Arteries, Veins, Notch, and VEGF

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Arteries and veins are the two most fundamental blood vessel types: Higher-pressure, oxygenated blood flows outward through arteries, and lower-pressure, deoxygenated blood returns via veins. The existence of these two intertwined yet distinct vascular networks has been appreciated for thousands of years. The difference between arteries and veins was noted by the Greek anatomists Praxagoras and Herophilus in the third century B. C. (Wiltse and Pait 1998). In the 17th century, William Harvey established the functional definition of arteries and veins as vessels that carry blood away from the heart and toward the heart in a continuous circulation (Harvey 1970). Since Harvey's time, there has been a great deal of additional description of arteries and veins and the morphological differences that distinguish them. These morphological differences include the presence of a thicker vascular smooth muscle-containing wall around arterial vessels, and the presence of valves and other specialized structures found primarily within venous vessels (Fig. 1). The origins of the morphological differences between arteries and veins have generally been attributed to physiological factors such as blood flow and hemodynamic pressure (Clark 1918; Gonzales-Crussi 1971; Girard 1973), and it has been assumed that the endothelial cells lining both types of vessels are essentially naive with respect to their arterial or venous identity, at least initially. In recent years, however, information has begun to be forthcoming about the molecular aspects of blood vessel determination challenging prevailing views on the nature and origins of arteries and veins.

ARTERIAL AND VENOUS VASCULAR ENDOTHELIA HAVE DISTINCT MOLECULAR IDENTITIES

Just within the last four years, it has become clear that the vascular endothelial cells that line arteries and veins do in fact have distinct molecular identities, and that this distinction precedes the initiation of blood flow, or even the assembly of a morphologically defined, lumenized vascular tube (Fig. 2). The first such evidence arrived in 1998 with a report describing arterial- and venous-restricted gene expression in mice. During murine embryonic development, the ephrinB2 and EphB4 genes are reciprocally expressed in arteries and veins, respectively (Wang et al. 1998). The functional importance of this distinction was highlighted by targeted disruption of the ephrinB2 locus, which resulted in improper morphogenesis of both arterial and venous blood vessels and defects in remodeling the arterial–venous vascular interface (Wang et al. 1998). The symmetrical results of targeted disruption of the EphB4 locus suggested that EphB4 and ephrinB2 act as a defined ligand–receptor pair (Gerety et al. 1999). Introduction of a cytoplasmic deletion allele of ephrinB2 into mice suggested further that signaling between these molecules is bidirectional and that ephrinB2 can act not only as a ligand but also as a receptor (Adams et al. 2001). The idea that ephrin ligands can also have receptor activity has precedent in other studies of ephrin and Eph genes, particularly in relation to nervous system

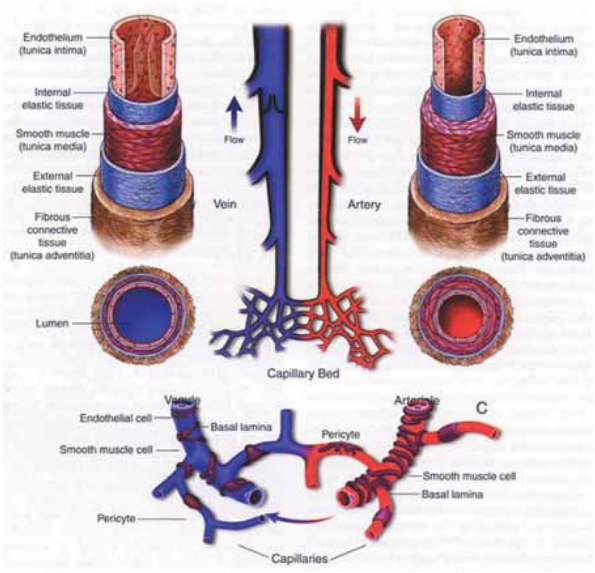

Figure 1. Blood vessels come in two fundamental flavors—arteries and veins. Both types of vessels are composed of an inner endothelium (tunica intima) surrounded by internal elastic tissue, smooth muscle cell layer (tunica media), external elastic tissue, and fibrous connective tissue (tunica adventitia). Larger-caliber arteries have thicker smooth muscle cell layers, and larger veins possess specialized structures such as valves. The two networks of tubes are completely separate at the level of the larger vessels but are linked distally through a system of fine capillaries. (Reprinted, with permission, from Cleaver and Krieg 1999 [copyright Academic Press].)

