Arterial Venous Specification During Development

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This Review is part of a thematic series on **Arterial Specification: A Finishing School for the Endothelium,** which includes the following articles:
Role of Crosstalk Between Phosphatidylinositol 3-Kinase and Extracellular Signal-Regulated Kinase/Mitogen-Activated Protein Kinase Pathways in Artery–Vein Specification [2008;103:573–579]
Branching Morphogenesis [2008;103:784–795]
Brothers and Sisters: Molecular Insights Into Arterial–Venous Heterogeneity [2008;103:929–939]
Shared Circuitry: Developmental Signaling Cascades Regulate Both Embryonic and Adult Coronary Vasculature [2009;104:159–169]
Guidance of Vascular Development: Lessons From the Nervous System [2009;104:428–441]

Arterial–Venous Specification During Development

Michael Simons, Guest Editor

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Matthew R. Swift, Brant M. Weinstein

Abstract—The major arteries and veins of the vertebrate circulatory system are formed early in embryonic development, before the onset of circulation, following de novo aggregation of "angioblast" progenitors in a process called vasculogenesis. Initial embryonic determination of artery or vein identity is regulated by variety of genetic factors that work in concert to specify endothelial cell fate, giving rise to 2 distinct components of the circulatory loop possessing unique structural characteristics. Work in multiple in vivo animal model systems has led to a detailed examination of the interacting partners that determine arterial and venous specification. We discuss the hierarchical arrangement of many signaling molecules, including Hedgehog (Hh), vascular endothelial growth factor (VEGF), Notch, and chicken ovalbumin upstream-transcription factor II (COUP-TFII) that promote or inhibit divergent pathways of endothelial cell fate. Elucidation of the functional role of these genetic determinants of blood vessel specification together with the epigenetic factors involved in subsequent modification of arterial–venous identity will allow for potential new therapeutic targets for vascular disorders. **(*Circ Res*. 2009;104:576-588.)**

Key Words: arterial–venous specification ■ Hh ■ VEGF ■ Notch ■ COUP-TFII

The vertebrate cardiovascular system, consisting of the heart, blood, and blood vessels, is the first organ to function during embryogenesis. The circulatory system plays many essential roles, including transporting oxygenated blood, metabolites, and waste products, serving as a conduit for hormonal communication between distant tissues and facilitating rapid deployment of immune responses to distal sites within the body. Further organogenesis during development is totally dependent on the delivery of oxygen and nutrients facilitated by a functional circulatory system, and major defects in the developing vasculature lead to early embryonic lethality. The proper functioning of the circulatory system as a closed loop continually recirculating blood to and from peripheral tissues is itself dependent on the fundamental division of the circulatory system into 2 distinct and separate yet completely intertwined and interconnected networks of arterial and venous blood vessels. Although the existence of these 2 distinct types of blood vessels has been appreciated for hundreds if not thousands of years, we have only begun to appreciate the functional and molecular differences between the cells that line these 2 types of vessels, how these differences are acquired, and how these 2 separate yet intimately associated vascular networks are assembled.

During early development cardiac contraction begins high-pressure blood flow through large diameter arterial vessels, which acquire an extensive supporting system of smooth

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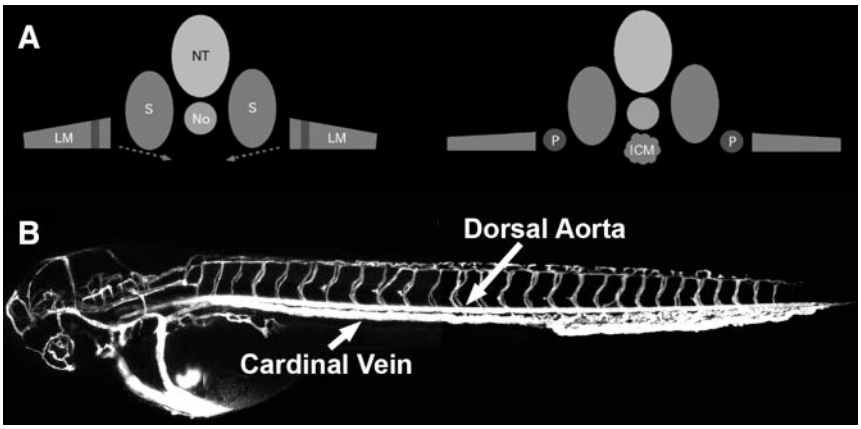

Figure 1. A, ICM formation and vasculogenesis. In this schematic cross-section of the developing zebrafish trunk, blood and vascular progenitors delaminate from medial potions of the lateral plate mesoderm (LM) and migrate to the trunk midline beneath the somites (S), taking up position to form the ICM just below the notochord. ICM angioblasts assemble the zebrafish axial vessels (dorsal aorta and cardinal vein) by vasculogenesis. B, Confocal microangiography of the circulatory system of a 2.5-day-old zebrafish reveals the 2 unpaired longitudinally aligned axial vessels, the dorsal aorta and the cardinal vein.

muscle cells and extracellular matrix components. Blood returns under lower pressure to the heart through the venous system, which includes specialized valve structures to maintain proper directional flow. The distinct hemodynamic forces found in arteries and veins, such as blood flow rate, direction, and pressure, were long thought to be the key factor driving differentiation of vessels to an arterial or venous fate, and a number of studies have supported the idea that hemodynamic forces have the capacity to program or redirect the specification of blood vessel type during development. However, other studies have demonstrated that arteries and veins possess distinct molecular identities from a very early stage. Even at the level of the smallest capillaries, arterial and venous blood vessels are distinguishable from one another by their differential expression of these molecular markers. The fact that these molecular distinctions are evident even before the initiation of circulatory flow suggests that genetic determinants play a critical role in dictating at least the initial steps of arterial/venous fate determination during development. Through extensive characterization of endothelial cells (ECs) in culture and endothelial differentiation and blood vessel assembly in in vivo models such as chick, mouse, frog, and zebrafish, a picture of the interacting signaling complexes that comprise the molecule program for arterial–venous specification has begun to emerge. In this review, we discuss some of the recent literature on the physiological and molecular factors that regulate the acquisition of arterial and venous differentiated identity.

