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Blood Vessel Patterning at the Embryonic Midline

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The reproducible pattern of blood vessels formed in vertebrate embryos has been described extensively, but only recently have we obtained the genetic and molecular tools to address the mechanisms underlying these processes. This review describes our current knowledge regarding vascular patterning around the vertebrate midline and presents data derived from frogs, zebrafish, avians, and mice. The embryonic structures implicated in midline vascular patterning, the hypochord, endoderm, notochord, and neural tube, are discussed. Moreover, several molecular signaling pathways implicated in vascular pattering, VEGF, Tie/tek, Notch, Eph/ephrin, and Semaphorin, are described. Data showing that VEGF is critical to patterning the dorsal aorta in frogs and zebrafish, and to patterning the vascular plexus that forms around the neural tube in amniotes, is presented. A more complete knowledge of vascular patterning is likely to come from

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the next generation of experiments using ever more sophisticated tools, and these results promise to directly impact on clinically important issues such as forming new vessels in the human body and/or in bioreactors.

I. Introduction

Developmental vascular biology presents an interesting paradox. Blood vessels are easy to see because of the blood within. In 1672, Marcello Malpighi first described that blood coursed through specific tubes in chick embryos (Gilbert, 2003), and much subsequent embryology described the development and elaboration of blood vessels. These early studies reached an apex with the publication of careful, descriptive studies of blood vessel formation by Herbert Evans, E. R. Clark, and Florence Sabin (Clark, 1918; Evans, 1909; Sabin, 1917, 1920). Yet until very recently, developmental blood vessel formation has been understudied relative to other developmental processes such as limb formation and neural development. This was partially due to the ubiquitous presence of blood vessels in almost all tissues, which prevented extensive molecular analysis until these studies could be carried out at the single-cell level. Vascular pattern formation has been even more refractory to mechanistic analysis, even though these patterns have been described for hundreds of years. However, the recent surge in interest in vascular patterning has resulted in much new information and models for further testing. Moreover, beyond the basic developmental questions are applications to diseases and therapies that also motivate investigations of vascular pattern formation. For example, if we understand how the embryo coordinates the pattern of vessels with the development of other organs and tissues, we may be able to apply this information to the reconstruction of functional vasculature in the adult or even in an artificial setting. It is an exciting time to work in this field. The genetic and analytic tools are available and the questions are compelling.

II. Vascular Development and Patterning

A. Overview

The embryonic vasculature is formed via the coordination of multiple cellular processes. These include the specification of mesodermal precursor cells called angioblasts, their differentiation into endothelial cells, and the migration and assembly of angioblasts and endothelial cells into vessels (reviews: Cleaver and Krieg, 1999; Daniel and Abrahamson, 2000; Drake and Little,

1999; Jain, 2003; Folkman, 2003; Risau, 1997; Poole *et al*., 2001; Weinstein, 2002; Yancopoulos *et al*., 2000). These processes must be synchronized within the vascular lineage, and they must also interface with the developmental programs of other embryonic lineages. This coordination is called vascular patterning, and it results in a primary vessel network that is reproducible in both time and space. Signals produced by other tissues impinge on angioblasts and endothelial cells to pattern the embryonic vasculature (Coffin and Poole, 1991; Noden, 1988; Poole and Coffin, 1989). However, vascular patterning signals have been identified only recently, and the list is very incomplete. Moreover, little is known about where and how these signals act to pattern vessels.

This review combines information from zebrafish, frogs, avians, and mice and focuses on the migration and assembly of vessels guided by axial structures that straddle the embryonic midline. We describe the molecular signaling pathways implicated in vascular patterning, then describe the evidence for involvement of specific axial structures in vessel patterning around the midline: the hypochord, endoderm, notochord, and neural tube. Due to space constraints, we will not discuss in detail the short-range patterning of vessels that occurs in the limb and retina (Mukouyama *et al*., 2002; Otani *et al*., 2002; Stone *et al*., 1995; Zhang *et al*., 1999). Likewise, other interesting aspects of patterning, such as how endothelial cells signal to tissues (reviewed in Cleaver and Melton, 2003) and arterial/venous differentiation (reviewed in Lawson and Weinstein, 2002a), are the subject of recent excellent reviews and will not be covered in detail here.

B. Blood Vessel Formation

Blood vessels in the embryo form through a combination of two developmental processes—vasculogenesis and angiogenesis. The coalescence and differentiation of mesodermal precursor cells to form vessels de novo is termed vasculogenesis. Angiogenesis involves the migration and division of already differentiated endothelial cells to form new vessels. The work of several labs, including ours, demonstrates that many vessels in the embryo form by a combination of both processes (Ambler *et al*., 2001; Brand-Saberi *et al*., 1995; Childs *et al*., 2002; Feinberg and Nolden, 1991). Historically, both normal and pathological neovascularization were thought to occur solely by angiogenic processes in the adult. However, recent studies show that bone-marrow-derived circulating endothelial cells contribute to adult neoangiogenesis, suggesting that both vasculogenesis and angiogenesis occur throughout the life of an organism (Asahara *et al*., 1997, 1999; Otani *et al*., 2002).

C. Blood Vessel Patterning

Early descriptive studies of blood vessel patterning were based on live observations of vessel development in chick embryos and in the tails of frog tadpoles, complemented by analysis of ink injections and histological sections of embryos at different developmental stages (Clark, 1918; Evans, 1909; Sabin, 1917, 1920). These early studies were built on by the Clarks, who elegantly described vessel formation and remodeling in frog tails by examining living specimens (Clark and Clark, 1939). Subsequently, several groups carefully described vessel formation and patterning in the developing quail using a vascular cell-specific antibody (Coffin and Poole, 1988; Pardanaud *et al.*, 1987). These studies in aggregate led to a model of blood vessel patterning in which an early primitive vascular plexus is first formed by either vasculogenesis or angiogenesis at the site of a future vessel or vessel bed, and it is then extensively remodeled to form the final vascular pattern (review: Drake *et al.*, 1998). Experimental analysis of the migratory behavior and origin of angioblasts was then carried out by several groups using quail transplants in chick hosts (Noden, 1989; Pardanaud *et al.*, 1989; Poole and Coffin, 1989). Noden determined that most mesodermal tissues, with the exception of the prechordal plate, contain cells with angiogenic potential that can migrate large distances to form vessels in the embryo (Noden, 1989). It is important that Noden as well as and Poole and Coffin, observed that angioblasts that normally form vessels in the trunk were able to participate in the formation of blood vessels unique and appropriate to the head (Noden, 1989; Poole and Coffin, 1989). These observations continue to influence the way we think about vascular patterning, and subsequent work supports a model in which the pattern of blood vessels is guided by environmental cues rather than intrinsic to endothelial cells or their progenitors. The identification of an embryonic structure as the source of a vascular patterning signal came from Cleaver and Krieg, who first showed that the vertebrate midline produced a vascular patterning signal for dorsal aorta formation and suggested that hypochord-derived VEGF was important for this signal (Cleaver and Krieg, 1998; Cleaver *et al.*, 1997). These landmark studies have set up important questions in vascular patterning that are currently under investigation, such as: What are additional embryonic sources of vascular patterning cues? What is the molecular composition of the signals that emanate from these sources? How are these signals coordinated with ongoing development and patterning in the rest of the embryo?

D. Vascular Patterning Information from Different Model Organisms

An interesting evolutionary question is: When did blood vessels first arise? A bona fide vasculature is associated with vertebrates, although many

productive analogies have been made between vertebrate blood vessel formation and *Drosophila* tracheal development (Ghabrial *et al*., 2003). Current models of embryonic vascular patterning rely on information derived from several different vertebrate model organisms, and each of these different model organisms has provided unique opportunities to dissect aspects of vascular patterning.

1. *Xenopus laevis* (tropical frog)

Blood and lymphatic vessels in frog tails were some of the earliest specimens to be analyzed by live observation. The modern advantages of this well-used embryological model are the large size and free-living status of the early embryo. This allows for molecular manipulation via injection of individual early cells with RNAs that misexpress proteins or morpholinos that block expression. Unfortunately, significant vascular patterning occurs later in development when molecular manipulation of the *Xenopus* embryo is more difficult. Moreover, there are few markers for *Xenopus* vascular cells. However, the first studies defining midline vascular patterning signals were carried out by Cleaver and Krieg in the *Xenopus* embryo (Cleaver and Krieg, 1998; Cleaver *et al*., 1997). Recently, vascular development in *Xenopus* was documented by visualization of vessels using injection of DiI-Ac-LDL, a compound that selectively binds to endothelial cells in many organisms (Levine *et al*., 2003).

2. Zebrafish (*Danio rerio*)

There are several advantages to this rather new model of vertebrate development. The embryo is transparent and free-living, facilitating the acquisition of descriptive information. The relatively short life cycle and small size permit forward genetic screens to uncover novel genes important in vascular development and patterning. The recent finding that morpholino injection can provide information on the phenotypic consequences of reduced function of specific genes has added an important tool to analysis of zebrafish development. These advantages have been exploited by a number of investigators to study aspects of vascular patterning, such as arterial-venous differentiation (Lawson *et al*., 2001, 2002; Zhong *et al*., 2001) and sprouting of intersomitic vessels (Childs *et al*., 2002). Moreover, analysis of the *gridlock* mutation in zebrafish showed that only vessels in specific parts of the embryo were compromised (Weinstein *et al*., 1995; Zhong *et al*., 2000), suggesting that local cues from surrounding tissues pattern vessels and supporting the work of Noden (described in Section II.C). The midline structures of the zebrafish embryo are well characterized and amenable to disruption by mutations. Recently, Weinstein and colleagues injected vessels

of zebrafish embryos with fluorescent microspheres (microangiography), and they subsequently generated transgenic zebrafish that express green fluorescent protein (GFP) in the developing vasculature. They then used state-of-the-art live imaging to describe vascular development in the zebrafish embryo (Isogai *et al.*, 2001; Lawson and Weinstein, 2002b). These studies have significantly increased our understanding of vessel development and patterning and promise to further our knowledge even more in the future.

3. Avians (chick and quail)

The avian embryo has historically had a central place in investigations of vascular patterning. Early investigators used the chick embryo for live observation and ink injections, so by 1920 it was the best-described embryological model of vascular development. Subsequently, the accessibility of the avian embryo was exploited for surgical manipulations, and the observation that quail cells could be distinguished from chick cells by nuclear morphology was used in early graft experiments to follow angioblast migration and patterning. An important refinement was development of the QH1 antibody that recognizes an epitope specific to quail endothelial cells and progenitors, but does not recognize chick endothelial cells (Pardanaud *et al.*, 1987). A series of elegant studies analyzed quail grafts placed into chick hosts and provided a rich source of data that led to our current model of vascular patterning around the avian midline (Klessinger and Christ, 1996; Pardanaud and Dieterlen-Lievre, 1995; Pardanaud *et al.*, 1996; Wilting *et al.*, 1995). Others have used the quail to experimentally manipulate molecules implicated in vessel formation and patterning such as VEGF, bFGF, and integrins (Cox and Poole, 2001; Drake and Little, 1995; Drake *et al.*, 2000; Finkelstein and Poole, 2003). Recently, Little and colleagues have devised protocols for dynamic image analysis of vessel development and patterning in the avian embryo, and analytical tools for quantitative assessments of vessel behavior (Rupp *et al.*, 2003).