development (Cowan and Henkemeyer 2002). Vascular expression of other ephrin and Eph genes was also reported, and functional roles for some of these genes in vessel differentiation and patterning were also established (Adams et al. 1999; Helbling et al. 2000). The molecular distinction between arteries and veins represented by arterial-specific expression of ephrinB2 extends to the finest capillaries and includes expression in both the endothelial cells and the vascular smooth muscle cells that surround the arteries (Gale et al. 2001; Shin et al. 2001). Although the original ephrinB2 knockout constructs did not distinguish between vascular endothelial and vascular smooth muscle-specific functions of ephrinB2, a more recent endothelial-specific knockout of ephrinB2 showed that, at least for the early, lethal phenotypes of loss of ephrinB2, it is the expression within vascular endothelial cells that is critical (Gerety and Anderson 2002). This idea is also supported by evidence that ephrinB2 expression in endothelial cells precedes expression in adjacent smooth muscle cells, and suggests that smooth muscle cell expression may depend on signals from endothelium (Gale et al. 2001; Shin et al. 2001).

Although the results above indicate that the arterial–venous (A-V) identity of early blood vessels is genetically programmed and precedes circulatory flow, other recent work suggests that this fate choice is not irreversible and that maintenance of differentiated A-V identity might require components of the vascular wall. Two separate groups recently performed quail–chick grafting experiments to examine the plasticity of A-V endothelial cell fate (Moyon et al. 2001; Othman-Hassan et al. 2001). Portions of embryonic arteries or veins were grafted from quail donors at various stages of development into chick hosts, and the A-V identity of donor cells contributing to different host vessels was assessed using artery- or vein-specific molecular markers. Up until approximately E7, donor cells populate both types of vessels and assume the appropriate molecular identity, but after E7, this plasticity is progressively lost. However, isolated endothelial cells or isolated dissected endothelia were still plastic even in older vessels, suggesting that components of the vascular wall are necessary to maintain, or are sufficient to redirect, the A-V identity of adjacent endothelial cells.

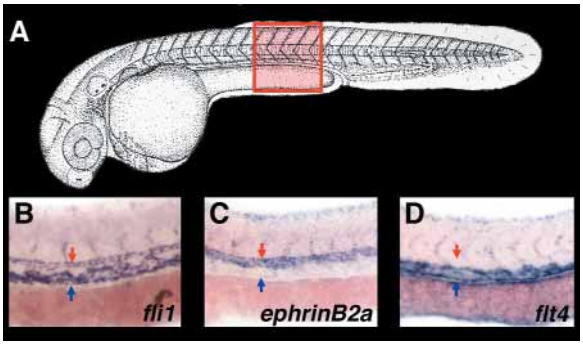

Figure 2. Arterial and venous endothelial cells have molecularly defined identities that are evident prior to circulatory flow or even tubulogenesis. In the zebrafish, the expression of artery markers such as *ephrinB2a* (*C*) and vein markers such as *flt4* (*D*) is evident by in situ hybridization of 25-somite-stage embryos, several hours before circulation begins in the trunk. In fact, expression of *ephrinB2a* within the dorsal aorta begins just as the endothelial cells that have migrated from the lateral mesoderm are aggregating into a cord of cells at the trunk midline. Expression of the pan-endothelial marker *fli1* is shown for comparison (*B*). Box *A* shows approximate location of in situ images, for reference. (*Red arrows*) Dorsal aorta. (*Blue arrows*) Posterior cardinal vein. (*A*, modified, with permission, from Kimmel et al. 1995 [copyright Wiley Interscience].)

SPECIFICATION OF A-V IDENTITY: THE ROLE OF NOTCH SIGNALING

It is clear from the phenotypes of targeted disruption of ephrin and Eph genes in mice that arterial or venous expression of these genes is critical for the proper formation of arteries and veins (Wang et al. 1998; Adams et al. 1999; Gerety et al. 1999). However, it is equally clear that these genes are not themselves involved in the initial selection of arterial and venous endothelial cell populations. Mice homozygous for an ephrinB2 lacZ knock-in allele still expressed β-galactosidase appropriately in the arterial endothelial compartment, at least initially, although boundaries between arterial and venous endothelial populations were not as well defined and the cell populations did not interact properly (Wang et al. 1998). This indicates that specification of arterial and venous domains must depend on other, upstream factors. What are the factors that lie upstream of ephrinB2 and EphB4?