The Emergence of Endothelium and Arterial–Venous Identity

Two major processes have been described for formation of blood vessels during development, as well as postnatally. First, major embryonic vessels form by coalescence of individual endothelial progenitor cells or "angioblasts" that arise de novo from extraembryonic and embryonic lateral mesoderm. These progenitors form vesicles and cords of attached vascular ECs that undergo further morphogenesis to form epithelial tubes. This process, called "vasculogenesis," is thought to be mainly restricted to early vascular development. Most later developmental and postnatal blood vessel formation, however, occurs via "angiogenesis," defined as the formation of new vessels from preexisting vessels, either by sprouting and elongation of new vessels from these existing vessels (sprouting angiogenesis) or by remodeling of existing vessels via internal division of preexisting vessels in the capillary plexus (intussusceptive angiogenesis). In later development and adult life, these 2 types of vessel formation processes often occur together and the distinction between them is frequently not so clear. In addition to the earliest extraembryonic and lateral mesoderm, angioblasts are also thought to arise de novo at later stages of development from other mesodermal tissues such as the paraxial/somitic mesoderm, mesodermal mesenchyme, and possibly even hematopoietic progenitors.

A proposed mesodermally derived progenitor cell, the hemangioblast, is thought to be the precursor to both pluripotent hematopoietic stem cells, which produce all of the different blood cell lineages, and angioblasts, which give rise to the first vascular ECs.[1] In zebrafish, a caudal population of progenitor cells originating in the posterior lateral plate mesoderm migrate to the midline of the trunk to form the intermediate cell mass (ICM) (Figure 1). The ICM is positioned ventral to the notochord and is flanked laterally by the somites.[2–5] Lateral mesoderm-derived progenitors give rise to both blood and ECs in the ICM. Fate-mapping studies of mesodermal progenitors labeled at midblastula and gastrula stages suggest that at least some of the progenitor cells are bipotential, capable of giving rise to cells of both lineages, although the majority of labeled cells give rise to only 1 of the 2 lineages.[1] ICM angioblasts assemble via vasculogenesis into 2 longitudinally aligned trunk axial vessels: the dorsal aorta (DA), which lies ventral to the notochord, and the posterior cardinal vein (PCV), found just below and immediately juxtaposed to the DA. In zebrafish there is only a single unpaired DA and PCV, but in many other vertebrates one or the other of these vessels are initially bilaterally paired on either side of the midline and only fuse together to form a single tube at later stages of development (in mammals the DA and PCV are initially both paired, whereas in *Xenopus*, the PCV are initially paired but the DA assembles as a single tube). As noted above, the initial expression of markers of arterial and venous identity occurs very early, before the initiation of a heart beat and circulatory flow (Figure 2). Indeed, fate-mapping studies in the zebrafish have suggested that angioblasts become restricted to either an arterial or venous cells fate as, or even before, they begin to migrate to the trunk midline to form the DA and PCV.[6] These data

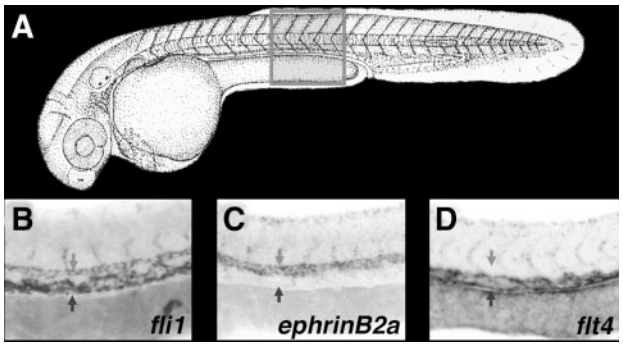

Figure 2. Arterial and venous ECs have molecularly defined identities that are evident before circulatory flow or even tubulogenesis. Expression of artery markers such as *ephrinB2a* (C) and vein markers such as *flt4* (D) is evident by in situ hybridization of 25 somite stage zebrafish embryos, several hours before circulation begins in the trunk. Expression of *ephrinB2a* within the dorsal aorta begins just as the migratory ECs arrive at the trunk midline from the lateral mesoderm and begin to aggregate into a cord of cells. B, Expression of the pan-endothelial marker *fli1* is shown for comparison. Box in upper diagram (A) shows approximate location of in situ images, for reference. Light arrows indicate dorsal aorta; dark arrows, posterior cardinal vein.

suggest that the acquisition of early arterial–venous cell fate by endothelium is genetically programmed, and other recent studies have begun to define many of the molecular players that regulate this process.

Genetic Determinants of Arterial–Venous Identity

After the initial identification of molecular differences between arterial and venous ECs, described above, further research has identified many additional factors differentially expressed between arterial and venous ECs. More importantly, the functional roles of some of these factors and the signaling pathways that they participate in have been explored in some detail, and we are now beginning to acquire a picture, albeit still highly incomplete, of how some of these pathways act together in the determination of arterial–venous fate. Below, we review some of the pathways and factors implicated in arterial–venous differentiation and recent data on their respective roles.

EphrinB2/EphB4

The first genes discovered to be differentially expressed in arterial and venous endothelium were ephrinB2 and EphB4, members of the Eph-ephrin subclass of receptor tyrosine kinases. The Eph receptors are the largest of the fourteen subfamilies of receptor tyrosine kinases and are activated by ligands of the equally large ephrin family. The receptors and their cognate ligands are usually, although not always, present in adjacent populations of cells.[7] Eph receptors and their ephrin ligands regulate a variety of morphogenetic processes in different tissues, including hindbrain and paraxial mesoderm segmentation, axonal guidance and fasciculation during the formation of topographical maps in the vertebrate embryonic nervous system, and regulation of cell movement in both vertebrates and invertebrates, including *Xenopus* gastrulation, avian and rodent neural crest migration, and neuroblast and epidermal cell movement in nematodes.[8–10] Ephs and ephrins are both transmembrane proteins and Eph-ephrin signaling requires cell-to-cell contact. One somewhat unusual aspect of Eph-ephrin signaling is that it can be bidirectional. Both forward (ephrin ligand to Eph receptor) and reverse (Eph receptor to ephrin ligand) signaling has been documented. Forward signaling is initiated by ephrin ligand engagement by an Eph receptor dimer, which leads to transphosphorylation of the short intracellular juxtamembrane region. This results in a conformational change in the receptor that activates its kinase domain and enables a variety of proteins implicated in the regulation of mitogenesis, cell substrate interactions, and cytoskeletal dynamics to be phosphorylated by the kinase domain or to interact with the other regions of the cytoplasmic tail. On the other hand, reverse signaling by ephrins of the B subclass involves the phosphorylation of conserved tyrosine residues in the small, approximately 85-aa-long cytoplasmic domain upon contact with the ectodomain of cognate EphB receptors, or by an Eph-independent mechanism. This leads to recruitment of a variety of SH2 (Src homology-2) domain containing adaptor proteins and their SH3 (Src homology-3) binding partners and results in cytoskeletal changes usually associated with cell repulsion. A short PDZ-binding motif YKV at the end of the carboxyl terminus also appears to mediate reverse signaling via a phosphorylation-independent mechanism (reviewed elsewhere[11]).

