependent enhanced secretion of VEGF was reported in retinal pigment epithelial cells (Deckers *et al.*, 2002; Kozawa *et al.*, 2001; Vogt *et al.*, 2006; Yeh and Lee, 1999). However, BMPs also induce endothelial migration, filopodia formation, and tube formation independent of VEGF activity, via activation of the transcription factor Id1 through SMADs or through ERK signaling (Valdimarsdottir

et al., 2002; Pi *et al.*, 2007). Moreover, there appear to be distinct requirements for VEGF vs. BMP signaling in blood vessel formation in zebrafish, as VEGF perturbations selectively affect the dorsal aorta and intersegmental vessels, while BMP perturbations selectively affect the axial vein and venous plexus immediately ventral to the aorta (D. M. Wiley, J. Hao, C. C. Hong, V. L. B. and S-W. Jin, submitted). These studies suggest that in some cases these pathways allow closely apposed vessels to expand networks in different directions.

3.3. BMP mutations

3.3.1. In development

Genetic evidence supports a role for BMP signaling in vascular development. Loss of the Type I receptor Alk 1 leads to lethality with angiogenesis defects in both mice and zebrafish (Oh *et al.*, 2000; Roman *et al.*, 2002; Urness *et al.*, 2000), while endothelial specific deletion of Alk 1 leads to arteriovenous malformations (Park *et al.*, 2006). Loss of the Type II receptor BMPRII is lethal early in development, and conditional loss in endothelial cells was associated with pulmonary arterial hypertension (PAH) later in life (Beppu *et al.*, 2004; Hong *et al.*, 2008). However, another group found that shRNA knockdown of the same receptor leads to vascular defects in multiple vascular beds, and signaling from isolated endothelial cells was attenuated, along with defects in smooth muscle investment (Liu *et al.*, 2007). Genetic deletion of co-receptors is lethal with effects on vessels. Loss of β-glycan leads to lack of coronary vessel development, while loss of endoglin results in both vascular and smooth muscle defects and arteriovenous malformations (Arthur *et al.*, 2000; Li *et al.*, 1999; Sorensen *et al.*, 2003). BMP ligands are proangiogenic by several criteria. BMP2, BMP4, and BMP6 stimulate endothelial cells in culture, and BMP-expressing beads stimulate angiogenesis in embryos (de Jesus Perez *et al.*, 2009; Nimmagadda *et al.*, 2005; Pi *et al.*, 2007; Ren *et al.*, 2007; Teichert-Kuliszewska *et al.*, 2006; Yang *et al.*, 2005).

3.3.2. In human disease

The strongest genetic evidence linking BMP to angiogenesis in humans is a set of mutations called hereditary hemorrhagic telangiectasia (HHT). These mutations are characterized by vessel malformations including dilated and fragile vessels and arteriovenous shunts (Abdalla and Letarte, 2006; Fernandez *et al.*, 2006). *Alk-1* and *endoglin* mutations primarily contribute to the development of HHT (Johnson *et al.*, 1996; McAllister *et al.*, 1994), while defective *BMPRII* and *Smad4* may contribute to disease progression and associated pulmonary artery disease (Abdalla *et al.*, 2004; Gallione *et al.*, 2004; Harrison *et al.*, 2003; Trembath *et al.*, 2001). PAH, a condition

characterized by a decrease in the small, distal pulmonary arteries and excessive muscularity of remaining arteries (Rabinovitch, 2008), is associated with mutations in *BMPR2* (Deng *et al.*, 2000; Lane *et al.*, 2000; Machado *et al.*, 2006). The penetrance of *BMPR2* mutations is only about 20%, suggesting that additional genetic lesions in BMP signaling may contribute to the phenotype (Morrell, 2006).

There is still much to learn regarding the role of BMP signaling in vascular development. The number of pathway components, the role of BMP in early development, and potential redundancy have all contributed to the lack of a consensus model to date. In this regard, a recent study in zebrafish provides strong evidence for context-dependent requirements for BMP in vascular development, as described above (Wiley *et al.*, submitted). The elucidation of vascular BMP signaling requirements in the relatively simple fish embryo may inform hypotheses regarding the role of BMP signaling in vessel formation in higher vertebrates.