4. Mouse

The mouse is the least tractable model for visual and surgical manipulations, but in contrast, both gain- and loss-of-function genetic experiments in the mouse have shed light on genetic pathways important in vessel development and patterning in mammals. The earliest manipulations were of the VEGF and Tie/tek signaling pathways, and both are clearly crucial to vessel formation (Carmeliet *et al.*, 1996; Dumont *et al.*, 1994; Ferrara *et al.*, 1996; Fong *et al.*, 1995; Sato *et al.*, 1995; Shalaby *et al.*, 1995). However, the early and profound effects of mutations in these and a number of other pathways have somewhat hindered analysis of effects on vascular patterning in mammals.

The recent explosion in analysis of mutations of genes in specific cell types of the mouse by tissue-specific excision using the Cre-lox system promises to circumvent this problem and lead to a more sophisticated understanding of vascular patterning in mammals. Our group has attempted to utilize the advantages of both avian and mouse models by analyzing mouse grafts placed into avian hosts (Ambler *et al*., 2001, 2003; Hogan *et al*., 2004). This work will be described in more detail in Section IV.D.

The analysis of vascular patterning events and mechanisms in the models described above has been useful in revealing universal aspects of vascular patterning while defining differences among different organisms. Examples of universal features of vascular pattern formation are: (1) the use of both vasculogenesis and angiogenesis to form the embryonic vasculature; (2) the midline aggregation of vascular cells to form the dorsal aorta; and (3) the importance of the VEGF signaling pathway in vascular development. Examples of differences are: (1) the formation of large vessels by remodeling of an initial plexus in amniotes versus formation of the large vessels de novo in zebrafish and (2) formation of the dorsal aorta via signals from the hypochord, a VEGF-secreting structure at the midline of frogs and zebrafish but lacking in avians and mammals.

III. Signaling Pathways Implicated in Vascular Patterning

A. Overview

There are numerous signaling pathways thought to be important in aspects of vascular patterning. This has been one of the most exciting areas of research recently; the genetic and molecular tools available have permitted not only the testing of hypotheses about specific pathways, but the identification of mutations that affect patterning. Here we describe only the subset of pathways for which evidence exists for a role in vessel patterning. We do not discuss in detail interesting mutations, such as *out-of-bounds,* that have not yet been cloned. We also do not describe important signaling pathways whose primary effect is thought to be at the level of vessel stability and remodeling, such as the SIP/EDG, TGF beta superfamily, and PDGF pathways.

B. Signaling Pathways Implicated in Vascular Patterning

1. VEGF (Vascular endothelial growth factor)

The VEGF family of ligands and their receptors play a central role in many aspects of blood vessel formation, including vascular patterning. The

preponderance of evidence for effects on vascular patterning is restricted to the most studied family member, VEGF-A. Thus we will discuss the role of VEGF-A (VEGF) and its receptors flk-1 (VEGFR-2) and flt-1 (VEGFR-1) in vascular patterning. An interacting set of coreceptors, neuropilin-1 and neuropilin-2, will be covered in Section III.B.5, as they were initially identified as Semaphorin receptors.

VEGF-A is a multi-functional protein involved in differentiation, proliferation, and migration of endothelial cells (reviews: Cross *et al.*, 2003; Ferrara *et al.*, 2003). It is expressed early in development in vertebrates, and its expression coincides temporally and spatially with blood vessel formation at numerous embryonic sites (Cleaver *et al.*, 1997; Dumont *et al.*, 1995; Flamme *et al.*, 1995; Miquerol *et al.*, 1999). A requirement for VEGF in vascular development is demonstrated by the paucity of vasculature and the embryonic lethality when one VEGF-A allele is deleted in mice, and the almost complete lack of vessels in embryos and embryonic stem cells lacking VEGF-A (Bautch *et al.*, 2000; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). In zebrafish, morpholino knockdown of VEGF indicates that the initial establishment of axial vasculature does not require VEGF-A, although it is required for patterning intersegmental vessels (Nasevicius *et al.*, 2000). In addition, modest increases or decreases in VEGF-A levels in mice also disrupt vessel development and lead to embryonic lethality (Damert *et al.*, 2002; Miquerol *et al.*, 2000), indicating that tight dosage control of the VEGF-A signal is important.

However, as mentioned in Section II.D.4, the pleiotropic effects of VEGF-A on vascular development have impeded a direct assessment of its role in vascular patterning until quite recently (see Section IV.A, D). Gain-of-function experiments indicate that VEGF-A is involved in patterning, since injection of VEGF or placement of VEGF-coated beads into avian embryos results in ectopic and mis-patterned vessels (Bates *et al.*, 2003; Drake and Little, 1995; Drake *et al.*, 2000; Flamme *et al.*, 1995; Finkelstein and Poole, 2003). VEGF-A RNA is alternatively spliced to produce three major isoforms of 120, 164, and 188 amino acids (Park *et al.*, 1993). These isoforms have different biochemical properties, suggesting that they may be differentially deposited in the embryo: VEGF120 is predicted to be freely diffusible; VEGF188 is predicted to be matrix bound; and VEGF164 is predicted to be intermediate in these properties. Mice that are genetically engineered to express only VEGF120 or VEGF188 have consistent differences in the caliber and branching of vessels, suggesting that isoform composition affects local assembly and patterning of the vascular plexus (Carmeliet *et al.*, 1999; Gerhardt *et al.*, 2003; Ng *et al.*, 2001; Ruhrberg *et al.*, 2002; Stalmans *et al.*, 2002).

The VEGF receptor tyrosine kinases, flk-1 and flt-1, both bind VEGF-A with high affinity. VEGF-A binding to flk-1 induces receptor tyrosine phosphorylation, and endothelial cells respond with downstream signaling

that leads to proliferation, migration, survival, and permeability changes (reviews: Cross *et al*., 2003; Ferrara *et al*., 2003). Deletion of *flk-1* in mice or embryonic stem cells is embryonic lethal with lack of organized blood vessels (Schuh *et al*., 1999; Shalaby *et al*., 1995, 1997). The similarity between the loss-of-function phenotypes for VEGF-A and flk-1 suggest that most VEGF-A signaling is mediated by the flk-1 receptor. Evidence that signaling through the flk-1 receptor is important in vascular patterning around the amniote axial midline comes from analysis of embryonic stem-cell-derived embryoid bodies placed into the presomitic mesoderm cavity of quail hosts (Ambler *et al*., 2003). Although wild-type angioblasts migrated and patterned properly in this model, angioblasts genetically deleted for *flk-1* did not respond to avian patterning cues to migrate to specific embryonic locations. Moreover, in zebrafish, a *flk-1* mutation that severely down regulates flk mRNA did not disrupt initial vasculogenesis, but prevented sprouting of intersomitic and other vessels (Habeck *et al*., 2002). Taken together, these results strongly indicate that VEGF signaling through flk-1 mediates vascular patterning around the embryonic midline.

The role of the flt-1 receptor in vascular development and patterning appears more complex, and it has been somewhat controversial. Flt-1 is clearly necessary for proper vascular development, since deletion of *flt-1* leads to vessel overgrowth and embryonic lethality (Fong *et al*., 1995). It was subsequently suggested that flt-1 normally modulates the cell fate decision that induces hemangioblasts, progenitor cells capable of giving rise to both hematopoietic and endothelial cells, to form from mesoderm (Fong *et al*., 1999). However, corroboration of that model has been lacking. Recently, we showed that vascular cells lacking flt-1 have a higher rate of cell division than wild-type controls, suggesting that flt-1 normally negatively modulates cell division (Kearney *et al*., 2002). This is consistent with a model of flt-1 action in which its ability to act as a sink for the VEGF-A ligand is important developmentally, likely through a soluble form of the receptor that is generated via alternative splicing (Kendall and Thomas, 1993; Kendall *et al*., 1996). However, these basic questions regarding the cellular phenotype and mechanism of flt-1 action have precluded extensive analysis of its role in vascular patterning. We recently used dynamic image analysis of embryonic stem-cell-derived vessels to show that flt-1 is a positive modulator of vascular sprout formation and branching; that is to say, in the absence of flt-1, the vascular plexus is less branched (Kearney *et al*., 2004). The defect of the *flt-1* mutants could be largely rescued with a transgene that only expressed the soluble form of flt-1, suggesting that the morphogenetic effect is largely mediated by soluble flt-1. These findings suggest that flt-1 does affect vascular patterning at the local level. It will be interesting to determine if flt-1 is also involved in modulation of midline vascular patterning mediated by VEGF-A.

2. Tie/Tek Pathway

Tie2 (also called tek) and Tie1 (also called Tie) are receptor tyrosine kinases expressed on endothelial cells during development (reviews: Loughna and Sato 2001a; Ward and Dumont 2002). Angiopoietins (Ang) are the ligands for Tie2, while the Tie1 ligand(s) is unknown. Deletion of *Tie2* in mice is embryonic lethal at midgestation, with extensive vascular defects that include decreased sprouting, simplified vessel branching, and lack of pericyte recruitment (Dumont *et al*., 1994; Patan, 1998; Puri *et al*., 1999; Sato *et al*., 1995). *Tie1* mutant mice also die as embryos, but at later stages and with reduced vessel integrity (Puri *et al*., 1995; Sato *et al*., 1995). Deletion of *Ang1* is also embryonic lethal, but the vasculature is less affected than in embryos lacking Tie2, suggesting that other ligands for Tie2 exist *in vivo* (Suri *et al*., 1996). Ang2 has complex effects on vessel development that appear to be context dependent. That is to say, Ang2 can act as either an agonist or an antagonist of Tie2 signaling in different situations. Although most evidence suggests that Tie/tek signaling is involved in local patterning and remodeling, an interesting study suggests that it may impinge on global patterning. Loughna and Sato generated embryos that were deleted for both Ang1 and Tie1, the receptor without identified ligands (Loughna and Sato, 2001b). These double knockout mice had disruption of the venous system only on the righthand side of the embryo. This phenotype correlated with the expression of Ang1 on the right side veins at this time, although Tie1 is more generally expressed in vessels. These findings suggest that these pathways intersect to specify veins in a particular location of the embryo.