The zebrafish, a genetically tractable vertebrate with a physically accessible, optically clear embryo, has proven to be a highly useful model for studying vascular development (Roman and Weinstein 2000; Vogel and Weinstein 2000). Zebrafish studies have given us important new insights into the molecular signals regulating A-V identity. In particular, the well-studied Notch signaling pathway (Artavanis-Tsakonas et al. 1999) has been shown to have an important new role in regulating vascular endothelial A-V cell fate determination (Lawson et al. 2001). A variety of Notch signaling genes are expressed in the vasculature. In mouse, Notch1 and 2 are expressed in endothelial cells (Del Amo et al. 1992; Zimrin et al. 1996), and the expression of Notch4 is restricted to endothelial cells (Uyttendaele et al. 1996). Vascular expression of these and other Notch receptors and ligands has been reported to be restricted to arteries in mouse and zebrafish embryos (Shutter et al. 2000; Smithers et al. 2000; Lawson et al. 2001; Villa et al. 2001), suggesting a role for this pathway during arterial differentiation. Mice that lack Jagged1 or Notch1 have abnormal vascular development (Xue et al. 1999; Krebs et al. 2000), whereas expression of an activated form of Notch4 specifically in the vasculature results in defective angiogenic blood vessel growth (Uyttendaele et al. 2001). Although phenotypic studies of targeted disruption of different Notch pathway receptors and ligands in mice have pointed to the importance of the Notch pathway in vascular morphogenesis, these studies have not addressed the specific function of Notch in the vasculature or its role in A-V differentiation.

Several recent studies in the zebrafish (Lawson et al. 2001, 2002) have shown that Notch signaling promotes arterial differentiation at the expense of venous differentiation during vascular development. As in other vertebrate species, the artery-specific expression of Notch signaling genes such as *notch5* (Kortschak et al. 2001) and *deltaC* (Smithers et al. 2000) in zebrafish blood vessels suggested that Notch might be playing an important role in artery formation. The ability to readily visualize the vasculature and carry out defined functional manipulation of developing embryos permitted a direct test of this idea. Notch signaling was repressed in zebrafish embryos either genetically, using the neurogenic *mindbomb (mib)* mutant, or experimentally, by injecting mRNA encoding a dominant-negative DNA-binding mutant of *Xenopus suppressor of hairless* protein (Lawson et al. 2001). In either case, repression of Notch signaling resulted in loss of *ephrinB2a* expression from arteries accompanied by ectopic expansion of normally venous-restricted markers into the arterial domain (Fig. 3). Conversely, activation of Notch signaling suppressed the expression of vein-restricted markers and promoted ectopic expression of *ephrinB2a* and other arterial markers in venous vessels. This activation was accomplished either by heat-shock promoter-driven ubiquitous expression of the Notch1a intracellular domain (Notch1a-ICD) or by Fli1-promoter-driven vascular-specific expression of Notch5-ICD. The latter set of experiments demonstrated the vascular endothelial cell autonomy of Notch-ICD effects, confirming that Notch is in fact acting directly at the level of the vascular endothelial cell itself and not via indirect signals from some other, adjacent Notch-responsive cells or tissues.

What are the functional consequences of a change in A-V molecular identity? In *mib* mutants or DN-Su(H)-injected embryos, reduction in Notch signaling causes the major trunk axial vessels (dorsal aorta and posterior cardinal vein) to display defects in morphogenesis and remodeling, and to form with poorly defined A-V boundaries (Lawson et al. 2001). These phenotypes are similar to those observed in mice with targeted disruption of Notch receptors or ligands (Xue et al. 1999; Krebs et al. 2000). In addition, the ability to visualize circulatory flow patterns in living zebrafish embryos permitted observation of prominent A-V shunts between the dorsal aorta and posterior cardinal vein in embryos with reduced Notch signaling. This reinforces the idea that the formation of distinct, well-demarcated arterial and venous cell populations is perturbed in these embryos. The strong similarity between Notch and ephrin/eph gene knockout phenotypes in mice suggests that ephrinB2 and ephB4 are probably playing a major role in arterial differentiation downstream from Notch signaling.