In a landmark study on the molecular basis of arterial–venous cell fate, ephrinB2 and EphB4 tau-lacZ "knockins" were used to show that the 2 genes are differentially expressed in the arterial and venous endothelium of the mouse embryo before the initiation of circulation.[12,13] This provided the first evidence demonstrating that the initial acquisition of molecular differences between arteries and veins during development is not dependent on circulatory flow. Because these were null alleles, they were also used to explore the functional consequences of loss of ephrinB2-EphB4 signaling in the vasculature. Animals lacking ephrinB2 undergo vasculogenesis of the major arterial and venous trunk vessels in a reasonably normal fashion but have defects in the remodeling of both arteries and veins.[12] Notably, there is a failure of proper intercalation of arterial and venous vessels. Overexpression of a dominant negative form of EphB4 in *Xenopus* embryos gives rise to defects in vascular remodeling within intersomitic vessels (ISVs).[14] These observations suggest that the complementary expression of the ephrinB2 ligand and its EphB4 receptor in arterial and venous endothelium mediates signaling between these 2 cell types required for the proper remodeling of arterial and venous vascular networks and for the establishment of complex but distinct boundaries between them. Although ephrinB2 is also expressed in vascular smooth muscle cells, subsequent EC-specific knockouts demonstrated that the gene is critically required in ECs, at least for its earliest vascular functions.[15] The functional importance of ephrinB2 in vascular development was further confirmed by a later study in which it was also shown that mice in which the EphB4 locus is inactivated by inserting a tau-LacZ reporter display vascular defects that parallel those found in mice lacking ephrinB2.[16] EphB4 is highly enriched in veins,

and is the only EphB receptor that specifically binds to ephrinB2. The symmetrical nature of the ephrinB2 and EphB4 phenotypes and the strong and specific affinity between this ligand-receptor pair indicates that EphB4 is the principal functional partner for ephrinB2 in this system and suggests that the angiogenic defects and failures to intercalate arteries and veins observed in ephrinB2 and EphB4 loss-of-function mutants result from defective arterial–venous communication.

The respective functional roles of forward versus reverse ephrinB2-EphB4 signaling have been somewhat more difficult to parse out. Expression of ephrinB2 full-length and cytoplasmic domain deletion mutants in *Xenopus* results in the identical ISV defects as is observed with dominant negative EphB4 mutants, indicating that remodeling occurs through forward, not reverse signaling.[14] Conversely, mice designed to be specifically impaired in ephrinB2 reverse signaling because of lack of the entire cytoplasmic domain (deletion of approximately 85 C-terminal amino acids) exhibited similar vascular remodeling defects to those displayed by mice completely deficient for ephrinB2 or EphB4, suggesting a potentially mammalian specific essential requirement for bidirectional communication.[17] Furthermore, increased EphB4 expression in B16 Melanoma cells impairs the survival of arterial ECs in mouse tumor xenograft model systems by reverse signaling via ephrinB2.[18]

It is worth noting that although the expression of ephrinB2 and EphB4 expression "labels" arterial and venous ECs and their precursors, the function of these genes is not required for specification of these cell fates during vasculogenesis. Appropriate spatially partitioned early expression of knocked-in lacZ transgenes is still observed in mice homozygous null for either ephrinB2 or EphB4, although these mice subsequently exhibit defects in interdigitation and remodeling of arterial and venous vascular networks, suggesting these genes are required to define and maintain the arterial–venous interface. In contrast to ephrinB2 and EphB4, some of the genes discussed in the sections below do appear to play an important role in the arterial–venous cell fate decision.

Notch

Notch receptors are evolutionarily conserved type I transmembrane receptors that interact with the DSL (Delta-Serrate-Lag2) ligands to direct cell-fate decisions during embryonic development.[19] Receptor–ligand interaction results in the translocation of the Notch intracellular domain (NICD) into the nucleus following proteolytic cleavage events. On nuclear transport, the NICD can bind the transcriptional regulator SuH (suppressor of hairless) in zebrafish and its mammalian ortholog Rbpj protein (the recombination signal-binding protein for immunoglobulin-κ J region) in mice. Notch signaling regulates a variety of events in addition to cell fate decisions, including proliferation, apoptosis, and maturation and is known to be involved in neuronal function, ventricular development, pancreatic specification, hematopoiesis, and osteoblastic differentiation.[20–23] Notch signaling also regulates tissue homeostasis and maintenance of stem cells in adults.[19,24,25] Owing to the complexity of the pathway, the consequences of Notch signaling are highly diverse and dependent on multiple variables including both the spatial and temporal characteristics of the signal and the cellular and developmental context (reviewed elsewhere[26]).

A variety of evidence from mammals has highlighted the importance of notch signaling for proper formation of the vasculature. The human vascular diseases Alagille syndrome and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) are caused by mutations in the Jag1 and Notch3 genes, respectively. In mice, the Notch 1, Notch4, Jag1, Jag2, and Dll4 genes are all expressed in arterial but not venous ECs.[27–36] Mice lacking Notch4 have no major defects in vessel formation, but Notch1 knockout mice exhibit a reduction in size of axial vessel, failure to properly remodel the vasculature, and die during embryonic development. Notch1/Notch4 double mutants show abnormal development in the axial vessels that is more severe than in Notch 1 mutants,[30] suggesting that the 2 genes are at least partially functionally overlapping. Mice lacking even 1 of the 2 copies of the Notch ligand Dll4 are not viable in at least some genetic backgrounds and exhibit severe vascular defects.[30,37] Dll4 heterozygous mice also show reduced ephrinB2 expression and increased EphB4 expression, consistent with a failure in arterial differentiation.[38] This defect is similar to that observed in Notch-deficient Rbpj and Mib mutant mice and Hey1/Hey2 double mutants.[39–42] These results implicate Dll4-Notch1/4 and their downstream effectors in proper vessel formation during murine development and suggest that Notch activity is important for promoting arterial cell fate.