3. Notch Pathway

Notch proteins are large trans-membrane receptors that are important to a variety of developmental processes, including blood vessel formation (review: Iso *et al*., 2003). Their ligands, Delta-like and Jagged proteins, are also largely membrane localized, leading to models of Notch signaling that involve neighboring cell types. Mice have four *Notch* genes, and deletion of *Notch1* or hypomorphism for *Notch2* in mice results in complex phenotypes with vascular abnormalities (McCright *et al*., 2001; Swiatek *et al*., 1994). Deletion of *Notch4* produces normal mice, but *Notch1–Notch4* double mutants have more severe vascular defects than *Notch1* mutant mice, suggesting functional overlap among Notch family members (Krebs *et al*., 2000). Further evidence that Notch signaling is important in vascular patterning comes from analysis of transgenic mice that express an activated Notch4 in the vasculature (Uyttendaele *et al*., 2001). The mutant embryos have numerous vascular patterning defects, including disorganized vessel networks and fewer smaller vessels. Mutations in *Notch3* in humans lead to a vascular defect associated with adult stroke and dementia called

CADASIL (Joutel *et al.*, 1996; Salloway and Hong, 1998). Deletion of Notch ligands *Jag1* or *Dll1* in mice results in vascular defects and hemorrhage in the head and yolk sac and further supports a role for the Notch pathway in vessel patterning (Hrabe de Angelis *et al.*, 1997; Xue *et al.*, 1999). Thus far, the phenotypes generated in mice via targeted mutations are complex, and it is not completely clear what role Notch signaling plays in vascular patterning, although vessel morphogenesis appears to be affected by some genetic manipulations. It will be interesting to determine the phenotypes of Notch mutations localized to the vasculature.

Notch signaling is also important for arterial differentiation in zebrafish. Embryos lacking Notch activity fail to express artery-specific markers in the dorsal aorta, and mutation of a downstream target of Notch signaling, *gridlock*, results in defective dorsal aorta patterning (Lawson *et al.*, 2001; Weinstein *et al.*, 1995; Zhong *et al.*, 2000, 2001). Further studies suggest a signaling cascade in which Shh can activate VEGF, which can in turn activate Notch signaling and arterial differentiation (Lawson *et al.*, 2002). The zebrafish studies suggest that one role for Notch signaling in vascular development is to control cell fate decisions, a model consistent with how Notch signaling affects other developmental processes. However, the data in mice also suggest that Notch signaling is important, at least in vessel morphology, and it may be important in how neighboring tissues pattern vessels locally.

4. Ephrins/Eph Receptor Pathway

Eph receptor tyrosine kinases and their membrane-bound ligands, ephrins, are required for the proper placement of tissues developmentally. For example, neural crest cells that emanate from the neural tube migrate from the hindbrain in specific stripes that are defined by ephrin/Eph expression patterns (reviews: Adams, 2002; Himanen and Nikolov, 2003). In mice, ephrin-B2 is expressed by arterial endothelial cells, and its receptor, EphB4, is expressed predominantly on veins (Adams *et al.*, 1999; Gerety *et al.*, 1999; Wang *et al.*, 1998). Deletion of either gene in mice results in defective angiogenic remodeling in veins and arteries of the yolk sac and head, with mid-gestational embryonic lethality. These data demonstrate a genetic component to the distinction between arteries and veins and suggest that disruption of the arterial/venous boundaries have severe consequences for vascular patterning. In many cases, cells that express Eph receptors and those that express ephrins are prevented from mixing, and endothelial cells clearly use this pathway (and likely others as well) to distinguish arteries from veins (review: Wilkinson, 2000). Although intersomitic vessels normally do not enter somites, the intersomitic vessels of the *ephrin-B2* null embryos often invade somites (Adams *et al.*, 1999). The role of ephrins and their receptors

in vascular development is likely to be complex, as other family members such as EphB3 and ephrin-B1 are also expressed in the vasculature. Moreover, some family members, such as EphB2, are also expressed in the mesenchyme and likely function in endothelial–mesenchymal cell signaling, which could affect vascular patterning (Adams *et al*., 1999). Gene targeting of ephrins in the vascular compartment will likely resolve some of these issues. However, current evidence clearly indicates that vessel patterns as well as vessel identity are influenced by ephrin-Eph receptor interactions, and further studies will likely uncover more important specific roles for these signals in vascular patterning.

5. Semaphorin/Neuropilin/Plexin Pathway

Semaphorins are another group of signaling molecules that have recently been implicated in the restriction of angioblast/endothelial cell migration. Semaphorins were first identified as guidance molecules for neurons, and the family includes both secreted and transmembrane signaling proteins (reviews: Goshima *et al*., 2002; Kolodkin, 1998; Tessier-Lavigne and Goodman 1996). Semaphorins bind to two different transmembrane-receptor families, neuropilin (NP) and plexin. It is now thought that NPs provide a ligand binding site and plexin proteins play a signaling role (Goshima *et al*., 2002). It is interesting that Sema3A binds NP1, which is also a coreceptor for the 165 isoform of VEGF-A expressed on endothelial cells (Soker *et al*., 1998). *In Vitro*, NP1/VEGF-A interactions appear to strengthen the ability of VEGF-A to promote chemotaxis of endothelial cells (Soker *et al*., 1998). In contrast, NP1/Sema3A interactions inhibit the motility of endothelial cells expressing NP1 (Miao *et al*., 1999). These findings suggest that differential or competitive binding of Sema3A or VEGF-A165 to NP1 might be involved in modulation of vascular patterning mediated by VEGF-A. NP2 is a second co-receptor that binds Sema3F with high affinity, and it is also expressed by endothelial cells and binds VEGF165 (Gluzman-Poltorak *et al*., 2000). A recent report showed that Sema3F effectively blocks tumor neoangiogenesis and interferes with VEGF signaling (Kessler *et al*., 2004). Thus, both Sema3A/NP1 and Sema3F/NP2 interactions may impact vascular patterning.

In Vivo experiments demonstrate the importance of this pathway and support a model whereby both NP coreceptors affect vascular patterning. Sema3A bead implantations into E4.5 avian forelimbs caused failure of blood vessel formation and formed vessels deviate away from the bead, while overexpression of Sema3A or Sema3F in the perineural vascular network of the head of chick embryos led to defects in vascular remodeling (Bates *et al*., 2003; Serini *et al*., 2003). In addition, Sema3A and Sema3C knockout mice both have cardiovascular defects (Feiner *et al*., 2001; Serini

et al., 2003). Overexpression or deletion of NP1 in mice leads to numerous vascular defects (Kawasaki *et al*., 1999; Kitsukawa *et al*., 1995), while deletion of NP2 does not (Chen *et al*., 2000; Giger *et al*., 2000). However, the combined deletion of NP1 and NP2 results in severe defects, resembling the *Vegfa* and *flk-1* knockouts (Takashima *et al*., 2002). Recently, Kolodkin and Ginty and colleagues reported a set of elegant studies showing that VEGF/NP1 but not Sema/NP1 signaling was required for general vascular development (Gu *et al*., 2003). To determine this, they first conditionally ablated NP1 in endothelial cells and found severe vascular defects. They then generated mice that expressed a mutant NP1 from the NP locus that could bind to VEGF but not to Sema, and bred it to the NP2-deficient background so that mutant NP1 was the sole NP. Vascular defects were not seen in these mice, showing that the vascular abnormalities were specific to the lack of VEGF/NP1 signaling in endothelial cells. Thus, the effects of the semaphorins on vascular development and endothelial function described above could result from competitive inhibition of VEGF/NP signaling, since although Semas and VEGF have distinct binding sites, it is thought that steric hindrance may prevent simultaneous binding.

Data from zebrafish also demonstrate an important role for this pathway. NP1 antisense morpholinos injected into zebrafish produce defects in the intersegmental vessels, and knockdown or ubiquitous expression of Sema3a1 impairs dorsal aorta formation (Lee *et al*., 2002; Shoji *et al*., 2003). Thus, the evidence to date implicates semaphorins in vascular patterning events, but exactly how and where semaphorins affect patterning remains to be elucidated.

IV. Axial Structures Implicated in Vascular Patterning

As mentioned in Section II, this review focuses on the axial midline structures implicated in vascular patterning, since these structures are presumably the source of vascular patterning signals described in Section III. Figure 1 shows a schematized cross section of an amphibian/zebrafish embryo (A) and an avian/mammalian embryo (B) to highlight the similarities and differences in the different model organisms. Both types of embryo have a dorsal neural tube and a notochord immediately ventral to the neural tube. Both also have a major axial artery(s) called the dorsal aorta, and a major axial vein(s) called the cardinal vein. However, in amphibians and zebrafish these structures are single vessels, while in avians and mammals the dorsal aorta initially forms as a set of paired vessels on either side of the midline (shown in Fig. 1), then fuses to a single vessel only in the mid-trunk region of the embryo. The cardinal veins form as paired vessels on either side of the midline and remain that way in avians and mammals. A second major difference is the formation

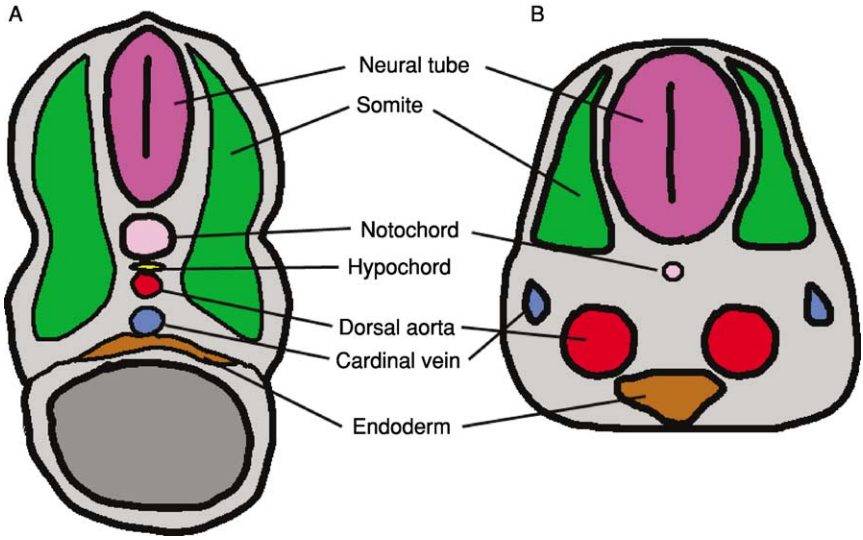

Figure 1 Schematic cross section through the trunk of midgestation vertebrate embryos at forelimb level. (A) Representative amphibian/zebrafish embryo. (B) Representative avian/mammalian embryo.

of the hypochord in amphibians and zebrafish that is lacking in amniotes. Finally, all embryos have endoderm ventral to the other structures that will form the gut and other endodermally derived organs. We now discuss each of the major non-vascular axial midline structures.