Potential downstream targets of Notch signaling have also been identified in the vasculature. Zebrafish embryos mutant for the *gridlock* (*grl*) gene possess cranial circulation but lack trunk circulation as a result of defective formation of the trunk lateral and dorsal aortae (Weinstein et al. 1995). Positional cloning of *grl* revealed that it is a basic helix-loop-helix protein of the *hairy/enhancer of split* family of transcriptional repressors (Zhong et al. 2000) similar to the murine HRT2 gene (Nakagawa et al. 1999). Like *notch5* and *ephrinB2*, *grl* is expressed in arterial but not venous blood vessels. HRT2 and *grl* are both targets of Notch signaling, at least in some cellular contexts (Nakagawa et al. 2000; Zhong et al. 2001). Injection of *grl* mRNA into wild-type zebrafish embryos can repress the expression of the vein-restricted marker *flt4* (Zhong et al. 2001), as does Notch activation in vivo (Lawson et al. 2001). Reduction of Grl activity by antisense morpholino oligonucleotides eliminates arterial expression of *ephrinB2a* (Zhong et al. 2001). However, *grl* continues to be expressed in the dorsal aorta in embryos lacking Notch activity despite the fact that arterial differentiation is suppressed in these and they display vascular morphogenesis defects (Lawson et al. 2001). Furthermore, *grl* and *flt4* are expressed simultaneously throughout early vascular development (Thompson et al. 1998; Zhong et al. 2001), and *grl* mutants continue to express *ephrinB2a* in the dorsal aorta (Lawson et al. 2001) despite their vascular morphogenesis defects (Zhong et al. 2000). It should also be noted that *grl* morpholino-injected embryos not only lose dorsal aorta *ephrinB2a* expression, but frequently lose the dorsal aorta entirely (Zhong et al. 2001), unlike *mib* mutants or dominant negative suppressor of hairless–injected embryos (Lawson et al. 2001). Taken together, these results indicate that *grl* plays an important role in formation of the dorsal aorta but suggest that its role as a regulator of A-V differentiation requires further clarification.

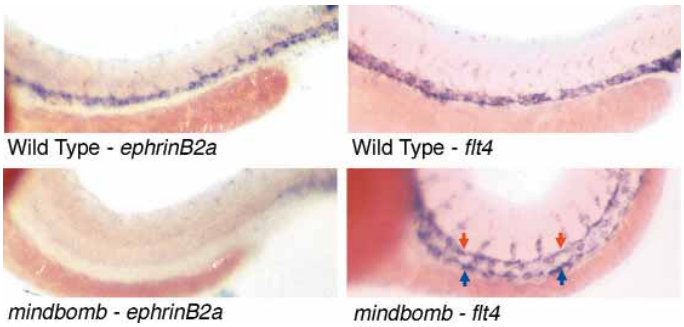

Figure 3. Reduction in Notch signaling perturbs A-V identity. In situ hybridization using artery markers in 30-somite-stage Notch-deficient *mindbomb* (mib^{ta52b}) mutant and wild-type sibling zebrafish embryos. (*A*) In wild-type siblings, *ephrinB2a* expression is apparent in the DA. (*B*) In mib^{ta52b} mutant embryos, this expression is absent. (*C*) In wild-type siblings, *flt4* expression is restricted to the PCV by the 30-somite stage. (*D*) In mib^{ta52b} mutant embryos, expression persists within both the PCV (*blue arrows*) and the DA (*red arrows*). All panels show lateral views of the trunk, dorsal up, anterior to the left. (Modified from Lawson et al. 2001.)

VASCULAR ENDOTHELIAL GROWTH FACTOR ACTS UPSTREAM OF NOTCH

What acts upstream of Notch? Recent studies in zebrafish and mice have revealed a surprising new role for vascular endothelial growth factor (VEGF) signaling upstream of Notch. VEGF-A is a key regulator of vascular development in vertebrates (Ferrara and Gerber 2001). It mediates vascular permeability and functions as an endothelial mitogen and angiogenic inducer. Loss of only a single allele of this gene is lethal in mouse, with severe defects in both vasculogenic and angiogenic blood vessel growth (Carmeliet et al. 1996; Ferrara et al. 1996). A number of studies in mice and zebrafish have provided evidence that another important role of VEGF is to specifically induce the differentiation of arterial blood vessels.