4. Notch/Delta/Jagged in Vascular Development

The Notch signaling pathway is essential for vascular development. Recent evidence implicates Notch signaling as a critical integration node for the establishment and/or maintenance of endothelial heterogeneity within developing vessels and as a pathway that intersects and perhaps integrates input from numerous other pathways critical to vascular development. There are several recent and comprehensive reviews, so this review will highlight experimental manipulations of Notch pathway genes and vascular pathologies associated with Notch genetic mutations (Hofmann and Iruela-Arispe, 2007; Phng and Gerhardt, 2009; Roca and Adams, 2007; Siekmann *et al.*, 2008).

4.1. Notch/Delta/Jagged signaling pathway

Briefly, the four Notch receptors (Notch1–4) are transmembrane proteins that engage with five different ligands – Delta-like (Dll)1, Dll3, and Dll4, and Jagged-1 and -2. All but Notch 2 are expressed in developing vessels. Several other regulators are essential for proper Notch signaling, including γ-secretase, which cleaves the Notch intracellular domain (NICD) following receptor binding of a ligand. Subsequently, the NICD enters the nucleus and forms a complex that triggers target gene activation (i.e., Hey-1, Hey-2, Hes, Nrarp) (Fischer *et al.*, 2004; Phng *et al.*, 2009). The Notch signaling system provides a means for cell–cell communication, because the Notch receptors and ligands interact at the interface between

two neighboring cells (Bray, 2006). Thus, adjacent cells can establish distinctions between themselves and their neighbors, which is an important mechanism for establishing and maintaining phenotypic heterogeneity within a group of cells (Ghabrial and Krasnow, 2006).

The Notch pathway is implicated at several stages of blood vessel development, including endothelial cell differentiation and arteriovenous specification. In the developing zebrafish, Notch signaling in the nascent mesoderm acts as a cell-fate switch for the specification of endothelial and hematopoietic progenitor cells (Lee *et al.*, 2009). Elevated levels of Notch signaling in zebrafish embryos led to fewer endothelial cells, while reduced Notch activity increased their number at the expense of cells specified for the hematopoietic lineage. In addition, vascular smooth muscle cell differentiation, recruitment, and investment are perturbed by disruptions in the Notch pathway (Domenga *et al.*, 2004; High *et al.*, 2008; Kim *et al.*, 2008). Endothelial-specific deletion of *Jagged1* in mice, for instance, results in deficient smooth muscle cell differentiation, cardiovascular defects, and embryonic lethality (High *et al.*, 2008). The Notch pathway also plays a critical role in arteriovenous specification. Several Notch receptors and ligands are specifically expressed by arterial endothelial cells (Mailhos *et al.*, 2001; Shutter *et al.*, 2000; Villa *et al.*, 2001), suggesting the importance of the Notch pathway in establishing broader artery vs. vein specification within the developing vasculature (Lawson *et al.*, 2001; Siekmann and Lawson, 2007). Notch signaling is implicated in the transcriptional regulation of artery specification (Zhong *et al.*, 2000, 2001) via upregulation of artery-specific markers such as ephrinB2 (Grego-Bessa *et al.*, 2007; Iso *et al.*, 2006; Lawson *et al.*, 2001). The VEGF pathway intersects with Notch signaling to regulate arterial endothelial cell identification during development (Lawson *et al.*, 2002). The intersection of these pathways may also be important after the onset of blood flow to reinforce arterial identity and promote subsequent expansion of arteries (Masumura *et al.*, 2009). However, the exact molecular crosstalk between the pathways following exposure to blood flow remains unknown.

4.2. Notch coordination of endothelial crosstalk and heterogeneity

4.2.1. Notch–VEGF

It is becoming increasingly clear that endothelial cells have heterogeneous responses to angiogenic stimuli, and that this functional heterogeneity is important for proper blood vessel formation. These differences in signaling between adjacent endothelial cells are organized by the Notch pathway to establish the proper relationships between these cells, and their subsequent behaviors. During sprouting angiogenesis, for example, Notch receptor–