A. Hypochord

The hypochord is a temporary, rodlike structure in amphibian and fish embryos located just ventral to the notochord and in close association with the dorsal aorta (Lofberg and Collazo, 1997) (see Fig. 1). The hypochord is thought to derive from the endoderm in frogs, but fate mapping shows it to be a mesodermal derivative in zebrafish (Cleaver *et al*., 2000; Latimer *et al*., 2002). Cleaver and Krieg demonstrated that the hypochord transiently expresses high levels of VEGF, and that this expression correlates with the formation of the dorsal aorta in *Xenopus* (Cleaver *et al*., 1997; Cleaver and Krieg, 1998). They showed that angioblasts originating in the lateral plate mesoderm migrate to the midline to form the dorsal aorta. Their data further suggests that a long-range diffusible form of VEGF (the 121 isoform) acts as a midline chemoattractant for the migrating angioblasts. It is unclear

whether VEGF is expressed by the zebrafish hypochord (Eriksson and Lofberg, 2000; Liang *et al*., 2001; Weinstein, 2002). However, fish hypochord expresses Ang1, a ligand of Tie/tek receptors that is important in vessel formation, and other genes such as *radar*, which are required for vascular integrity (Eriksson and Lofberg, 2000; Hall *et al*., 2002; Pham *et al*., 2001). Cleaver and Krieg suggest that the notochord may be responsible for induction of the hypochord that, in turn, patterns the dorsal aorta (Cleaver *et al*., 2000). It should be possible to test this model in zebrafish, as mutants exist in which notochord is formed at the expense of hypochord, and vice versa (Latimer *et al*., 2002). Regardless of how these structures affect axial vascular patterning in amphibians and fish, only these model organisms possess a hypochord, so another structure must guide dorsal aorta patterning in avians and mammals. This may be the endoderm.

B. Endoderm

There is evidence that endoderm guides the assembly of the axial vein in zebrafish. One-eyed pinhead (*oep*) mutant embryos lack several tissues, including most endoderm and endoderm derivatives (Hammerschmidt *et al*., 1996; Schier *et al*., 1996, 1997; Strahle *et al*., 1997). It is interesting that these embryos only have a single vessel formed immediately ventral to the notochord, which is most likely the dorsal aorta (Brown *et al*., 2000). Other zebrafish mutants with similar defects, yet with some endoderm (*sqt* and *cyc*), still retain axial veins. These data suggest that endoderm, which is found in close proximity to the axial vein, actually signals to migrating angioblasts to form the axial vein. Sonic hedgehog (Shh) is one candidate molecular mediator of the endoderm signal, since it is expressed in endoderm and is known to act as an induction/patterning signal at other embryonic sites (Krauss *et al*., 1993; Strahle *et al*., 1996). *Sonic-you* is a mutation in the zebrafish homolog of Shh, and these embryos resemble mice lacking Shh in many respects (Schauerte *et al*., 1998). However, unlike $Shh^{-/-}$ mutant mice, *sonic-you* zebrafish embryos do not form either the dorsal aorta or the axial vein. Because the other zebrafish mutants that lack endoderm lack the axial vein but not the dorsal aorta, the lack of an axial vein in the *sonic-you* embryos is likely due to a loss of Shh expression specifically from the endoderm (Brown *et al*., 2000).

The endoderm may also be important for dorsal aorta formation in avians and mammals. Historically, endoderm was implicated as the source of an angioblast induction signal. However, recently reported experiments show that removal of endoderm from frog or avian embryos does not alter specification of angioblasts, but angioblasts do not coalesce into tubes without endoderm (Vokes and Krieg, 2002). These data are consistent with

a role for endoderm in dorsal aorta formation. Moreover, endoderm is a potent site of VEGF expression in avians and mammals (Aitkenhead *et al.*, 1998; Dumont *et al.*, 1995; Flamme *et al.*, 1995; Miquerol *et al.*, 1999). Thus, VEGF expression from the definitive endoderm in avians and mammals may be analogous to the strong VEGF midline expression from the hypochord in amphibians. In addition, *VegfA* mutant embryos have abnormal dorsal aorta formation (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). However, since the expression data is correlative and the genetic experiments removed VEGF globally, targeted deletion of VEGF in the endoderm will be required to determine if endoderm-derived VEGF is critical to dorsal aorta formation in mammals.

C. Notochord

The notochord is a transient structure that lies ventral to the neural tube in all vertebrates, and it has a critical role in organizing the midline structures. Notochord signals induce differentiation of the floor plate and motor neurons of the neural tube, the sclerotome of the somites, and oligodendrocytes (Brand-Saberi *et al.*, 1993; Fan *et al.*, 1995; Halpern *et al.*, 1993; Placzek *et al.*, 1990; Trousse *et al.*, 1995; Yamada *et al.*, 1993). The notochord also patterns the endoderm (Cleaver *et al.*, 2000). Despite these known patterning interactions, the role of the notochord in patterning the vasculature is poorly understood. In fact, there is data to support a role for the notochord as a source of both positive and negative vascular patterning signals.

1. The Notochord as the Source of Positive Vascular Patterning Signal(s)

One area of recent focus has been the role of notochord in assembly of the dorsal aorta, which comes to lie ventral to the notochord. In zebrafish, angioblasts assemble at the midline by the 14 somite stage to form the dorsal aorta, and other angioblasts begin forming the axial vein (also known as the posterior cardinal vein) ventral to the dorsal aorta by the 14–20 somite stage (Fouquet *et al.*, 1997). Two zebrafish mutants have provided evidence that the notochord guides the initial assembly of angioblasts that will form the dorsal aorta at the midline. *Floating head* (*flh*) is a mutant that lacks notochord, has an un-patterned neural tube, and has fused somites (Halpern *et al.*, 1995). *No tail* (*ntl*) is a mutant that retains some notochord precursors, a neural tube that consists of a wider than normal floor plate, and unfused but abnormally shaped somites (Halpern *et al.*, 1993; Melby *et al.*, 1996). Both mutants lack trunk circulation, and only a single vessel forms between the notochord and endoderm. This vessel is likely to be the axial vein based on its position in the *flh* mutants (Fouquet *et al.*, 1997; Sumoy

et al., 1997). Mosaic embryos with both *flh* mutant cells and wild-type cells had patches of notochord and evidence of dorsal aorta assembly adjacent to these patches (Fouquet *et al.*, 1997). In sum, these findings strongly suggest that notochord signals are necessary for dorsal aorta formation, but do not rule out an indirect effect of the notochord in patterning other tissues that, in turn, relay directive signals to migrating angioblasts. For example, the *flh* mutants lack a hypochord as well as a notochord (Talbot *et al.*, 1995), and the hypochord seems critical to dorsal aorta development in amphibians (see Section IV.A). In addition, VEGF expression is missing in somites, and Sema3a1 is inappropriately expressed throughout the entire somite in *flh* mutants (Liang *et al.*, 2001; Shoji *et al.*, 1998). Reduced expression of either VEGF or Sema3a1 via morpholinos also leads to abnormalities in dorsal aorta development (Nasevicius *et al.*, 2000; Shoji *et al.*, 2003). More studies will be needed to determine whether the notochord acts directly or indirectly to pattern the angioblasts that form the dorsal aorta.

Our lab has recently examined the vasculature in mice mutant for *Brachyury*, which is a T-box gene that is the mammalian homologue of zebrafish *ntl*. The *Brachyury* mutant embryos (*T/T*) do not form a mature notochord in the posterior half of the embryo. It is interesting that the dorsal aorta forms throughout the A–P axis of these mutant embryos, although with more perturbations in the size and shape in the posterior of the embryo (Jenkins, T., K.A.H. and V.L.B., unpublished results). Thus, it appears that the mouse differs from the zebrafish in the requirement for the notochord for dorsal aorta assembly. These differences may reflect differences in the midline structure that is the source of important vascular patterning signals. Specifically, the endoderm in amniotes may provide the initial patterning signals for the dorsal aorta, and this role may be carried out by the notochord/hypochord in the amphibian and zebrafish.

The identity of notochord signals important in dorsal aorta formation is unknown. Both *flh* and *ntl* encode transcription factors, so their effects are likely to be indirect. Sonic hedgehog is the best-characterized signal emanating from the notochord, making it a prime candidate in the search for a notochord signal responsible for dorsal aorta assembly. In support of this hypothesis, the sonic hedgehog mutant, *sonic-you*, lacks both the dorsal aorta and the axial vein (Brown *et al.*, 2000). It is likely that lack of notochord-derived Shh is upstream of the dorsal aorta defect, while endoderm-derived Shh is upstream of the axial vein defect. These studies, once again, do not rule out an indirect effect of Shh signaling via other cell types that are no longer patterned when Shh is missing. Another member of the T-box family of transcription factors, *hrT*, is expressed in the dorsal aorta and heart, and mutants lacking *hrT* resemble *flh* mutants in that no dorsal aorta forms (Ahn *et al.*, 2000; Griffin *et al.*, 2000; Szeto *et al.*, 2002). The

zebrafish hedgehog signaling mutant *you-too* lacks hrT expression, suggesting that *hrT* is a downstream effector of hedgehog signaling (Szeto *et al.*, 2002). Additional studies in zebrafish demonstrate that Shh has a role in arterial identity acting upstream of both the VEGF and the Notch pathways (Lawson *et al.*, 2002).

The effects of Shh on vascular development in mammalian models appear to be indirect. Transgenic overexpression of Shh in the dorsal neural tube of mice results in hypervascularization of the tissue (Rowitch *et al.*, 1999). Shh administration to aged mice induces neovascularization in an ischemic hind limb model, and the same treatment induces angiogenesis with large branching vessels in the murine cornea (Pola *et al.*, 2001). However, direct treatment of human umbilical vein endothelial vein cells (HUVECs) with Shh had no effect, although fibroblast cells responded to Shh by upregulating VEGF and angiopoietins (Kanda *et al.*, 2003; Pola *et al.*, 2001). Thus, it was hypothesized that Shh upregulates VEGF in non-vascular cells such as fibroblasts, and this upregulation in turn positively affects blood vessel formation. Nonetheless, Shh can induce capillary morphogenesis of HUVECs in Matrigel through the activation of PI-3 kinase (Kanda *et al.*, 2003).

2. The Notochord as the Source of Negative Vascular Patterning Signal(s)

In mouse and avian embryos, the notochord is associated with negative patterning signals in two ways. First, early in gastrulation, the embryonic midline is set up, and subsequently, angioblasts/endothelial cells are largely prevented from crossing the midline to colonize the contralateral side of the embryo. Second, surrounding the notochord is an avascular zone that presents a striking contrast to most other embryonic tissues and structures that are highly vascularized (Fig. 2A).