Postnatal retinal angiogenesis was examined in mice selectively expressing individual VEGF isoforms (Stalmans et al. 2002). Most vertebrates have three predominant VEGF-A isoforms (molecular weights 120, 164, and 188 in mice). Mice engineered to express only VEGF164 were healthy and normal, but mice expressing only the VEGF120 or VEGF188 isoforms had reduced viability and exhibited defects in retinal arterial differentiation. The phenotype of VEGF188 mice was particularly interesting, since these mice had relatively normal venular outgrowth but greatly impaired retinal arterial development. In another study, mice were generated in which VEGF164 was overexpressed in cardiac muscle under the control of a myosin heavy chain (MHC) promoter (Visconti et al. 2002). These mice were crossed together with ephrinB2 or EphB4 τ-lacZ knock-in "reporter" mice in order to easily assay arterial or venous endothelial differentiation. MHC-VEGF164 mice had increased numbers of ephrinB2-positive capillaries at the expense of EphB4-positive vessels in the heart, indicating that VEGF expression had selectively promoted the formation of additional arterial vessels.

Another study, examining the role of sensory nerves in guiding the patterning of blood vessels, provided additional evidence for an arteriogenic role for VEGF (Mukouyama et al. 2002). Previous work had documented the fact that nerves and larger vessels co-align in the skin (Martin and Lewis 1989), and Mukouyama et al. examined the molecular basis for this phenomenon. They demonstrated that arteries, but not veins, specifically align with peripheral nerves in embryonic mouse limb skin. Loss of peripheral sensory nerves or Schwann cells leads to defects in arteriogenesis, whereas these same cells can induce arterial marker expression in isolated embryonic endothelial cells when they are co-cultured in vitro. Sensory neurons and glia both express VEGF, and additional in vitro experiments demonstrated that VEGF is necessary and sufficient to mediate the arterial induction effects of these cells. VEGF alone could induce arterial differentiation of isolated embryonic endothelial cells, and at lower doses it did so without inducing proliferation, demonstrating that the increase in *ephrinB2*-positive cells was not due to preferential growth of these cells but reflected direct induction of artery-specific gene programs. Soluble VEGFR2(Flk1)-Fc, a potent VEGF antagonist (Ferrara and Davis-Smyth 1997), blocked the in vitro arterial differentiation effects of peripheral sensory nerves and Schwann cells. Together, these data suggest that peripheral nerves provide a template for the formation of arteries in the skin via local secretion of VEGF.

The murine studies described above are consistent with recent work in the zebrafish demonstrating that VEGF is necessary and sufficient for arterial differentiation (Lawson et al. 2002). The zebrafish data also provide a link to Notch, showing that VEGF acts upstream of Notch signaling in arterial fate determination. Reduction of VEGF activity using antisense morpholino oligonucleotides caused loss of arterial marker expression from the trunk dorsal aorta, ectopic arterial expression of vein markers, and morphologic defects in the aorta and cardinal vein (Fig. 4). All of these phenotypes were similar to those noted for loss of Notch signaling (Lawson et al. 2001). Injection of VEGF mRNA induced ectopic expression of *ephrinB2a* in the posterior cardinal vein. The relationship between VEGF and Notch signaling during arterial differentiation was further tested in several additional experiments (Fig. 5). VEGF mRNA injected into Notch signaling-deficient *mindbomb* mutant embryos did not induce the expression of arterial markers such as *ephrinB2a* in the trunk. This did not reflect a general lack of VEGF responsiveness of the endothelial cells in *mindbomb* mutants, since up-regulation of the VEGF receptor *flk1*, a documented response to VEGF induction (Flamme et al. 1995; Kremer et al. 1997; Liang et al. 2001), was