ligand interactions are proposed to establish phenotypic heterogeneity among vessel endothelial cells by critically regulating their responsiveness to VEGF stimulation. Local heterogeneity in VEGF responsiveness causes endothelial cells that experience relatively higher levels of VEGF signaling to increase Dll4 expression. Elevated Dll4 in turn further increases the cell's sensitivity to VEGF (via upregulation of VEGFR2 and VEGFR3), and this cell becomes the tip cell selected for outward migration from the parent vessel (Hellstrom *et al.*, 2007; Lobov *et al.*, 2007; Sainson *et al.*, 2005; Siekmann and Lawson, 2007; Suchting *et al.*, 2007; Tammela *et al.*, 2008). Notch receptors on neighboring stalk cells are engaged by the Dll4 ligands, and this activation of Notch signaling results in decreased VEGFR2 expression that is thought to inhibit sprouting from these cells (Suchting *et al.*, 2007). Evidence for interactions between the Notch pathway and VEGFR1 also exists (Funahashi *et al.*, 2010; Harrington *et al.*, 2008; J.C.C. and V.L.B., unpublished results; Suchting *et al.*, 2007; Taylor *et al.*, 2002). Thus, it is intriguing to speculate that VEGFR1 expression in lateral base cells is induced by Notch signaling to reduce VEGF ligand availability, preventing their outward migration and guiding the sprouting tip cell (Chappell *et al.*, 2009). Elevated expression of Jagged1 on stalk cells also contributes to endothelial phenotypic heterogeneity during angiogenesis (Benedito *et al.*, 2009). Stalk cell Jagged1 antagonizes Dll4 activity and thus reduces the induction of Notch signaling in the adjacent tip cell. The tip cell therefore maintains its responsiveness to VEGF stimulation and migrates outward to establish a new branch (Benedito *et al.*, 2009). Endothelial cell proliferation is also regulated by Notch (Hellstrom *et al.*, 2007; Leslie *et al.*, 2007; Lobov *et al.*, 2007; Siekmann and Lawson, 2007; Suchting *et al.*, 2007). However, it remains unclear how stalk cells, which experience elevated Notch signaling (i.e., suppression of proliferation) (Liu *et al.*, 2006; Noseda *et al.*, 2004), still undergo increased proliferation for vessel lengthening (Gerhardt *et al.*, 2003).

Although there is strong genetic evidence that Notch signaling and endothelial heterogeneity are important in vessel formation, the endothelial expression patterns of Notch pathway components do not fully follow the predictions of the models for establishing heterogeneity. This lack of concordance may reflect temporal oscillations in Notch signaling that are thought to be involved in establishing endothelial heterogeneity (Bentley *et al.*, 2009). Moreover, the mechanisms responsible for the initial endothelial heterogeneity that allows endothelial cells to respond differentially to comparable levels of angiogenic stimulation are still unknown.

4.2.2. Notch–BMP

In contrast to the extensive information regarding Notch–VEGF intersections, little is known about if and how Notch intersects with BMP signaling.

Both Notch and BMP stimulate expression of ER71, an Ets family transcription factor that stimulates formation of Flk-1-positive mesoderm, giving rise to both blood and vessel progenitors (Lee *et al.*, 2008). However, each pathway seems to independently regulate ER71 expression, since combined blockade is additive. There are a few examples of pathway intersections (Kluppel and Wrana, 2005). In myogenic cells, Smad1 activation downstream of BMP4 acts in both Notch-dependent and Notch-independent ways to activate Notch target genes such as Hey-1, and a physical interaction between Smad1 and the NICD was reported (Dahlqvist *et al.*, 2003). In endothelial cells, lack of cell contact (and Notch signaling) allowed BMP to activate Smad1 and upregulate Id1, which led to endothelial cell migration. Cell–cell contact activated Notch signaling and this synergized with BMP signals (via a Smad1–NICD complex) to activate Herp2, another Notch target that blocks endothelial migration (Itoh *et al.*, 2004).