The midline barrier to angioblast–endothelial cell crossing was demonstrated experimentally through the use of quail chick chimeras. Somite and presomitic mesoderm grafts produced vascular cells that migrated cranially, caudally, and laterally, but never crossed the axial midline (Pardanaud *et al.*, 1996; Wilting *et al.*, 1995). However, removal of the notochord resulted in extensive midline crossing, showing that the notochord was required to maintain the midline barrier (Klessinger and Christ, 1996). Using mouse–avian chimeras, we demonstrated that mouse angioblast/endothelial cells also respect the midline barrier in the avian host (Ambler *et al.*, 2001). The molecular nature of this midline barrier is unknown.

The avascular zone surrounding the notochord has been observed but not experimentally manipulated. Initially, the notochord closely abuts both the neural tube dorsally and the dorsal aorta ventrally. However, the notochord soon becomes surrounded by cells, as the sclerotome cells of

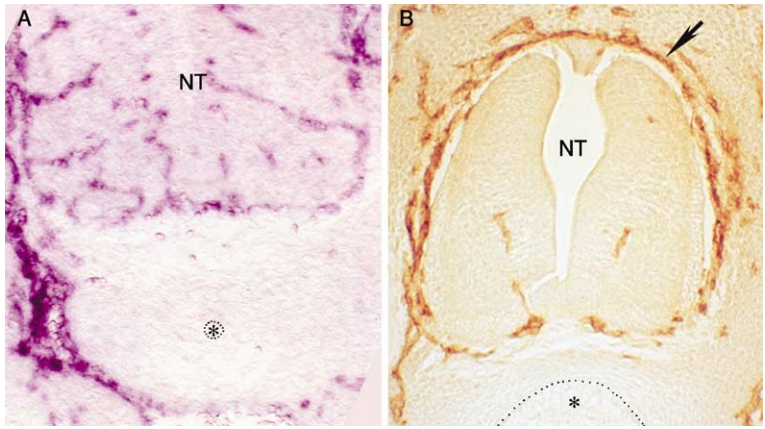

Figure 2 Vascular patterning at the embryonic midline of amniotes. (A) The perinotochordal area is avascular as visualized by *in situ* hybridization for flt-1 mRNA in an E11.5 mouse embryo. The notochord is denoted by an asterisk and surrounded by dotted lines, and the vessels are stained purple. (B) The peri-neural vascular plexus (PNVP) (arrow) surrounds the neural tube (NT) as shown in this HH stage 24 quail embryo. The vessels are reacted with QH1 antibody and appear brown.

the somites migrate around the notochord to form the peri-notochordal mesenchyme (review: Christ *et al.*, 2000). As the intersomitic vessels sprout from the dorsal aorta and migrate dorsally, they hug but do not invade the avascular zone. We have begun to investigate the avascular zone of the peri-notochordal mesenchyme, and preliminary experiments suggest that the avascular zone requires the notochord, because surgical placement of the notochord into somites resulted in the formation of an avascular zone around the ectopic notochord (Ambler, C. A., and V.L.B., unpublished results).

How do we reconcile the data showing that the notochord positively affects dorsal aorta assembly but prevents midline crossing and sets up an avascular zone? First, it is quite possible that the notochord has different midline effects depending on the organism being studied. To date, the notochord effects on dorsal aorta assembly are exclusive to amphibians and zebrafish, and in these models the distinction between notochord and hypochord effects is not completely clear. There may also be stage-specific effects of the notochord on vessel development. For example, initially, the notochord may impart positive patterning signals to recruit the dorsal aorta, and at later stages the notochord may up regulate a negative vascular patterning signal. Finally, it is quite likely that some or even all of the effects may be indirect, that is to say, that notochord-derived signals may induce expression of distinct sets of genes in different target cells. These notochord-activated

genes may encode proteins that mediate either positive or repulsive vascular patterning signals. Indeed, the tissues neighboring the notochord change dynamically during mid-gestation, suggesting a model whereby both positive and negative vascular patterning effects could result from the notochord-derived signals at different times as a result of indirect effects on adjacent cells. It will be interesting to dissect the role of the notochord in vascular patterning more completely.

D. Neural Tube

While the dorsal aorta is the first vessel formed in the embryo by midline signals, other vessels are also patterned at the midline at later stages. One such patterning event is the development of the perineural vascular plexus (PNVP), which comes to surround the neural tube (Fig. 2B). This plexus provides essential nutrients and oxygen to the developing neural tissue, and it is the source of vascular sprouts that subsequently invade and metabolically support the neural tissue. These PNVP-derived vessels go on to form the blood–brain barrier that is critical to proper CNS function in the adult (Bar, 1980; Bauer *et al*., 1993; Risau and Wolburg, 1990). Although the invasion of angiogenic sprouts into neural tissue has been described, the developmental processes that result in the formation of the PNVP at the proper location have not been investigated. Noden noted from his quail chick chimera studies that the neural tube itself does not have vascular potential, and thus it must become vascularized by exogenous endothelial precursors (Noden, 1989). Both quail-chick and mouse–quail chimera analysis identified somite-derived precursor cells as an important source of endothelial cells that comprise the PNVP (Ambler *et al*., 2001; Klessinger and Christ, 1996; Pardanaud *et al*., 1996; Pardanaud and Dieterlen-Lievre, 1999; Wilting *et al*., 1995). However, the source and nature of the signal(s) that act on somite-derived angioblasts to pattern the PNVP have only recently been investigated.

To formally prove that the neural tube is the source of a vascular patterning signal, our group placed mouse neural tubes ectopically in avian hosts and showed that a vascular plexus forms around the neural tube regardless of its position (Fig. 3A) (Hogan *et al*., 2004). We then did a graft experiment in which a buffer of avian tissue separated the mouse graft (presomitic mesoderm) from the avian host neural tube. The finding that graft-derived endothelial cells were found in the PNVP indicated that the neural tube signal could act at a distance to recruit endothelial cells to the PNVP. We next tested the role of VEGF-A in neural tube patterning through the use of a novel explant assay in which vessels form from presomitic mesoderm grafts in a VEGF-dependent manner. The neural tube

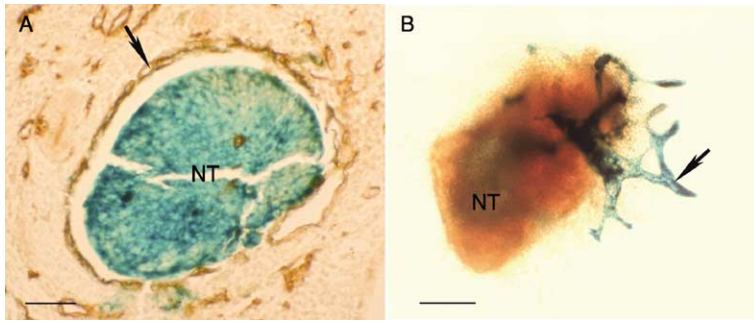

Figure 3 The neural tube directs PNVP formation. (A) Transverse section through the trunk of an HH stage 24 quail embryo containing a grafted *ROSA* +/− mouse neural tube, 3 days postsurgery. Chimeric embryos were whole-mount stained for β-galactosidase (blue), then sectioned and reacted with QH1 antibody (brown). The arrow points to a host-derived vascular plexus surrounding the grafted neural tube. NC, notochord; NT, neural tube. (B) *Flt1* +/− mouse presomitic mesoderm grafts from E8.5 mouse embryos were placed beside stage HH 10–13 quail neural tubes in collagen gels and cultured for 72 h in basal medium. Mouse vascular cells were visualized by β-galactosidase staining (blue). Arrow indicates vascular plexus. Scale bars are 50 μm in A and 200 μm in B.

could replace the requirement for VEGF in this system (Fig. 3B), and both pharmacological inhibition and genetic ablation of VEGF-A signaling showed that this pathway is required for the neural tube to pattern vessels from the presomitic mesoderm (Hogan *et al.*, 2004). Thus, not only is VEGF-A implicated in the first midline patterning events that lead to dorsal aorta formation, it is also important in later midline patterning mediated by the neural tube.

V. Conclusions and Future Directions

As with many endeavors, the journey to understand the mechanisms that underlie vascular patterning events has provided more questions than answers so far. The initial hypothesis that angioblasts respond to extrinsic vascular patterning cues to migrate and assemble in the embryo has been solidified by numerous experiments. Clearly, the axial midline of the vertebrate embryo acts to organize the developing vasculature, as it does other structures and tissues in the organism. Moreover, it does so in complex ways. There are differences in time; for example, at early times the hypochord and endoderm appear critical to patterning the dorsal aorta, while at later times the neural tube provides positive vascular patterning cues to promote the migration and assembly of vessels. There are differences in which structures are responsible for certain effects. For example, the hypochord of

amphibians and zebrafish is missing in avians and mammals, and the endoderm may provide similar patterning cues in the latter embryos. There are also differences in how the structures themselves affect patterning. The notochord seems to act as a source of positive vascular patterning cues in zebrafish that are critical to dorsal aorta formation, yet the notochord of amniotes prevents midline crossing of angioblasts and is surrounded by an extensive avascular zone. It is quite likely that further studies using the sophisticated molecular and genetic tools available will lead to a much more refined model of vascular patterning around the vertebrate midline.

There is no doubt that the VEGF signaling pathway presents an important nexus for vascular patterning events, as it is involved in almost every aspect of endothelial cell formation and function. To date, all clearly defined axial patterning events require VEGF signaling—the initial formation of the dorsal aorta, the specification of this vessel in zebrafish, and the formation of the plexus surrounding the neural tube in mouse and avian embryos. However, other signals and receptors are clearly important in vascular patterning, and it is quite likely that soon more studies will show defined roles for the Tie/tek, Notch, Eph/ephrin, and Semaphorin pathways in vascular patterning at the midline. The near future will likely see the development of models that effectively incorporate all of these molecular inputs to pattern vessels. The future will also see the integration of these signaling pathways with upstream inputs and downstream targets, such as transcription factors and cytoskeletal proteins.

We have also just begun to use the power of genetics to uncover novel pathways and genes involved in vascular patterning. In this realm, the zebrafish is clearly a star, as it is possible to perform forward genetic screens for novel genes. An interesting group of genes that affect vessel patterning, such as *out-of-bounds*, is sure to become larger with time and expand our knowledge of the molecular control of vascular patterning beyond the "usual suspects."

The future is very bright. The patterning of blood vessels presents a basic question of developmental biology: As embryos develop and form diverse cell types, how do these distinct groups of cells communicate with each other and influence each other's behavior? These processes have intrigued developmental biologists for hundreds of years, and we now have available the tools to address the mechanisms underlying vascular patterning events. In addition, understanding vascular patterning is relevant to potential therapeutic applications as never before. We now dream of inducing new vessels in the human body where and when we need them, and we even hope to be able to produce vessels *in vitro* for placement in the human body. Both of these clinical applications will move forward more quickly as we increase our knowledge of how the body regulates and patterns vessels.