A number of studies in the zebrafish have been critical for defining the important role that Notch signaling plays in arterial specification.[6,43,44] In zebrafish, the *notch5* (formerly *notch3*) gene is expressed in the DA but not the PCV during embryonic development. Embryos lacking Notch activity, either through the injection of a dominant negative Su(H) or in a notch-deficient mindbomb (*mib*) mutant, fail to induce arterial *ephrinB2* expression and exhibit ectopic expression of venous markers *EphB4* and *flt4* in the DA.[43] Constitutive activation of Notch signaling by ubiquitous expression of the "activated" NICD results in reduced venous marker expression. Expression of NICD specifically in endothelium of the zebrafish also suppresses venous marker expression, confirming that Notch functions cell-autonomously within the endothelium to repress venous fate. Like ephrinB2$^{-/-}$ and EphB4$^{-/-}$ mice, Notch-deficient zebrafish possess a properly located DA and PCV, again indicating that the observed phenotype is attributable to a defect in arterial specification and not angioblast migration or vascular tube morphogenesis.[43]

A role for Notch signaling in artery formation has also been suggested by analysis of another zebrafish mutant, gridlock (*grl*), defective in a zebrafish ortholog of mammalian *Hey2* (hairy and enhancer of split-related 2).[6,45] The *Hes* (hairy and enhancer of split) and *Hey* genes have been shown to be function in at least some contexts as downstream targets of the Notch pathway. Zebrafish embryos deficient in *grl* fail to properly form the dorsal aorta and lack circulation to the trunk and tail.[46,47] The *grl* gene is initially expressed in the posterior lateral mesoderm, becoming restricted to the DA

during later stages of development.[45] The expression of *grl* is induced by activated *notch1* and can be repressed by inhibitors of Notch signaling.[6] Blocking *grl* translation using antisense morpholino oligonucleotides (morpholinos) results in arterial defects, including reduction of *ephrinB2* expression and increase in *EphB4* expression in the DA. Overexpression of *grl* yields a smaller PCV with loss of *flt4* expression but has no effect of DA formation. Although the results above indicate that *grl* is needed for artery formation, other studies have shown that *grl* is not repressed in embryos injected with dominant negative Su(H) or in *mib* mutants,[43] suggesting that *grl* is not a direct Notch target, making the role of gridlock in arterial differentiation somewhat less clear.

Although arterial specification is disrupted in zebrafish embryos lacking Notch activity, some artery-specific markers are still expressed in Notch-deficient animals, suggesting that additional upstream factors may help to regulate arterial differentiation.[43] Sonic hedgehog (shh) and vascular endothelial growth factor (VEGF) have been shown to function upstream from Notch signaling in a pathway regulating early arterial differentiation.

Hedgehog

The Hedgehog (Hh) family of secreted morphogens, which includes sonic hedgehog (shh) and Indian hedgehog (ihh), has diverse roles in embryogenesis and patterning. Hh signals through the interaction with a transmembrane receptor Patched (ptc) to release ptc-mediated inhibition of the transmembrane protein Smoothened (smo), leading to downstream activation of target genes through the Gli family of transcription factors. A number of studies have indicated a role for Hh signaling in vascular development. Shh is expressed in the endoderm, proximal to the developing vascular network but not in the mesoderm. However, the ptc1 receptor is expressed on ECs.[48] Overexpression of shh in the mouse leads to hypervascularization of neuroectoderm, whereas decreased vascularization of lung tissue is observed in $shh^{-/-}$ mice.[49,50] Expression of the proangiogenic factor Angiopoeitin-1 and its cognate receptor, Tie2 is also decreased in lung tissue of embryonic day (E)11.5 to 12.5 $shh^{-/-}$ mouse embryos.[51] Furthermore, shh is able to induce the expression of vascular endothelial growth factor and the angiopoietins in mice, which can induce the development of coronary vessels.[48,52,53] Hh signaling is a critical instructive endodermal signal triggering the assembly of the first primitive vessels in the mesoderm of the mouse yolk sac,[54] activating the critical vasculogenic ligand Bmp4 via Foxf1.[55] $Ihh^{-/-}$ and $smo^{-/-}$ mouse embryos display the initiation of vasculogenesis in the yolk sac but exhibit defects in vascular remodeling, suggesting that Hh signaling promotes EC differentiation and vascular remodeling but is not required for cell-fate determination.[54] Hh signaling has also been implicated in hemangioblast and hematopoietic stem cell specification both in mice and in the zebrafish.[5,56] The steroidal alkaloid cyclopamine (CyA) can inhibit smo activation, thereby conditionally blocking Hh signaling. Yolk sac vessels in mouse embryos treated with CyA are underdeveloped and proangiogenic factors such as VEGF and Notch-1 are downregulated. CyA treatment also inhibits proper fusion of the paired DA assemblies perhaps because of downregulation of Bmp4 and VEGF.[57]

Studies in the zebrafish have been instrumental in demonstrating the important role that Hh signaling plays in early arterial specification. Zebrafish with mutations in *syu* (sonic you), the zebrafish ortholog of mammalian *shh*, lack normal trunk circulation, and have defects in differentiation of both the DA and PCV, although angioblasts do migrate normally from the lateral plate mesoderm to the trunk midline.[58,59] However, embryos lacking *shh* or treated with CyA only develop a single large axial vessel tube expressing markers of venous but not arterial identity.[44] In contrast, injection of *shh* mRNA induces formation of ectopic *ephrinB2*-expressing arterial vessels within the trunk. Upregulation of other arterial markers, including the Notch ligands *deltaC* and *notch5,* is also observed in *shh* mRNA–injected animals.[44,60] Together, these results place *shh* upstream of Notch in regulating arterial specification. *Shh* is expressed in the notochord at the midline of the developing zebrafish embryo, immediately above the developing DA, suggesting that it could be providing an important direct inductive signal for DA formation. As noted above, a number of studies have shown that Hh plays an important inductive role in yolk sac vasculogenesis,[54,55] acting as a direct signal promoting proper vascular morphogenesis and tube formation both in vitro and in vivo.[61] However, other data from the fish have shown that *shh* acts indirectly to promote arterial differentiation of the DA via induction of *vegf* expression in the adjacent somites. Additionally, studies in fish using CyA to study the timing of Hh requirement for DA formation show that Hh is also needed independently for the medial migration and arterial specification of DA progenitors and for angiogenic sprouting of primary ISVs from the DA.[5]