4.3. Notch mutations

4.3.1. In development

Genetic manipulations of components in the Notch signaling system reveal the importance of this pathway for normal vascular development. However, because the Notch pathway is important in numerous embryonic tissues for developmental decisions, vascular-specific gene manipulations have been helpful for interpreting observations from the global genetic knockouts. Defects in vascular remodeling, and particularly of the large caliber vessels, arise from mutations in the *Notch1* gene (Krebs *et al.*, 2000). This same study showed that, while vascular development proceeds normally in *Notch4*$^{-/-}$ mice, *Notch1*$^{-/-}$ *Notch4*$^{-/-}$ double mutant embryos had more severe abnormalities in angiogenesis and in the formation of major vessels. Furthermore, endothelial-specific deletion of Notch1 recapitulates the vascular defects of the global Notch1 knockout mouse, suggesting an endothelial-intrinsic role for Notch1 in blood vessel formation (Limbourg *et al.*, 2005). Eye, heart, and kidney vessels in *Notch2* mutant mice exhibited dysmorphogenesis, but perinatal death was attributed to aberrant glomerular development in the kidney (McCright *et al.*, 2001). *Notch3*$^{-/-}$ mice are viable despite marked arterial defects resulting from impaired smooth muscle cell differentiation and maturation (Domenga *et al.*, 2004).

4.3.2. In human disease

Disrupted expression of Notch signaling components has been identified in congenital disorders that have distinct vascular pathologies. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, or CADASIL, results from inherited missense mutations in *Notch3* (Joutel *et al.*, 1996). Congruent with the arterial defects seen in *Notch3*$^{-/-}$ mice (Ruchoux *et al.*, 2003), CADASIL patients suffer from irregularities in dermal and cerebral arteries (Joutel and Tournier-Lasserve, 1998).

Degeneration of vascular smooth muscle cells and fibrotic accumulation around vessels contribute to the reduction in artery lumen diameter, resulting in migraines, dementia, and stroke (Chabriat *et al.*, 1995). Vascular abnormalities in experimental *Notch3* disruption are consistent with the clinically observed defects in CADASIL patients, but they do not fully explain the underlying pathogenesis. Thus, compounding genetic defects are likely to be involved, highlighting the importance of further investigations of the Notch pathway in human disease.

4.4. Delta mutations

4.4.1. In development

Dll1$^{+/-}$ mice survive to adulthood, and these mice show defects in postnatal arteriogenesis when challenged by an arterial occlusion (Limbourg *et al.*, 2007). By contrast, disruption of one allele of *Dll4* is lethal owing to irregularities in arterial branching, the specification of arterial endothelial cells, and vessel remodeling (Domenga *et al.*, 2004; Duarte *et al.*, 2004; Gale *et al.*, 2004). Analysis of heterozygous *Dll4*$^{+/-}$ embryos and early postnatal mice provided insight into some of these defects, which included increased angiogenic sprouting and excessive endothelial proliferation (Hellstrom *et al.*, 2007; Lobov *et al.*, 2007; Suchting *et al.*, 2007). Thus, lethality associated with *Dll4* haploinsufficiency demonstrates a strong dose-dependent regulation of vascular morphogenesis by Notch signaling, similar to that seen by VEGF signaling. Genetic disorders associated with *Dll4* mutations have yet to be identified, perhaps because misregulation of this gene is likely to be embryonic lethal owing to aberrant vascular development.

4.5. Jagged mutations

4.5.1. In development

Genetic loss of *Jagged1* does not compromise formation of the primary vascular networks, but remodeling of these vessels in the yolk sac and embryo is perturbed and leads to lethality at E10 (Xue *et al.*, 1999). Recent observations of mice with endothelial-specific manipulations of *Jagged1* suggest that deficient remodeling may arise from loss of both Jagged1-regulated angiogenesis and defective vascular smooth muscle cell differentiation (Benedito *et al.*, 2009; High *et al.*, 2008). As described previously, Jagged1 is proposed to antagonize Dll4 signaling and may activate intracellular signaling events within the ligand-presenting cell itself; and therefore, loss of *Jagged1* likely leads to aberrant Notch pathway activation in both the endothelial and smooth muscle cell compartments.

4.5.2. In human disease

Alagille syndrome (AGS) is a hereditary disorder arising from mutations in *Jagged1* (Li *et al.*, 1997; Oda *et al.*, 1997). Haploinsufficiency or truncations in *Jagged1* contribute to frequently observed abnormalities in blood vessel formation, including the narrowing of the aorta (coarctation) and other major arteries (stenosis) (Kamath *et al.*, 2004). *Notch2* mutations may also exacerbate the AGS phenotype. *Jagged1* heterozygous mice carrying a *Notch2* hypomorphic allele displayed several developmental defects consistent with AGS (McCright *et al.*, 2002), although significant vascular abnormalities were not reported. Nevertheless, mutations in *Notch2* have been found in AGS patients who lack *Jagged1* mutations (McDaniell *et al.*, 2006). Experimental evidence for the role of Jagged1 in blood vessel development coincides with the observed vascular deformities in AGS, and mutations in other Notch components such as *Notch2* have been implicated as modifiers of *Jagged1* perturbations. Further investigation will be necessary to determine which Notch signaling molecules contribute to and exacerbate the pathogenesis of this disorder.

