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Choose your fate: artery, vein or lymphatic vessel?

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The specification of cell fate is integral to embryonic development. Recent research has identified several molecules that are involved in the development of the embryonic vasculature. Their combined actions are required for the specification and development of the arteries, veins and lymphatic vessels; vascular networks that are vital for embryonic and adult survival, and whose malfunction causes major pathological disorders. Recent discoveries have impacted our understanding of the embryonic origins of arterial, venous and lymphatic endothelial cells and the signals that mediate their navigation into mature, functional circulatory systems.

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Abbreviations

Ang	angiopoietin
BMP	bone morphogenetic protein
CV	cardinal vein
LS	lymph sac
Nrp	neuropilin
Shh	Sonic hedgehog
SLP	src homology 2 (SH2) domain-containing leukocyte phosphoprotein
VEGF	vascular endothelial growth factor

Introduction

In vertebrates, two specialized vascular systems have evolved for effective circulation: the blood vasculature, which delivers oxygen and nutrients and carries away waste products for detoxification and replenishment; and the lymphatic vasculature, which returns the protein-rich extruded fluid to the bloodstream. In addition, these vascular networks are important conduits for the cellular trafficking that is vital to immune surveillance and responsiveness. The cellular components of each network have evolved for specific functions. The structure of arteries and veins is designed to fulfil the pressure requirements of directional blood flow; that of lymphatic vessels facilitates their ability to sense tissue pressure and transport lymph. The disruption of either vascular network can have devastating consequences; therefore, the study of vascular development will undoubtedly contribute to the improvement of therapeutics for vascular diseases. This review focuses on recent advances in embryonic vascular development research, particularly the elucidation of molecular mechanisms that are involved in the development of specialized arteriovenous or lymphatic cell lineages.

What is required for vascular network development?

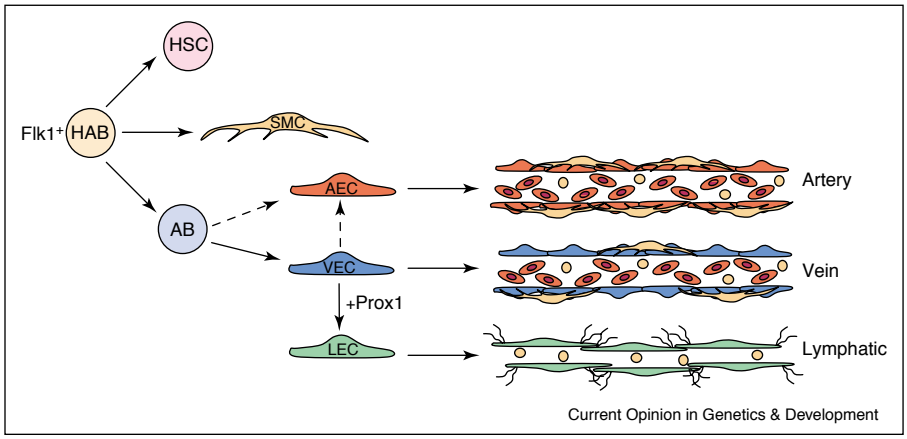
Vascular network formation can be separated into several phases. First, vasculogenesis: embryonic endothelial cells are specified, they then undergo proliferation, guided migration, coalescence, and finally, lumen formation. Second, during the subsequent process of angiogenesis, the primary vascular plexus is remodeled into an arborized network of large and small vessels. Several genes have been identified that are vital to one or both of these processes; those best characterized include members of the *vascular endothelial growth factor* (*VEGF*) family and their tyrosine kinase receptors, the *angiopoietins* and their tyrosine kinase *Tie* receptors, and some members of the *ephrin* family and their *Eph* tyrosine kinase receptors [1].

The specification of endothelial cells

Endothelial and hemopoietic cells arise from a common mesodermal-derived progenitor cell, the hemangioblast (Figure 1). Genetic ablation of the VEGF receptor *Flk1* results in the absence of endothelial and hemopoietic cells in the mouse embryo and lethality between E8.5 and E9.5 [2]. This finding indicates an essential role for *Flk1* and members of the *VEGF* family in the development of both cell lineages. Heterozygous inactivation of *VEGF* also results in arrested vasculogenesis and embryonic lethality [3,4], indicating that the expression of VEGF is required for vascular patterning and assembly.