Vascular Endothelial Growth Factor

The secreted growth factor VEGF-A, found in multiple isoforms in mammals, including VEGF120, VEGF164, and VEGF188, has been implicated in cell differentiation, proliferation, migration, and survival (the murine isoforms listed possess one less amino acid than their human counterparts). VEGF-A signals through multiple receptor tyrosine kinases including fetal liver kinase 1 (flk1) (also known as VEGFR2), fms-like tyrosine 1 (flt1) (also known as VEGFR1), flt4 (also known as VEGFR3), and neuropilin (NP)1 and NP2.[62] VEGF-A is produced by multiple cell types, including macrophages and smooth muscle cells, and regulates differentiation, proliferation, and survival of ECs.[63] VEGF-A is essential for embryonic vasculogenesis and angiogenesis and its expression localizes to the sites of blood vessel development throughout the embryo.[64–67] The 3 major VEGF-A isoforms differ in their diffusion properties. The lowest-molecular-weight VEGF120 isoform is freely diffusible, whereas the highest-molecular-weight VEGF188 isoform is tightly bound to cell surface heparan sulfate proteoglycans. VEGF164 possesses characteristics intermediate to the other 2 isoforms.[63,68] The multiple VEGF isoforms appear to act in concert to independently guide aspects of EC migration and proliferation.[69] Mice engineered to express only VEGF120 or VEGF188 possess impaired postnatal myocardial angiogen-

esis and impaired renal and retinal blood vessel branching.[70–72] Mice expressing only VEGF164 display no vascular defects.[73] VEGFR1 and -2 are expressed in vascular ECs, whereas VEGFR3 in mainly confined to lymphatic endothelium but is an early marker for venous cell fate in zebrafish. The neuropilins may act in concert with VEGFR2 as a coreceptor to facilitate downstream signaling of the VEGF164 (but not VEGF120) isoform for VEGF-A. NP1 is expressed by arterial ECs, whereas NP2 is expressed in venous and lymphatic ECs.[74–80]

Mice either homozygous or heterozygous for VEGF-A are embryonic lethal between E10 and E12 because of complications in cardiac development and dorsal aorta morphogenesis and an overall reduction in vascularization.[81,82] The haploinsufficient lethal phenotype of VEGF-A highlights the critical importance of this ligand and its proper expression for vascular development. Transgenic overexpression of VEGF-A in the murine heart results in an overall increase in cardiac arterial vessels.[83] VEGFR1$^{-/-}$ mice are embryonic lethal at E8.5 to E9.5 because of impaired vascular development, and VEGFR2$^{-/-}$ mice die at the same stage from defects in vasculogenesis and hematopoiesis.[84,85] Interestingly, VEGFR3$^{-/-}$ mice also are lethal at E9.5 and display large, unorganized, and poorly lumenized vessels, leading to cardiovascular failure, indicating an early role for VEGFR3 in vascular development before becoming specialized to the lymphatics.[86,87] NP1$^{-/-}$ mice die between E12.5 and 13.5 with defects in yolk sac and embryonic vascular formation.[88,89] Although NP2$^{-/-}$ display only minor defects in lymphatic development, NP1/NP2 double knockouts display a much more severe vascular phenotype than NP1 alone, including lack of capillary formation and blood vessel branching, and, in some regions, complete lack of vascularization, a phenotype similar to that observed in VEGF-A/VEGFR2 double knockout mice.[90]

A great deal of evidence has shown that VEGF-A is critical for vascular patterning through its effects on arterial specification, proliferation, and migration. Again, studies in the zebrafish have been important for demonstrating the role of *vegf* in arterial differentiation. During embryogenesis, zebrafish express the VEGF-A isoforms VEGF120 and VEGF164 in the axial vasculature, medial regions of the somites, the central nervous system, and mesoderm.[91] The high-molecular-weight VEGF180 isoform is encoded by a separate gene in zebrafish.[92] Zebrafish VEGFR1 and VEGFR2 are specifically expressed in blood vessels.[2,93–95] Zebrafish also possess duplicated copies of each of the neuropilin genes, and all 4 neuropilins are expressed in the embryonic vasculature and nervous system.[96,97] Somitic expression of *vegf* is dependent on Hh signals from the notochord. *Syu* mutants or CyA-treated animals fail to express vegf in the somites, whereas injection of *shh* mRNA results in an upregulation of somitic *vegf*.[44] Injection of morpholinos targeting *vegf* into zebrafish embryos results in a strong reduction in arterial *ephrinB2* expression and arterial cell fate, with concomitant upregulation of the venous marker *flt4*. Expression of *notch5* is also specifically reduced in the DA. Furthermore, injection of *vegf* mRNA rescues vascular *ephrinB2* expression in *shh*-deficient embryos. Together, these results indicate that *vegf* functions downstream from *shh* to nonautonomously promote arterial fate determination by its expression in the somites. Notch ligands and receptors, in contrast, are expressed autonomously within the endothelium, suggesting that Notch may function downstream from *vegf* in arterial fate determination. Additional functional studies carried out in the fish verified that this is indeed the case. In contrast to the ability of *vegf* to rescue arterial differentiation in Hh signaling–deficient animals, injection of *vegf* mRNA does not promote arterial differentiation in Notch-deficient embryos. Instead, expression of activated Notch ICD in zebrafish is able to promote *ephrinB2* expression and arterial differentiation in *vegf* morpholino-injected animals.[44] Together, all of these results reveal a hierarchical pathway for establishment of arterial cell fate (Figure 3). Expression of *shh* in the notochord induces expression of *vegf* in the adjacent somites, which in turn induces Notch signaling in the endothelium of the assembling DA, promoting arterial and suppressing venous cell fate. Of course, in addition to its role in arterial differentiation VEGF signaling also plays an important role in vasculogenesis and angiogenesis in the fish, as it does in other vertebrates. Exogenous addition of a VEGFR2 kinase inhibitor to one cell stage zebrafish embryos results in the complete lack of axial vessel development, whereas treatment at 24 hours postfertilization impedes ISV sprouting,[98] underscoring the temporal requirement of VEGF signaling for both vasculogenesis and angiogenesis. Zebrafish embryos injected with morpholinos targeting *np1* also show vascular and circulation defects at 36 hours postfertilization, similar to the later effect of disrupted VEGF signaling observed in NP1 null mice.[99]

Additional work carried out in mice and in cell culture has verified the important role that VEGF plays in establishing arterial fate. Transgenic mice expressing VEGF164 in cardiac muscle display increased numbers of ephrinB2-positive and a reduction in EphB4-positive capillaries.[83] In contrast, mice that express only VEGF180 but lack the lower-molecular-weight isoforms have normal outgrowth of veins but deficient arterial development in the retina,[73] suggesting that VEGF180 alone is capable of supporting significant vessel formation but that the lower-molecular-weight isoforms are required for arterial differentiation. Mouse embryonic angioblasts cultured in vitro with VEGF120 or VEGF164 can be induced to express artery markers and undergo arterial specification.[79] Local tissue sources may promote vascular remodeling of primitive capillary networks via VEGF-A signaling. In vivo, VEGF-A is highly expressed in peripheral nerve of mouse limb skin and local secretion of VEGF-A may promote blood vessel association and arteriogenesis and act as permissive inducing signal rather than an instructive determinant of arterial cell fate.[79] At this level, arteriogenesis is dependent on an NP1-mediated positive feedback loop involving the preferential recruitment of VEGF164.[100] These results, together with the discovery in human arterial but not venous ECs that exogenous VEGF induces expression of Notch1 and Dll4, suggest that the hierarchal arrangement of VEGF and Notch remains intact in mammalian arterial differentiation. NP1 is also expressed specifically in the arteries of avian embryos, suggesting that VEGF plays a similar role in avian