5. Perspectives

The significant crosstalk among signaling pathways involved in vascular development is increasingly evident (Holderfield and Hughes, 2008; Jakobsson *et al.*, 2009). However, despite the defined vascular pathologies associated with the individual pathways described above, there is surprisingly little genetic evidence for pathway crosstalk in human pathogenesis. If signaling intersections are indeed important aspects of human disease, further understanding the signaling crosstalk among these pathways may enhance treatment for certain vascular defects and conditions.

It is likely that rigorous dissection of both the pathways and the crosstalk among the pathways in vascular development will benefit from computational modeling approaches that supplement the experimental data derived from model systems (Bentley *et al.*, 2008; Mac Gabhann and Popel, 2008; Peirce, 2008). For example, one important question is how these pathways initiate and maintain phenotypic heterogeneity in endothelial cells during blood vessel formation. Appropriate computational models will allow us to input different pathway relationships and simulate how these relationships affect endothelial heterogeneity and vessel morphogenesis.

Another important future goal in the field is to combine high-resolution imaging with signaling readouts to better understand how information conveyed by signaling pathways is organized both spatially and temporally. This review highlights the importance of spatial

organization of information in developing vessel networks; yet we have little information regarding the dynamic temporal regulation of regional signaling that likely overlies the static images produced so far. The use of reporter genes that reflect pathway activity at the single cell level, along with fluorescence resonance energy transfer (FRET)-based tools to evaluate protein interactions stimulated by signaling pathways, will allow us to add temporal information to our understanding of vascular development. For example, a recent study followed PI3 kinase activation in individual migrating zebrafish neutrophils and used a photoactivatable Rac to show that Rac activation is sufficient to direct neutrophil migration *in vivo* (Yoo *et al.*, 2010). It will be exciting to apply these innovative approaches to outstanding questions in vascular development.

Finally, it will be informative to extend the concept of endothelial heterogeneity and pathway crosstalk beyond current models. For example, extrinsic angiogenic factors are often not set up in obvious gradients (Czirok *et al.*, 2008; Damert *et al.*, 2002; Kearney and Bautch, 2003; Keller, 2005), and so vessel-intrinsic heterogeneity may be important for proper vessel patterning in numerous scenarios. We recently showed that soluble Flt-1 released from cells adjacent to emerging sprouts modulates local VEGF distribution for proper sprout guidance away from the parent vessel (Chappell *et al.*, 2009). Notch signaling, which can be induced by VEGF signaling, regulates Flt-1 expression (Harrington *et al.*, 2008; Suchting *et al.*, 2007) and may represent a means for modulating numerous signals within the local microenvironment. Other pathways have negative regulators that may exhibit vessel-intrinsic phenotypes. For example, the BMP antagonist BMPER is expressed in some endothelial cells (Moser *et al.*, 2003) and may provide local cues to modulate BMP signaling during vessel development.

Endothelial heterogeneity is potentially relevant in maturing regions of the vasculature as well. Mazzone *et al.* recently demonstrated the importance of the endothelial "phalanx" phenotype in promoting vessel quiescence through increased cell adhesions and dampened response to VEGF (Mazzone *et al.*, 2009). Phenotypic heterogeneity likely exists in these regions of the endothelium such that recruited pericytes interact with more quiescent endothelial cells, forming localized contacts that enhance vessel stability (Armulik *et al.*, 2005; Gaengel *et al.*, 2009; Gerhardt and Betsholtz, 2003; Jain and Booth, 2003). Thus, the signaling pathways described in this review likely influence endothelial heterogeneity in both actively sprouting and remodeling regions of developing vessels.

In summary, extrinsic cues are likely complemented by endothelial cell signaling networks that establish an integrated and heterogeneous response to angiogenic stimuli. Some vascular pathologies likely arise from genetic mutations that perturb the normal establishment and maintenance of endothelial heterogeneity in vascular development and remodeling.

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