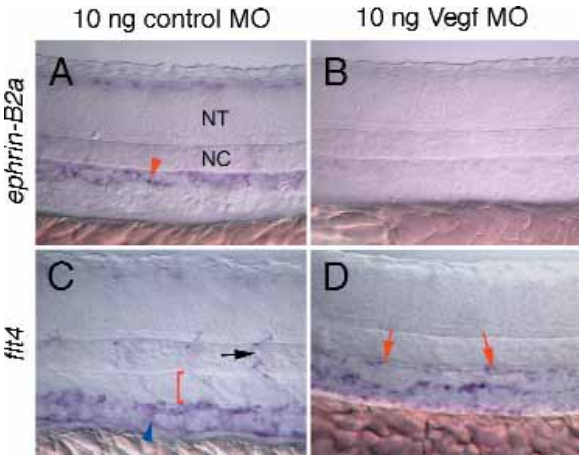

Figure 4. Loss of VEGF function perturbs A-V identity. (*A–D*) Whole-mount in situ hybridization of zebrafish embryos at 26 hpf. Lateral views of the trunk, dorsal up, anterior to the left. (*A*) Normal *ephrinB2a* expression in the dorsal aorta (*red arrowhead*) of an embryo injected with control morpholino; (NC) notochord, (NT) neural tube. (*B*) Loss of DA *ephrinB2a* expression in an embryo injected with 10 ng of a morpholino targeting VEGF. (*C*) Posterior cardinal vein-restricted *flt4* expression (*blue arrowhead*) in control morpholino-injected embryos (*black arrow* indicates normal expression within a segmental vessel). Expression is absent from the region of the dorsal aorta (indicated by *red bracket*). (*D*) Ectopic *flt4* expression in the dorsal aorta in VEGF morpholino-injected embryos. Red arrows indicate expression of *flt4* in cells of the dorsal roof of the dorsal aorta. (Modified from Lawson et al. 2002.)

still observed in VEGF-injected mutants. In contrast, activated Notch induced arterial differentiation just as efficiently in VEGF morpholino-injected embryos as in their control-injected siblings. Taken together, these experiments indicated that VEGF acts upstream of Notch signaling during arterial differentiation.

SPECULATION ON THE ASSEMBLY OF A-V NETWORKS

How can we reconcile this new arterial differentiation role for VEGF with its other well-documented roles as an endothelial mitogen, promigratory factor, and vascular permeability factor (Ferrara and Gerber 2001)? All of these previously documented activities are associated with new vessel growth. Induction of endothelial cell proliferation and migration are necessary to generate the cells that contribute to new vessels and allow them to take up positions in new, sometimes distant locales. Permeabilization of vessels facilitates deposition of new basement membrane, providing a suitable matrix for new vessel growth. Perhaps preferential induction of arterial fate is also an intrinsic feature of new vessel growth in response to VEGF. The results described above suggest a two-phase model for formation of new vascular networks (Fig. 6, panel A). In the first phase, new arteries would be formed in response to VEGF signaling, either by selection from a preexisting vascular plexus or by de novo (angiogenic) growth. In the second phase, venous vessels would emerge to provide return for arterial blood flow and allow the system to function.

This model has a number of attractive features. It is consistent with the observed anatomical colocalization of larger arteries and veins—there are few locales in the body where larger caliber veins and arteries are not found in pairs. It also provides a built-in mechanism for ensuring that venous return routes are provided for blood flowing through arterial vessels and that a functional system is constructed (Fig. 2). This comes as a natural consequence of using a primary, arterial vascular network as the template for a secondary, venous network. Arterial vessels could serve as actual direct templates for the formation of juxtaposed veins via molecular signals transmitted from arterial vessels to venous endothelial progenitors. Alternatively, or in addition, morphogenesis of a venous return system from a surrounding "naive" vascular plexus could be driven by flow dynamics once a defined arterial tree has been generated.

Is there evidence for this two-step model in vivo? In fact, many vessels do form in the predicted manner. More than a century ago, Popoff (1894) published a staged description of the development of the vasculature of the avian yolk sac, and the emergence of the venous system was described in detail by Ishida (1956). In exquisite hand-drawn illustrations, these authors detailed how the vitelline arteries appear first within the vitelline capillary network. The remaining capillaries in the plexus lose their connections to the vitelline artery, and the detached capillaries on either side of the artery then sprout and remodel to form intermedial and then collateral veins above and alongside the artery (Fig. 6, panels B–D). A more recent study showed that initial vascular innervation of the XY gonad in mice appears to occur almost exclusively via immigration of ephrinB2- and Notch-positive arterial progenitors (Brennan et al. 2002). EphB4-positive venous progenitors are not apparent until later stages. Other recent work has shown that in the zebrafish the primitive intersegmental vessels in the trunk form in two distinct steps, with emergence of arterial and venous sprouts in temporally separate waves (S. Isogai et al., unpubl.). The studies described above in which ephrinB2-τ-lacZ knock-