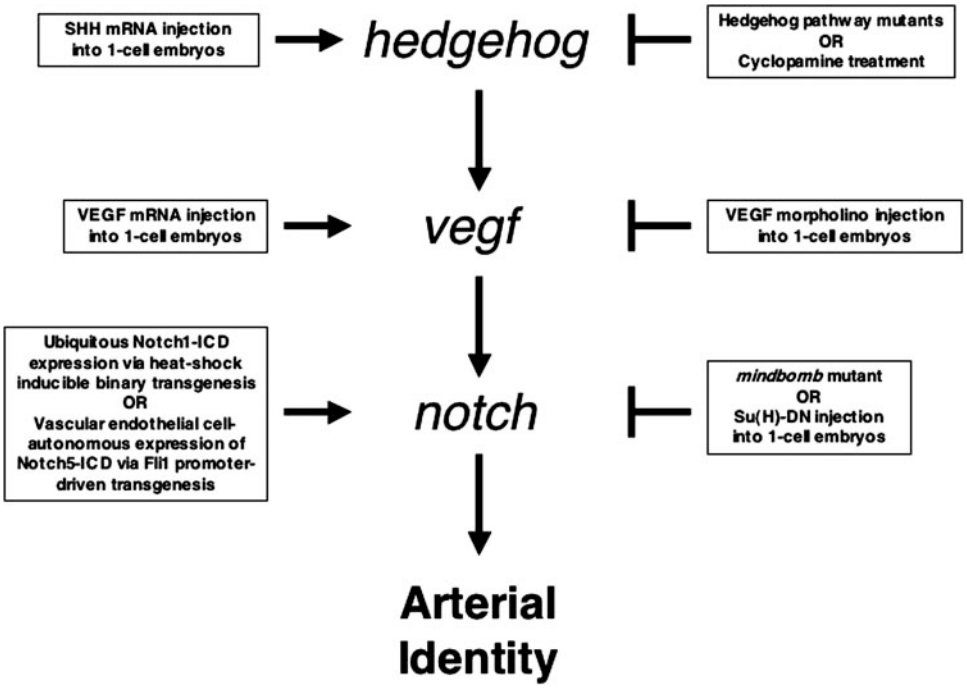

Figure 3. A molecular pathway for arterial–venous fate determination. Studies in the zebrafish have shown that vascular endothelial growth factor (vegf) acts downstream of shh and upstream of the Notch pathway to determine early arterial cell fate. A variety of different methods were used to either increase (left side) or decrease (right side) the levels and/or activities of each of these signaling pathways, as shown here and described in the text. Loss of Notch, Vegf, or Shh signaling results in loss of arterial identity, whereas exogenous activation or overexpression of these factors causes ectopic expression of arterial markers. "Molecular epistasis" experiments were performed by combining different methods to assemble these components into an ordered pathway. For example, microinjection of *vegf* mRNA into embryos homozygous mutant for hedgehog pathway genes can rescue their arterial differentiation defect. Likewise, inducible transgenic activation of the Notch pathway in zebrafish embryos rescues the loss of arterial marker gene expression caused by "knock-down" of vegf signaling.

arterial specification.[75] A number of different intracellular signaling pathways are known to function downstream from the VEGF receptors, and additional studies have begun to dissect the involvement of some of these pathways in arterial differentiation.

Extracellular Signal-Regulated Kinase/Phosphatidylinositol-3 Kinase Pathways

A phenotype-based small-molecule chemical screen performed on zebrafish embryos identified compounds that activate the VEGF pathway and can rescue the loss of DA specification in *grl* mutants.[101] In this screen, chemical regulators of extracellular signal-regulated kinase (ERK) or phosphatidylinositol-3 kinase (PI3K), 2 competing downstream signaling molecules that are activated by VEGF/VEGFR signaling, were shown to regulate Notch activation by promoting arterial or venous cell-fate specification in ECs.[101] A second small-molecule chemical screen identified 2 classes of structurally unrelated PI3K/Akt inhibitors that are capable of suppressing the *grl* arterial defect phenotype. The rescue of arterial specification in *grl* mutants requires partial inhibition of PI3K thus driving preferential activation of ERK, suggesting an upstream mediator of the Ras/ERK pathway can advance arterial cell fate, whereas PI3K/Akt signaling suppresses ERK activation and promotes venous cell fate.[102]

Phospholipase C (PLC)γ-1 is an immediately downstream mediator of VEGFR2 signaling and activates ERK in the VEGF/VEGFR signal transduction cascade.[103] Plc-$\gamma^{-/-}$ mice are embryonic lethal and show severe defects in embryonic erythropoiesis and vasculogenesis.[104] Forward genetic screens have identified a zebrafish Plc-γ1 mutant that displays defects in the formation of arteries, but not veins, demonstrating that VEGF-dependent activation of the Raf/ERK signaling cascade is necessary for proper arterial specification.[105]

Activated phosphorylated ERK is preferentially expressed in zebrafish angioblasts fated to become arteries and is localized to arterial ECs in later axial vessel development. Inhibition of an upstream activator of ERK, mitogen-activated or extracellular signal-related protein kinase kinase (MEK), leads to loss of arterial ECs and the improper formation of the DA. Blockade of either the Hh pathway with CyA or the VEGF pathway with a VEGFR inhibitor results in an overall reduction in ERK activation and defects in arterial differentiation.[102] Similarly, constitutive active Akt induces venous cell fate. PI3K thus appears to promote venous fate by inhibiting ERK activation, but the site and manner in which PI3K is able to block Raf/ERK signaling and beyond are unclear.[102] Cell culture experiments suggests PI3K signaling acts to induce Notch and Dll4 activation in direct contrast to in vivo data.[106,107] The differing results from cell culture and zebrafish in establishing the hierarchy of signaling molecules involved in arterial specification are perhaps attributable to epigenetic factors found in vivo but not present in vitro. A

more detailed examination of ERK/PI3K signaling in vessel development is reviewed in Hong et al in this series.