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References

Adams, R. H. (2002). Vascular patterning by Eph receptor tyrosine kinases and ephrins. *Semin. Cell Dev. Biol.* **13,** 55–60.

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W., and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev.* **13,** 295–306.

Ahn, D. G., Ruvinsky, I., Oates, A. C., Silver, L. M., and Ho, R. K. (2000). tbx20, a new vertebrate T-box gene expressed in the cranial motor neurons and developing cardiovascular structures in zebrafish. *Mech. Dev.* **95,** 253–258.

Aitkenhead, M., Christ, B., Eichmann, A., Feucht, M., Wilson, D. J., and Wilting, J. (1998). Paracrine and autocrine regulation of vascular endothelial growth factor during tissue differentiation in the quail. *Dev. Dyn.* **212,** 1–13.

Ambler, C. A., Nowicki, J. L., Burke, A. C., and Bautch, V. L. (2001). Assembly of trunk and limb blood vessels involves extensive migration and vasculogenesis of somite-derived angioblasts. *Dev. Biol.* **234,** 352–364.

Ambler, C. A., Schmunk, G. A., and Bautch, V. L. (2003). Stem cell–derived endothelial cells/progenitors migrate and pattern in the embryo using the VEGF signaling pathway. *Dev. Biol.* **257,** 205–219.

Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., Kearne, M., Magner, M., and Isner, J. M. (1999). Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ. Res.* **85,** 221–228.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J. M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275,** 964–967.

Bar, T. (1980). The vascular system of the cerebral cortex. *Adv. Anat. Embryol. Cell Biol.* **59,** 1–62.

Bates, D., Taylor, G. I., Minichiello, J., Farlie, P., Cichowitz, A., Watson, N., Klagsbrun, M., Mamluk, R., and Newgreen, D. F. (2003). Neurovascular congruence results from a shared patterning mechanism that utilizes Semaphorin3A and Neuropilin-1. *Dev. Biol.* **255,** 77–98.

Bauer, H. C., Bauer, H., Lametschwandtner, A., Amberger, A., Ruiz, P., and Steiner, M. (1993). Neovascularization and the appearance of morphological characteristics of the blood–brain barrier in the embryonic mouse central nervous system. *Brain Res. Dev. Brain Res.* **75,** 269–278.

Bautch, V. L., Redick, S. D., Scalia, A., Harmaty, M., Carmeliet, P., and Rapoport, R. (2000). Characterization of the vasculogenic block in the absence of vascular endothelial growth factor-A. *Blood* **95,** 1979–1987.

Brand-Saberi, B., Ebensperger, C., Wilting, J., Balling, R., and Christ, B. (1993). The ventralizing effect of the notochord on somite differentiation in chick embryos. *Anat. Embryol.* **188,** 239–245.

Brand-Saberi, B., Seifert, R., Grim, M., Wilting, J., Kuhlewein, M., and Christ, B. (1995). Blood vessel formation in the avian limb bud involves angioblastic and angiotrophic growth. *Dev. Dyn.* **202,** 181–194.

Brown, L. A., Rodaway, A. R., Schilling, T. F., Jowett, T., Ingham, P. W., Patient, R. K., and Sharrocks, A. D. (2000). Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. *Mech. Dev.* **90,** 237–252.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380,** 435–439.

Carmeliet, P., Ng, Y. S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V. V., Stalmans, I., Mattot, V., Perriard, J. C., Dewerchin, M., Flameng, W., Nagy, A., Lupu, F., Moons, L., Collen, D., D'Amore, P. A., and Shima, D. T. (1999). Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Nat. Med.* **5,** 495–502.

Chen, H., Bagri, A., Zupicich, J. A., Zou, Y., Stoeckli, E., Pleasure, S. J., Lowenstein, D. H., Skarnes, W. C., Chedotal, A., and Tessier-Lavigne, M. (2000). Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. *Neuron* **25,** 43–56.

Childs, S., Chen, J. N., Garrity, D. M., and Fishman, M. C. (2002). Patterning of angiogenesis in the zebrafish embryo. *Development* **129,** 973–982.

Christ, B., Huang, R., and Wilting, J. (2000). The development of the avian vertebral column. *Anat. Embryol.* **202,** 179–194.

Clark, E. R. (1918). Studies on the growth of blood vessels in the tail of the frog larvae. *Am. J. Anat.* **23,** 37–88.

Clark, E. R., and Clark, E. L. (1939). Microscopic observations on the growth of blood capillaries in the living mammal. *Am. J. Anat.* **64,** 251–299.

Cleaver, O., and Krieg, P. (1998). VEGF mediates angioblast migration during development of the dorsal aorta in *Xenopus*. *Development* **125,** 3905–3914.

Cleaver, O., and Krieg, P. A. (1999). Molecular mechanisms of vascular development. *In* "Heart Development" (R. P. Harvey and N. Rosenthal, Eds.), pp. 221–252. Academic Press, New York.

Cleaver, O., and Melton, D. A. (2003). Endothelial signaling during development. *Nat. Med.* **9,** 661–668.

Cleaver, O., Seufert, D. W., and Krieg, P. A. (2000). Endoderm patterning by the notochord: development of the hypochord in *Xenopus*. *Development* **125,** 869–879.

Cleaver, O., Tonissen, K. F., Saha, M. S., and Krieg, P. A. (1997). Neovascularization of the *Xenopus* embryo. *Dev. Dyn.* **210,** 66–77.

Coffin, J. D., and Poole, T. J. (1988). Embryonic vascular development: Immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development* **102,** 735–748.

Coffin, J. D., and Poole, T. J. (1991). Endothelial cell origin and migration in embryonic heart and cranial blood vessel development. *Anat. Rec.* **231,** 383–395.

Cox, C. M., and Poole, T. J. (2001). Angioblast differentiation is influenced by the local environment: FGF-2 induces angioblasts and patterns vessel formation in the quail embryo. *Dev. Dyn.* **218,** 371–382.

Cross, M. J., Dixelius, J., Matsumoto, T., and Claesson-Welsh, L. (2003). VEGF-receptor signal transduction. *Trends. Biochem. Sci.* **28,** 488–494.

Damert, A., Miquerol, L., Gertsenstein, M., Risau, W., and Nagy, A. (2002). Insufficient VEGFA activity in yolk sac endoderm compromises haematopoietic and endothelial differentiation. *Development* **129,** 1881–1892.

Daniel, T. O., and Abrahamson, D. (2000). Endothelial signal integration in vascular assembly. *Ann. Rev. Physiol.* **62,** 649–671.

Drake, C. J., Hungerford, J. E., and Little, C. D. (1998). Morphogenesis of the first blood vessels. *In* "Morphogenesis: Cellular Interactions" (R. Fleischmajer, R. Timpl, and Z. Werb, Eds.), Vol. 857, pp. 155–180. New York Academy of Sciences, New York.

Drake, C. J., and Little, C. D. (1995). Exogenous vascular endothelial growth factor induces malformed and hyperfused vessels during embryonic neovascularization. *Proc. Natl. Acad. Sci. USA* **92,** 7657–7661.

Drake, C. J., and Little, C. D. (1999). VEGF and vascular fusion: Implications for normal and pathological vessels. *J. Histochem. Cytochem.* **47,** 1351–1355.

Drake, C. J., LaRue, A., Ferrara, N., and Little, C. D. (2000). VEGF regulates cell behavior during vasculogenesis. *Dev. Biol.* **224,** 178–188.

Dumont, D. J., Gradwohl, G., Fong, G. H., Puri, M. C., Gertsenstein, M., Auerbach, A., and Breitman, M. L. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8,** 1897–1909.

Dumont, D. J., Fong, G. H., Puri, M. C., Gradwohl, G., Alitalo, K., and Breitman, M. L. (1995). Vascularization of the mouse embryo: A study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev. Dyn.* **203,** 80–92.

Eriksson, J., and Lofberg, J. (2000). Development of the hypochord and dorsal aorta in the zebrafish embryo (Danio rerio). *J. Morphol.* **244,** 167–176.

Evans, H. M. (1909). On the development of the aortae, cardinal and umbilical veins, and other blood vessels of the vertebrate embryos from vertebrates. *Anat. Rec.* **3,** 498–518.

Fan, C. M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A., and Tessier-Lavigne, M. (1995). Long-range sclerotome induction by sonic hedgehog: Direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. *Cell* **81,** 457–465.

Feinberg, R. N., and Noden, D. M. (1991). Experimental analysis of blood vessel development in the avian wing bud. *Anat. Rec.* **231,** 136–144.

Feiner, L., Webber, A. L., Brown, C. B., Lu, M. M., Jia, L., Feinstein, P., Mombaerts, P., Epstein, J. A., and Raper, J. A. (2001). Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. *Development* **128,** 3061–3070.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J., and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380,** 439–442.

Ferrara, N., Gerber, H. P., and LeCouter, J. (2003). The biology of VEGF and its receptors. *Nat. Med.* **9,** 669–676.

Finkelstein, E. B., and Poole, T. J. (2003). Vascular endothelial growth factor: A regulator of vascular morphogenesis in the Japanese quail embryo. *Anat. Rec.* **272A,** 403–414.

Flamme, I., Breier, G., and Risau, W. (1995). Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (flk-1) are expressed during vasculogenesis and vascular differentiation in the quail embryo. *Dev. Biol.* **169,** 699–712.

Flamme, I., von Reutern, M., Drexler, H. C., Syed-Ali, S., and Risau, W. (1995). Overexpression of vascular endothelial growth factor in the avian embryo induces hypervascularization and increased vascular permeability without alterations of embryonic pattern formation. *Dev. Biol.* **171,** 399–414.

Folkman, J. (2003). Fundamental concepts of the angiogenic process. *Curr. Mol. Med.* **3,** 643–651.

Fong, G. H., Rossant, J., Gertsenstein, M., and Breitman, M. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376,** 66–70.

Fong, G. H., Zhang, L., Bryce, D. M., and Peng, J. (1999). Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development* **126,** 3015–3025.

Fouquet, B., Weinstein, B. M., Serluca, F. C., and Fishman, M. C. (1997). Vessel patterning in the embryo of the zebrafish: Guidance by notochord. *Dev. Biol.* **183,** 37–48.

Gerety, S. S., Wang, H. U., Chen, Z. F., and Anderson, D. J. (1999). Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol. Cell* **4,** 403–414.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D., and Betsholtz, C. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J. Cell Biol.* **161,** 1163–1177.