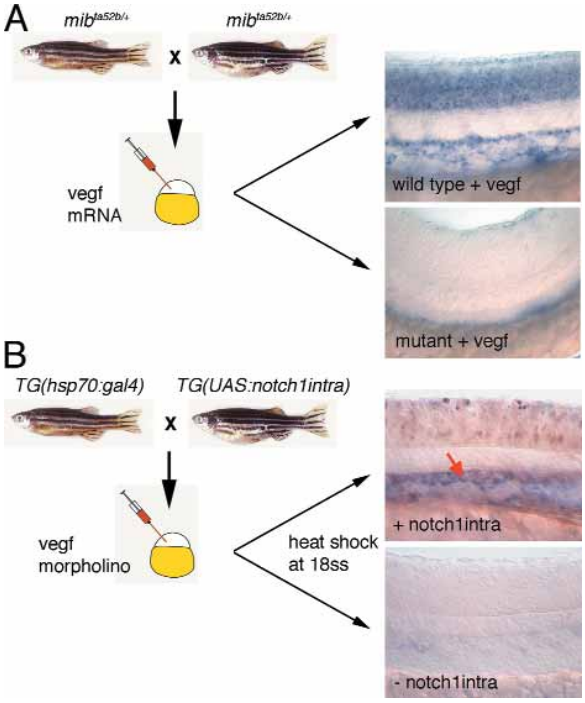

Figure 5. VEGF acts upstream of Notch signaling during arterial differentiation. (*A*) Notch signaling is required downstream of VEGF. Embryos that were derived from *mib*^{ta52b} heterozygous fish are injected with *vegf* mRNA (*left*). In wild-type embryos, artery marker gene expression is expanded in response to VEGF (*asterisks*), but is absent in *mib*^{ta52b} mutant embryos, indicating that Notch signaling is required downstream of VEGF for this expansion to occur. (*B*) Activation of the Notch pathway can induce arterial differentiation in the absence of VEGF. Adult fish that carry a heat-inducible *gal4* transgene (TG) (Scheer et al. 2001) are crossed to fish that express an intracellular form of zebrafish *notch1* (*notch1-intra*) (Scheer and Campos-Ortega 1999) that is constitutively active. Notch1-intra is tagged with an epitope to allow subsequent visualization by immunostaining and is driven by a Gal4-responsive promoter (UAS). Embryos from these fish are injected with a morpholino oligonucleotide to reduce VEGF protein levels (Nasevicius et al. 2000) and subsequently heat-shocked at the 18-somite stage to induce expression of the Notch1-intra. All embryos will have reduced VEGF, but a proportion of the embryos will also have activated the Notch pathway because they carry both transgenes. In embryos that have reduced VEGF expression but no exogenous Notch1-intra, *ephrinB2a* is not expressed (*top* panel). Activation of the Notch pathway as a result of exogenous Notch1-intra expression rescues *ephrinB2a* expression (Lawson et al. 2002) (*bottom* panel, *asterisk*). Together, these results indicate that the Notch pathway functions downstream of VEGF to promote arterial differentiation. (Modified from Lawson et al. 2002.)