Coup-TFII and Venous Identity

Previously, Notch had been determined to be a regulator of venous cell fate by controlling EphB4 expression. As a result, it was suggested that because arterial specification is driven by the preferential activation of the Shh/VEGF/Notch pathways, venous identity is the default differentiation pathway of ECs.[108] Recently, however, the existence of an upstream genetic factor regulating venous identity has also been identified. Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) (also known as nr2f2) acts as a positive mediator of venous specification. COUP-TFII, a member of the orphan nuclear receptor superfamily, is specifically expressed in venous but not arterial ECs, and can preferentially induce venous cell fate.[109,110] COUP-TFII$^{-/-}$ mice die at approximately E10.5 and undergo server hemorrhage and edema by E9.5 resulting from enlarged blood vessels, improper development of the atria and sinus venosus, and malformed cardinal veins.[111] Targeted disruption of COUP-TFII in mouse ECs results in venous acquisition of arterial markers ephrinB2, Jag1, Notch1, and NP1. Additionally, ectopic expression of COUP-TFII in ECs results in the fusion of arteries and veins, similar to phenotypes observed in NP1$^{-/-}$ or Notch1$^{-/-}$ mice.[88,112] COUP-TFII is proposed to establish venous identity by downregulating Notch signaling, potentially at the level of NP1, thus releasing factors such as EphB4 and flt4 from Notch-mediated repression.[110] Loss of COUP-TFII does not completely abolish EphB4 expression in ECs, however, suggesting more factors may be involved in venous specification. More investigation is required to properly establish the role COUP-TFII in regulating Notch activation.

In addition to COUP-TFII, the G protein–coupled receptor APJ is also expressed preferentially in venous ECs during early mouse embryogenesis. It is detected specifically in the venules of the developing mouse retinal vasculature and may represent an early and specific marker for venous phenotype.[113,114] The functional role of APJ and its high-affinity ligand, apelin, in the vasculature is still not clear. APJ$^{-/-}$ mice show no obvious vascular phenotype,[115] although morpholino knockdown of APJ and apelin in *Xenopus* results in defects in ISV development.[116] However, no other studies have thus far indicated a role for apelin/APJ involvement in venous identity. Rather, the apelin/APJ pathway may regulate hemodynamics and their potential epigenetic effects on arterial and venous specification.[117] Recently, studies in zebrafish indicate the apelin/APJ may be vital in the differentiation of the myocardial lineage.[118,119]

Additional Factors: Forkhead, Sox, clcr, and snrk-1

The forkhead box (fox) proteins are evolutionary conserved transcription factors that are involved in regulating gene expression and are known to be important for embryonic development. Two family members of the foxc subclass of transcription factors, foxc1 and foxc2, regulate arterial specification in mice. Foxc1 and foxc2 are expressed in the ECs and smooth muscle cells of the mouse aorta and targeted disruption of foxc1 and foxc2 results in arterial–venous malformations and aberrant expression of arterial-specific markers including Dll4 and Notch1. Venous makers COUP-TFII and EphB4 are unaffected by foxc1or foxc2 inactivation.[120,121] Notably, Foxc-binding sites are located in the upstream promoter region of the Dll4 gene and thus foxc appears to act to positively regulate Notch signaling by activating the Dll4 promoter during arterial specification. VEGF expression levels are altered in foxc null mutants, suggesting that foxc1 and foxc2 may act as downstream mediators of VEGF signaling to activate Notch pathway components and determine arterial cell fate.[121]

Members of the Sry-related HMG box (Sox) family of transcription factors have also been identified as contributing to arterial/venous differentiation in the zebrafish embryo. Sox7 and sox18 are both expressed in the posterior lateral plate mesoderm, migrating angioblasts, and developing axial vessels during embryonic development. Loss of either sox7 or sox18 has minimal effect on vasculogenesis; however, double knockdown of sox7 and sox18 results in severe arteriovenous malformations characterized by vessels fusion and blockage of trunk-tail circulation, suggesting a redundant role for sox7 and sox18 in vasculogenesis.[122–124] It is unclear whether ablation of sox7 and sox18 preferentially disrupts arterial or venous cell fate in the zebrafish embryo or at what level sox7 and sox18 act to regulate the complex of signaling molecules that drives arterial/venous differentiation.

Recently, additional new regulators of EC specification have been nominated and their potential role in the canonical model for specification of arterial and venous fates is under investigation. A microarray screen for differentially regulated genes in zebrafish vascular development identified a member of the sucrose nonfermenting kinase family (snrk-1) that may act upstream of *grl* and in parallel or downstream of notch to promote arterial cell fate.[125] Somite expression of calcitonin receptor-like receptor (crlr), a known receptor for adrenomedullin, may be under the regulation of shh, and control arterial differentiation upstream of VEGF.[126] Potential effectors of arterial/venous specification such as these will undoubtedly be located as the biology of artery–vein specification continues to be studied.

Plasticity of Arterial–Venous Identity

Although it is now clear that defined molecular pathways help regulate the acquisition of arterial–venous identity, does this mean that flow dynamics and circulation do not play a role? And once differentiated arterial–venous identity is acquired, is an EC permanently committed to this fate or can it be "reprogrammed"? This question is of critical medical importance in clinical settings where vessels of different identity are grafted together, such as during dialysis treatment or bypass surgery. Changes in the transplanted vessels after grafting,[127–131] and the significant risk of graft failure involved in these therapies,[132] does suggest a limited degree of plasticity in EC arterial–venous identity.

Two separate groups performed quail chick grafting experiments to test the plasticity of arterial–venous EC fate during early development.[75,133] Portions of embryonic arteries or veins were grafted from quail donors at various stages of

development into chick hosts, and the arterial–venous identity of donor cells contributing to different host vessels was assessed using artery- or vein-specific molecular markers. Using expression of NP1 and ephrinB2 as arterial markers and Tie2 as a vein-specific probe, both groups found that arterial or venous ECs from young donors can populate both types of vessels in host embryos and assume the appropriate molecular identity in their new locales, but this plasticity becomes progressively lost in ECs grafted from donors older than E7. However, when isolated ECs or dissected endothelial epithelia were grafted from these older donors instead of intact vascular segments, the older ECs were able to colonize both types of vessels as well as younger ECs. These results indicate that initial specification of arterial or venous cell fate is reversible and that additional inputs may influence or be required to maintain a specific arterial–venous identity. It is likely that ephrinB/EphB-mediated communication between arterial and venous cells is important for maintaining arterial–venous identity. This has been demonstrated in patent vein grafts in both humans and aged rats, in which EphB4 transcripts and immunodetectable protein are downregulated in ECs and smooth muscle cells (SMCs). This loss of EphB4 is associated with intima-media thickening during vein graft adaptation to the arterial environment. Interestingly, neither ephrinB2 transcripts, nor arterial markers such as dll4 and notch, are not strongly induced.[134] Although it does not exactly duplicate the genetic regulation of EC determination in embryogenesis, vascular adaptation as viewed by vein grafts in adult mammals maintains an adequate subset of those genes to mediate plasticity.