Ghabrial, A., Luschnig, S., Metzstein, M. M., and Krasnow, M. A. (2003). Branching morphogenesis of the *Drosophila* tracheal system. *Ann. Rev. Cell Dev. Biol.* **19,** 623–647.

Giger, R. J., Cloutier, J. F., Sahay, A., Prinjha, R. K., Levengood, D. V., Moore, S. E., Pickering, S., Simmons, D., Rastan, S., Walsh, F. S., Kolodkin, A. L., Ginty, D. D., and Geppert, M. (2000). Neuropilin-2 is required *in vivo* for selective axon guidance responses to secreted semaphorins. *Neuron* **25,** 29–41.

Gilbert, S. F. (2003). "Developmental Biology." Sinauer, Sunderland, MA.

Gluzman-Poltorak, Z., Cohen, T., Herzog, Y., and Neufeld, G. (2000). Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165 [corrected]. *J. Biol. Chem.* **275,** 18040–18045.

Goshima, Y., Ito, T., Sasaki, Y., and Nakamura, F. (2002). Semaphorins as signals for cell repulsion and invasion. *J. Clin. Invest.* **109,** 993–998.

Griffin, K. J., Stoller, J., Gibson, M., Chen, S., Yelon, D., Stainier, D. Y., and Kimelman, D. (2000). A conserved role for H15-related T-box transcription factors in zebrafish and *Drosophila* heart formation. *Dev. Biol.* **218,** 235–247.

Gu, C., Rodriguez, E. R., Reimert, D. V., Shu, T., Fritzsch, B., Richards, L. J., Kolodkin, A. L., and Ginty, D. D. (2003). Neuopilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev. Cell* **5,** 45–57.

Habeck, H., Odenthal, J., Walderich, B., Maischein, H., and Schulte-Merker, S. (2002). Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. *Curr. Biol.* **12,** 1405–1412.

Hall, C. J., Flores, M. V. C., Davidson, A. J., Crosier, K. E., and Crosier, P. S. (2002). Radar is required for the establishment of vascular integrity in the zebrafish. *Dev. Biol.* **251,** 105–117.

Halpern, M. E., Ho, R. K., Walker, C., and Kimmel, C. B. (1993). Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. *Cell* **75,** 99–111.

Halpern, M. E., Thisse, C., Ho, R. K., Thisse, B., Riggleman, B., Trevarrow, B., Weinberg, E. S., Postlethwait, J. H., and Kimmel, C. B. (1995). Cell-autonomous shift from axial to paraxial mesodermal development in zebrafish floating head mutants. *Development* **121,** 4257–4264.

Hammerschmidt, M., Pelegri, F., Mullins, M. C., Kane, D. A., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C. P., Jiang, Y. J., Kelsh, R. N., Odenthal, J., Warga, R. M., and Nusslein-Volhard, C. (1996). Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, Danio rerio. *Development* **123,** 143–151.

Himanen, J. P., and Nikolov, D. B. (2003). Eph receptors and ephrins. *Int. J. Biochem. Cell Biol.* **35,** 130–134.

Hogan, K. A., Ambler, C. A., Chapman, D. L., Bautch, V. L. (2004). The neural tube patterns vessels developmentally using the VEGF signaling pathway. *Development* **131**, 1503–1513.

Hrabe de Angelis, M., McIntyre II, J., and Gossler, A. (1997). Maintenance of somite borders in mice requires the *Delta* homologue *Dll1*. *Nature* **386,** 717–721.

Iso, T., Hamamori, Y., and Kedes, L. (2003). Notch signaling in vascular development. *Arterioscler. Thromb. Vasc. Biol.* **23,** 543–553.

Isogai, S., Horiguchi, M., and Weinstein, B. M. (2001). The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev. Biol.* **230,** 278–301.

Jain, R. K. (2003). Molecular regulation of vessel maturation. *Nat. Med.* **9,** 685–693.

Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabriat, H., Mouton, P., Alamowitch, S., Domenga, V., Cecillion, M., Marechal, E., Maciazek, J., Vayssiere, C., Cruaud, C., Cabanis, E. A., Ruchoux, M. M., Weissenbach, J., Bach, J. F., Bousser, M. G., and Tournier-Lasserve, E. (1996). Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* **383,** 707–710.

Kanda, S., Mochizuki, Y., Suematsu, T., Miyata, Y., Nomata, K., and Kanetake, H. (2003). Sonic hedgehog induces capillary morphogenesis by endothelial cells through phosphoinositide 3-kinase. *J. Biol. Chem.* **278,** 8244–8249.

Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., and Fujisawa, H. (1999). A requirement for neuropilin-1 in embryonic vessel formation. *Development* **126,** 4895–4902.

Kearney, J. B., Ambler, C. A., Monaco, K. A., Johnson, N., Rapoport, R. G., and Bautch, V. L. (2002). Vascular endothelial growth factor receptor Flt-1 negatively regulates developmental blood vessel formation by modulating endothelial cell division. *Blood* **99,** 2397–2407.

Kearney, J. B., Kappas, N. C., Ellerstrom, C., DiPaola, F. W., Bautch, V. L. (2004). The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. *Blood* **103**, 4527–4535.

Kendall, R. L., and Thomas, K. A. (1993). Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc. Natl. Acad. Sci. USA* **90,** 10705–10709.

Kendall, R. L., Wang, G., and Thomas, K. A. (1996). Identification of a natural soluble form of the vascular endothelial growth factor receptor. FLT-1, and its heterodimerization with KDR. *Biochem. Biophys. Res. Comm.* **226,** 324–328.

Kessler, O., Shraga-Heled, N., Lange, T., Gutmann-Raviv, N., Sabo, E., Baruch, L., Machluf, M., and Neufeld, G. (2004). Semphorin-3F is an inhibitor of tumor angiogenesis. *Cancer Res.* **64,** 1008–1015.

Kitsukawa, T., Shimono, A., Kawakami, A., Kondoh, H., and Fujisawa, H. (1995). Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development* **121,** 4309–4318.

Klessinger, S., and Christ, B. (1996). Axial structures control laterality in the distribution pattern of endothelial cells. *Anat. Embryol.* **193,** 319–330.

Kolodkin, A. L. (1998). Semaphorin-mediated neuronal growth cone guidance. *Prog. Brain Res.* **117,** 115–132.

Krauss, S., Concordet, J. P., and Ingham, P. W. (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75,** 1431–1444.

Krebs, L. T., Xue, Y., Norton, C. R., Shutter, J. R., Maguire, M., Sundberg, J. P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., Smith, G. H., Stark, K. L., and Gridley, T. (2000). Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev.* **14,** 1343–1352.

Latimer, A. J., Dong, X., Markov, Y., and Appel, B. (2002). Delta-Notch signaling induces hypochord development in zebrafish. *Development* **129,** 2555–2563.

Lawson, N. D., Scheer, N., Pham, V. N., Kim, C. H., Chitnis, A. B., Campos-Ortega, J. A., and Weinstein, B. M. (2001). Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* **128,** 3675–3683.

Lawson, N. D., Vogel, A. M., and Weinstein, B. M. (2002). Sonic hedgehog and vascular endothelial growth factor act upstream of the notch pathway during arterial endothelial differentiation. *Dev. Cell* **3,** 127–136.

Lawson, N. D., and Weinstein, B. M. (2002a). Arteries and veins: Making a difference with zebrafish. *Nat. Rev. Genet.* **3,** 674–682.

Lawson, N. D., and Weinstein, B. M. (2002b). *In Vivo* imaging of embryonic vascular development using transgenic zebrafish. *Dev. Biol.* **248,** 307–318.

Lee, P., Goishi, K., Davidson, A. J., Mannix, R., Zon, L., and Klagsbrun, M. (2002). Neuropilin-1 is required for vascular development and is a mediator of VEGF-dependent angiogenesis in zebrafish. *Proc. Natl. Acad. Sci. USA* **99,** 10470–10475.

Levine, A. J., Munoz-Sanjuan, I., Bell, E., North, A. J., and Brivanlou, A. H. (2003). Fluorescent labeling of endothelial cells allows *in vivo*, continuous characterization of the vascular development of *Xenopus laevis*. *Dev. Biol.* **254,** 50–67.

Liang, D., Chang, J. R., Chin, A. J., Smith, A., Kelly, C., Weinberg, E. S., and Ge, R. (2001). The role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development. *Mech. Dev.* **108,** 29–43.

Lofberg, J., and Collazo, A. (1997). Hypochord, an enigmatic embryonic structure: Study of the axolotl embryo. *J. Morphol.* **232,** 57–66.

Loughna, S., and Sato, T. N. (2001a). Angiopoietin and Tie signaling pathways in vascular development. *Matrix Biol.* **20,** 319–325.

Loughna, S., and Sato, T. N. (2001b). A combinatorial role of angiopoietin-1 and orphan receptor TIE1 pathways in establishing vascular polarity during angiogenesis. *Mol. Cell* **7,** 233–239.

McCright, B., Gao, X., Shen, L., Lozier, J., Lan, Y., Maguire, M., Herzlinger, D., Weinmaster, G., Jiang, R., and Gridley, T. (2001). Defects in development of the kidney, heart and eye vasculature in mice homozygous for a hypomorphic Notch2 mutation. *Development* **128,** 491–502.

Melby, A. E., Warga, R. M., and Kimmel, C. B. (1996). Specification of cell fates at the dorsal margin of the zebrafish gastrula. *Development* **122,** 2225–2237.

Miao, H. Q., Soker, S., Feiner, L., Alonso, J. L., Raper, J. A., and Klagsbrun, M. (1999). Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. *J. Cell Biol.* **146,** 233–242.

Miquerol, L., Gertenstein, M., Harpal, K., Rossant, J., and Nagy, A. (1999). Multiple developmental roles of VEGF suggested by a lacZ-tagged allele. *Dev. Biol.* **212,** 307–322.

Miquerol, L., Langille, B. L., and Nagy, A. (2000). Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development* **127,** 3941–3946.

Mukouyama, Y. S., Shin, D., Britsch, S., Taniguchi, M., and Anderson, D. J. (2002). Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell* **109,** 693–705.

Nasevicius, A., Larson, J., and Ekker, S. C. (2000). Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant. *Yeast* **17,** 294–301.

Ng, Y. S., Rohan, R., Sunday, M. E., Demello, D. E., and D'Amore, P. A. (2001). Differential expression of VEGF isoforms in mouse during development and in the adult. *Dev. Dyn.* **220,** 112–121.

Noden, D. M. (1988). Interactions and fates of avian craniofacial mesenchyme. *Development* **103,** 121–140.

Noden, D. M. (1989). Embryonic origins and assembly of blood vessels. *Am. Rev. Respir. Disease* **140,** 1097–1103.