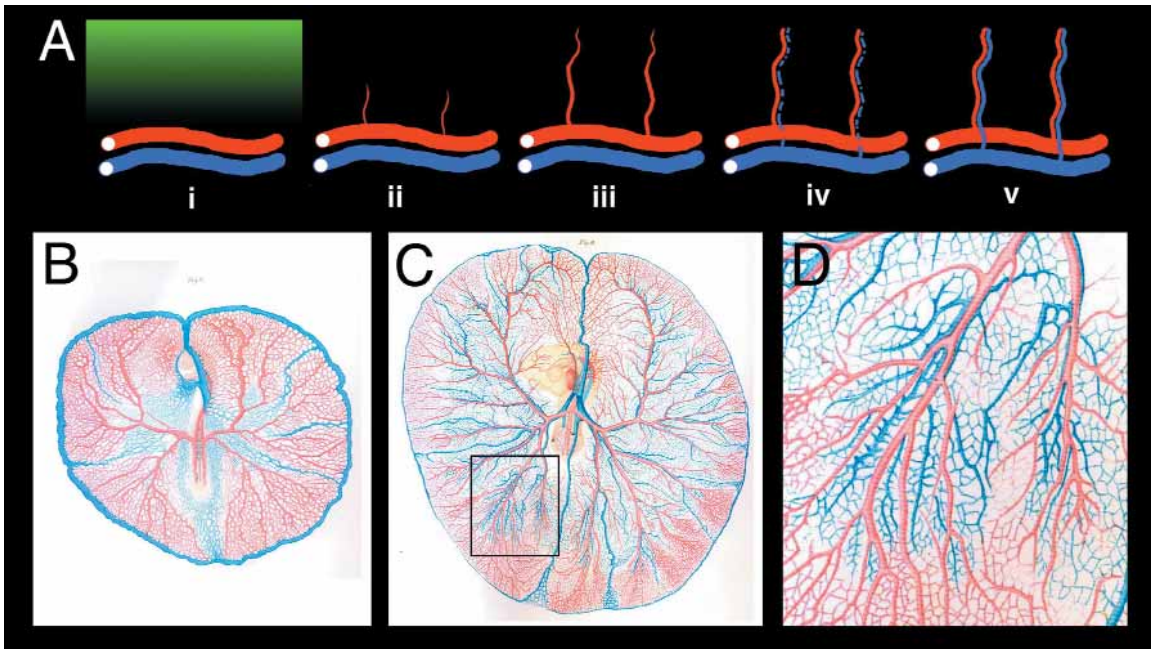

Figure 6. A model for VEGF-dependent vascular development. (*A*) In response to VEGF (*green* shaded gradient in panel *i*), arterial vessels appear (*ii*) and establish an initial defined arterial network (*iii*). A venous vascular network (*blue*) then coalesces adjacent to the arterial vessels (*iv*, *v*) to provide a venous circulatory return system. Evidence that vessels form in this way has existed in the literature for many years. Descriptive studies of the vasculature of the chick yolk sac showed that the yolk sac vasculature develops in a manner strikingly similar to the predictions of this model. (*B*) Initially a complex branched arterial network emerges from the vascular plexus, with venous drainage from much of this system primarily centrifugal, via the marginal vein at the rim of the area vasculosa. (*C*) At later stages, new intermedial and centripetally draining collateral veins emerge. (*D*) Higher magnification of the boxed region in *C* shows how this new venous drainage system takes shape via remodeling of the plexus of vessels surrounding the arteries. (*B–D*, modified from Popoff 1894.)

in mice were used to probe the expression profile of ephrinB2 (Gale et al. 2001; Shin et al. 2001) also revealed that ephrinB2 is selectively expressed "at sites of secondary angiogenesis in the embryo as well as sites of normal and pathological angiogenesis in the adult" (Gale et al. 2001). The vessels in these locales were not exclusively ephrinB2-positive, however; many PECAM-positive but ephrinB2-negative vessels were also seen. The presence of non-arterial vessels likely indicates that the two phases of artery and vein formation occur asynchronously throughout adjacent areas of a tissue. In addition, as noted above for the yolk sac, the emerging arterial system may be surrounded by a plexus of other vessels that lack arterial character, but that are not necessarily part of a morphologically defined venous network. In fact, recent evidence suggests that venous identity may represent a "ground state" in A-V differentiation (Lawson et al. 2001, 2002). When clear temporal separation between artery and vein formation is noted, this might represent situations where the arterial system has an alternative means of drainage initially, or where functioning of the system is not immediately necessary or desirable. The alignment of nerves and arteries in the skin and the role of VEGF in bringing this about (Mukouyama et al. 2002) help to explain how this model can also be used to form a vascular network with a defined and reproducible pattern. VEGF expressed from particular cells, tissues, and organs could direct artery formation in reproducible positions with respect to preexisting anatomical structures. In the skin, peripheral nerves provide VEGF; in the case of other organs and tissues, a VEGF-expressing "template" for artery formation might be provided by other cell types. In the retina, for example, astrocytes expressing VEGF induce vascular outgrowth, with migrating astrocytes leading an outward "front" of vessel assembly (Stone et al. 1995; Zhang et al. 1999).

MANY NEW QUESTIONS

Although we now understand a great deal more about how veins and arteries differ and how these differences come about than we did a few years ago, what we have learned has raised more questions than it has answered. How widely generalizable is the artery-specific role of VEGF, and the two-step model for vessel formation that we have proposed? What are the relative roles of different VEGF isoforms in arterial differentiation and the other functions of VEGF? Are there factors other than VEGF that play a similar arterial differentiation role in different contexts? In this regard, it is interesting to note the recent discovery of a novel endocrine-specific vascular growth factor, EG-VEGF. Does this factor have an arterial inducing activity similar to that of VEGF? If VEGF is primarily driving arterial patterning, what is responsible for the pattern of veins? Are flow dynamics the driving force, or are there novel artery-specific signals for

vein assembly? These and many other questions are sure to occupy the minds and experimental activities of vascular biologists in the years to come.

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