In vivo time lapse imaging of developing chick embryos demonstrates the plasticity of the capillary network on the onset of flow. Because the arterial network expands during development, a subset of capillary side branches of the aorta disconnect from the arterial network and appear to reconnect to the venous plexus, whereupon they lose their arterial identity in favor of a distinct venous identity.[80] The results of the avian studies also suggest that additional components of the vascular wall are necessary to maintain and/or sufficient to redirect the arterial–venous identity of adjacent ECs. SMCs or mural cells may be important for this function of the vessel wall. It may also be that the vessel wall serves simply to isolate ECs from other extrinsic inputs that influence arterial–venous identity and thus in a relatively passive way help to stabilize endothelial fate choice. Experimental studies have also shown that hemodynamic forces can alter EC identity following the onset of the circulation. Ligation of specific arteries in the avian embryo can result in the reversible switch from arterial-specific markers to venous markers.[80] Similarly, altered oxygen tension levels can modify artery and vein specification in the developing mouse retina vasculature.[135] Additional studies will be needed to determine whether SMC or other components of the vessel wall, hemodynamics, and oxygen tension actually play an instructive role in endothelial arterial–venous fate. A proposed system in which genetic predetermination controls initial arterial–venous specification and environmental inputs regulate subsequent vascular remodeling may resolve the debate between genetic versus epigenetic regulation of EC differentiation (reviewed elsewhere[136]).

Postnatal Neovascularization

In addition to their roles in embryonic vascular development, many of the molecular determinants of embryonic arterial–venous differentiation have been identified as potential regulators of adult angiogenesis. Dll4 expression is high in the capillaries and small vessels of developing ovarian follicles in sexually mature female mice but not neighboring ovarian blood vessels, suggesting a specific role for the Notch pathway in adult blood vessel remodeling and growth.[137] Examination of clear cell-renal cell carcinoma (CC-RCC) tissue samples indicates that dll4 expression is upregulated concomitant with increases in vegf in the tumor vasculature.[138] Furthermore, antibody-specific blockade of dll4 inhibits tumor growth in multiple models but does not appear to effect normal adult vascularization as observed in mouse retina.[139]

Similarly, Hh pathway components drive the revascularization of muscle tissue following mouse hindlimb ischemia, and ephrinB2 has a functional role in postnatal angiogenic response to ischemic injury.[140,141] Interestingly, the complementary expression of ephrinB2 and EphB4 in arteries and veins persists into adulthood, suggesting that the reciprocal expression of these genes may be important not only during development but also for continued maintenance of proper arterial–venous differentiation. Analysis of ephrinB2 expression in a variety of adult angiogenic settings also showed that ephrinB2 expression is strongly associated with adult neovascularization. EphrinB2 expression is highly enriched in the endothelium of angiogenic vessels and their sprouts during wound healing, follicular maturation and corpus luteum formation in the ovary, tumor angiogenesis, and experimentally induced VEGF-driven corneal neovascularization. These observations suggest that ephrinB2 may be important for both embryonic and adult angiogenic remodeling,[142,143] although further studies are need to confirm such a role in adults. Taken together, these studies indicate that the regulators of EC-fate determination during embryonic development can also promote specific subsets of adult vascularization and may offer unique targets for antiangiogenic therapy.

Conclusions

The genetic basis of arterial–venous determination has been examined in multiple animal model systems using a variety of experimental approaches, including target gene disruption, forward-genetic mutagenesis, phenotypic-based small-molecule chemical modifier screens, and gene microarrays. A variety of molecular factors involved in the establishment of arterial and venous identity have now been identified including well-studied morphogens (Hh), signaling molecules (Notch), and growth factors (VEGF) (Figure 4). Not surprisingly, this has led to a complex hierarchal arrangement of interacting signaling pathways that promote arterial cell fate at the expense of venous cell fate, or vice versa, and how these pathways are intertwined remains to be fully classified. Although the signaling pathways involved in EC determination appear to be conserved across vertebrate phyla, the

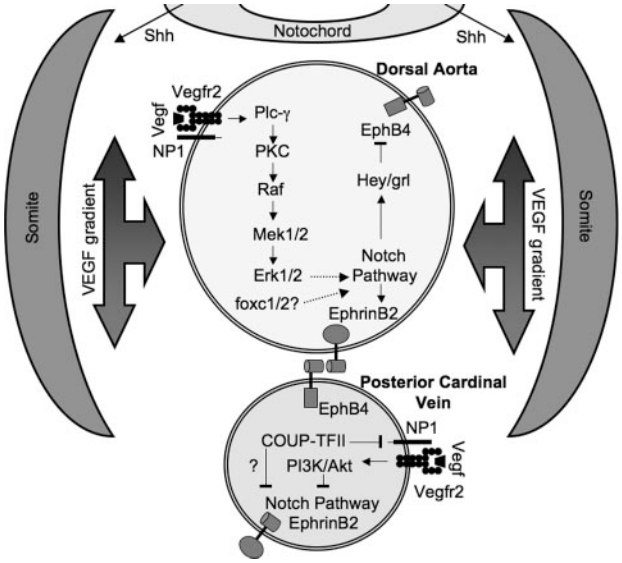

Figure 4. Cross-sectional schematic diagram illustrating proposed molecular pathways for arterial–venous specification in the trunk of a developing embryo. Shh expression in the notochord triggers expression of vegf in the surrounding tissue. High levels of local vegf interact with the VEGFR2-NP1 receptor complex and initiate the Plc-γ/Raf/ERK cascade in more dorsal ECs, which activates Notch signaling, inducing ephrinB2 at the expense of EphB4 and promoting arterial cell fate. Foxc1/2 proteins assist in Notch activation by inducing the Notch ligand Dll4. In more ventral ECs, where low levels of vegf are found, COUP-TFII and PI3K/Akt signaling suppress NP1 and Notch activation to promote venous cell fate. This figure is based, in part, on a model presented by Lamont and Childs.[144]

inherent genetic differences that arise from using animal model systems from mammals, birds, fish, and frogs have ensured that many unique aspects of cell-fate determination still need to be examined. This review has focused primarily on genetic factors involved in EC determination, although, as noted above, epigenetic factors such as the hemodynamics of blood pressure and flow also impact the global genetic programming. A more detailed investigation into other factors that influence the local environment of ECs, such as SMCs and pericytes, may also provide clues into arterial and venous specification. The continued emergence of ever more refined models for understanding how blood vessel formation is organized will be of vital importance in understanding the pathogenesis of congenital disorders and tumor angiogenesis.

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Disclosures

None.

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