Otani, A., Kinder, K., Ewalt, K., Otero, F. J., Schimmel, P., and Friedlander, M. (2002). Bone marrow-derived stem cells target retinal astrocytes and can promote or inhibit retinal angiogenesis. *Nat. Med.* **8,** 1004–1010.

Pardanaud, L., Altmann, C., Kitos, P., Dieterlen-Lievre, F., and Buck, C. A. (1987). Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* **100,** 339–349.

Pardanaud, L., and Dieterlen-Lievre, F. (1995). Does the paraxial mesoderm of the avian embryo have hemangioblastic capacity? *Anat. Embryol.* **192,** 301–308.

Pardanaud, L., and Dieterlen-Lievre, F. (1999). Manipulation of the angiopoietic/hemangiopoietic commitment in the avian embryo. *Development* **126,** 617–627.

Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L. M., Catala, M., and Dieterlen-Lievre, F. (1996). Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development* **122,** 1363–1371.

Pardanaud, L., Yassine, F., and Dieterlen-Lievre, F. (1989). Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. *Development* **105,** 473–485.

Park, J. E., Keller, G. A., and Ferrara, N. (1993). The vascular endothelial growth factor (VEGF) isoforms: Differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol. Biol. Cell* **4,** 1317–1326.

Patan, S. (1998). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Microvasc. Res.* **56,** 1–21.

Pham, V. N., Roman, B. L., and Weinstein, B. M. (2001). Isolation and expression analysis of three Zebrafish angiopoietin genes. *Dev. Dyn.* **221,** 470–474.

Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T., and Dodd, J. (1990). Mesodermal control of neural cell identity: Floor plate induction by the notochord. *Science* **250,** 985–988.

Pola, R., Ling, L. E., Silver, M., Corbley, M. J., Kearney, M. R., Blake, P., Shapiro, R., Taylor, F. R., Baker, D. P., Asahara, T., and Isner, J. M. (2001). The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat. Med.* **7,** 706–711.

Poole, T. J., and Coffin, J. D. (1989). Vasculogenesis and angiogenesis: Two distinct morphogenetic mechanisms establish embryonic vascular pattern. *J. Exp. Zool.* **251,** 224–231.

Poole, T. J., Finkelstein, E. B., and Cox, C. M. (2001). The role of FGF and VEGF in angioblast induction and migration during vascular development. *Dev. Dyn.* **220,** 1–17.

Puri, M. C., Rossant, J., Alitalo, K., Bernstein, A., and Partanen, J. (1995). The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *Embo. J.* **14,** 5884–5891.

Puri, M. C., Partanen, J., Rossant, J., Bernstein, A., and Alitalo, K. (1999). Interaction of the TEK and TIE receptor tyrosine kinases during cardiovascular development. *Development* **126,** 4569–4580.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386,** 671–674.

Risau, W., and Wolburg, H. (1990). Development of the blood–brain barrier. *Trends Neurosci.* **13,** 174–178.

Rowitch, D. H., St.-Jacques, B., Lee, S. M., Flax, J. D., Snyder, E. Y., and McMahon, A. P. (1999). Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. *J. Neurosci.* **19,** 8954–8965.

Ruhrberg, C., Gerhardt, H., Golding, M., Watson, R., Ioannidou, S., Fujisawa, H., Betsholtz, C., and Shima, D. T. (2002). Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev.* **16,** 2684–2698.

Rupp, P. A., Czirok, A., and Little, C. D. (2003). Novel approaches for the study of vascular assembly and morphogenesis in avian embryos. *Trends Cardiovasc. Med.* **13,** 283–288.

Sabin, F. R. (1917). Origin and development of the primitive vessels of the chick and of the pig. *Contrib. Embryol. Carnegie Inst.* **6,** 61–124.

Sabin, F. R. (1920). Studies on the origin of the blood vessels and of red blood corpuscles as seen in the living blastoderm of chick during the second day of incubation. *Contrib. Embryol. Carnegie. Inst.* **9,** 215–262.

Salloway, S., and Hong, J. (1998). CADASIL syndrome: a genetic form of vascular dementia. *J. Geriatr. Psychiatry Neurol.* **11,** 71–77.

Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376,** 70–74.

Schauerte, H. E., van Eeden, F. J., Fricke, C., Odenthal, J., Strahle, U., and Haffter, P. (1998). Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish. *Development* **125,** 2983–2993.

Schier, A. F., Neuhauss, S. C., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z., Yang, H., and Driever, W. (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* **123,** 165–178.

Schier, A. F., Neuhauss, S. C., Helde, K. A., Talbot, W. S., and Driever, W. (1997). The one-eyed pinhead gene functions in mesoderm and endoderm formation in zebrafish and interacts with no tail. *Development* **124,** 327–342.

Schuh, A. C., Faloon, P., Hu, Q. L., Bhimani, M., and Choi, K. (1999). *In Vitro* hematopoietic and endothelial potential of flk-1 −/− embryonic stem cells and embryos. *Proc. Natl. Acad. Sci. USA* **96,** 2159–2164.

Serini, G., Valdembri, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M., Tessier-Lavigne, M., Taniguchi, M., Puschel, A. W., and Bussolino, F. (2003). Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature* **424,** 391–397.

Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., Bernstein, A., and Rossant, J. (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89,** 981–990.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376,** 62–66.

Shoji, W., Isogai, S., Sato-Maeda, M., Obinata, M., and Kuwada, J. Y. (2003). Semaphorin3a1 regulates angioblast migration and vascular development in zebrafish embryos. *Development* **130,** 3227–3236.

Shoji, W., Yee, C. S., and Kuwada, J. Y. (1998). Zebrafish semaphorin Z1a collapses specific growth cones and alters their pathway *in vivo*. *Development* **125,** 1275–1283.

Soker, S., Takashima, S., Miao, H. Q., Neufeld, G., and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92,** 735–745.

Stalmans, I., Ng, Y. S., Rohan, R., Fruttiger, M., Bouche, A., Yuce, A., Fujisawa, H., Hermans, B., Shani, M., Jansen, S., Hicklin, D., Anderson, D. J., Gardiner, T., Hammes, H. P., Moons, L., Dewerchin, M., Collen, D., Carmeliet, P., and D'Amore, P. A. (2002). Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. *J. Clin. Invest.* **109,** 327–336.

Stone, J., Itin, A., Alon, T., Pe'er, J., Gnessin, H., Chan-Ling, T., and Keshet, E. (1995). Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *J. Neurosci.* **15,** 4738–4747.

Strahle, U., Blader, P., and Ingham, P. W. (1996). Expression of axial and sonic hedgehog in wildtype and midline defective zebrafish embryos. *Int. J. Dev. Biol.* **40,** 929–940.

Strahle, U., Jesuthasan, S., Blader, P., Garcia-Villalba, P., Hatta, K., and Ingham, P. W. (1997). one-eyed pinhead is required for development of the ventral midline of the zebrafish (Danio rerio) neural tube. *Genes Funct.* **1,** 131–148.

Sumoy, L., Keasey, J. B., Dittman, T. D., and Kimelman, D. (1997). A role for notochord in axial vascular development revealed by analysis of phenotype and the expression of VEGR-2 in zebrafish flh and ntl mutant embryos. *Mech. Dev.* **63,** 15–27.

Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87,** 1171–1180.

Swiatek, P. J., Lindsell, C. E., del Amo, F. F., Weinmaster, G., and Gridley, T. (1994). Notch1 is essential for postimplantation development in mice. *Genes Dev.* **8,** 707–719.

Szeto, D. P., Griffin, K. J., and Kimelman, D. (2002). HrT is required for cardiovascular development in zebrafish. *Development* **129,** 5093–5101.

Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., Niwa, H., Miyazaki Ji, J., Hirota, S., Kitamura, Y., Kitsukawa, T., Fujisawa, H., Klagsbrun, M., and Hori, M. (2002). Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc. Natl. Acad. Sci. USA* **99,** 3657–3662.

Talbot, W. S., Trevarrow, B., Halpern, M. E., Melby, A. E., Farr, G., Postlethwait, J. H., Jowett, T., Kimmel, C. B., and Kimelman, D. (1995). A homeobox gene essential for zebrafish notochord development. *Nature* **378,** 150–157.

Tessier-Lavigne, M., and Goodman, C. S. (1996). The molecular biology of axon guidance. *Science* **274,** 1123–1133.

Trousse, F., Giess, M. C., Soula, C., Ghandour, S., Duprat, A. M., and Cochard, P. (1995). Notochord and floor plate stimulate oligodendrocyte differentiation in cultures of the chick dorsal neural tube. *J. Neurosci. Res.* **41,** 552–560.

Uyttendaele, H., Ho, J., Rossant, J., and Kitajewski, J. (2001). Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. *Proc. Natl. Acad. Sci. USA* **98,** 5643–5648.

Vokes, S. A., and Krieg, P. A. (2002). Endoderm is required for vascular endothelial tube formation, but not for angioblast specification. *Development* **129,** 775–785.

Wang, H. U., Chen, Z. F., and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93,** 741–753.

Ward, N. L., and Dumont, D. J. (2002). The angiopoietins and Tie2/Tek: adding to the complexity of cardiovascular development. *Semin. Cell Dev. Biol.* **13,** 19–27.

Weinstein, B. M. (2002). Plumbing the mysteries of vascular development using the zebrafish. *Semin. Cell Dev. Biol.* **13,** 515–522.

Weinstein, B. M., Stemple, D. L., Driever, W., and Fishman, M. C. (1995). Gridlock, a localized heritable vascular patterning defect in the zebrafish. *Nat. Med.* **1,** 1143–1147.

Wilkinson, D. G. (2000). Eph receptors and ephrins: Regulators of guidance and assembly. *Int. Rev. Cytol.* **196,** 177–244.

Wilting, J., Brand-Saberi, B., Huang, R., Zhi, Q., Kontges, G., Ordahl, C. P., and Christ, B. (1995). Angiogenic potential of the avian somite. *Dev. Dyn.* **202,** 165–171.

Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster, G., and Gridley, T. (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum. Mol. Genet.* **8,** 723–730.

Yamada, T., Pfaff, S. L., Edlund, T., and Jessell, T. M. (1993). Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. *Cell* **73,** 673–686.

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., and Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature* **407,** 242–248.

Zhang, Y., Porat, R. M., Alon, T., Keshet, E., and Stone, J. (1999). Tissue oxygen levels control astrocyte movement and differentiation in developing retina. *Dev. Brain Res.* **118,** 135–145.

Zhong, T. P., Childs, S., Leu, J. P., and Fishman, M. C. (2001). Gridlock signalling pathway fashions the first embryonic artery. *Nature* **414,** 216–220.

Zhong, T. P., Rosenberg, M., Mohideen, M. A. P. K., Weinstein, B. M., and Fishman, M. C. (2000). Gridlock, and HLH gene required for assembly of the aorta in zebrafish. *Science* **287,** 1820–1824.