What are the upstream inductive signals that are required for the expression of the VEGF ligand and receptor family members? The homeobox gene *HoxB5* regulates the expression of Flk1 [5], suggesting that the induction of *HoxB5* by an unidentified signal might help to specify endothelial cell fate via the induction of Flk1 expression in primitive mesoderm. The oncoprotein c-Myc is also important for VEGF expression; *c-Myc*$^{-/-}$ embryos exhibit pronounced defects in vasculogenesis [6]. Some of the molecular components downstream of VEGF/Flk1 signaling that are involved in determining endothelial cell

Figure 1



The hemangioblast (HAB) is the precursor of hemopoietic stem cells (HSC), smooth muscle cells (SMC) and angioblasts (AB), the precursors of endothelial cells. Multiple lines of evidence suggest that venous endothelial identity (see VEC in figure) may be the basal ground state to which the expression of genes is gained, or repressed, to induce arterial or lymphatic cell fate. It is also possible that AECs might differentiate directly from angioblasts without proceeding via a venous phase. In mammals, LECs originate from the cardinal veins following the induction of expression of Prox1, a homeobox transcription factor that specifies LEC fate. Many structural differences exist between blood vessels and lymphatic vessels that correlate with their function; blood vessels have a much higher level of extracellular matrix and more associated perivascular support cells than lymphatic vessels, which have specialized anchoring filaments that are important for opening the vessel in conditions of high tissue pressure, thus facilitating the transport of lymph back to the bloodstream.

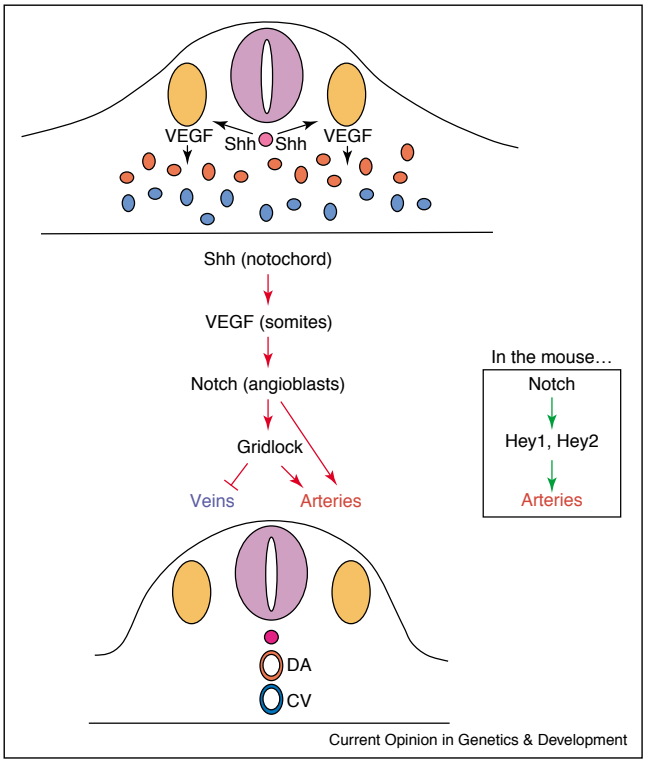
fate have also recently been identified. The basic helix-loop-helix protein Scl/Tal1 and the LIM (named from the Lin-11, Isl-1 and Mec-3 genes) domain protein Lmo2 induce the transcriptional program of endothelial differentiation [7,8]. Interestingly, the level of Scl/Tal1 expression influences the choice between hemopoietic, endothelial or smooth muscle cell fates [7]. The cytoplasmic tyrosine kinase Fps/Fes plays an important role downstream of VEGF-A/Flk1 signaling by mediating hemangioblast migration and proliferation [9]. Additionally, the VEGF co-receptors neuropilin (Nrp)1 and Nrp2 probably contribute to VEGF-mediated vasculogenic events, as demonstrated by the finding that embryos null for both genes display arrested vasculogenesis, similar to that observed in *VEGF* and *Flk1* knockout mice [10].

Multiple laboratories have demonstrated the importance of endoderm-derived signals for early stages of vascular development [11,12]. In particular, endoderm-derived Indian Hedgehog (Ihh) has been shown to be capable of, but not essential for, the specification of cells of the hemopoietic and vascular systems in the mouse, a process that is possibly mediated, at least in part, by BMP4 [11]. Interestingly, BMPER, a BMP-binding endothelial cell precursor–derived regulator, antagonizes BMP4 to regulate endothelial cell differentiation [13]. Using *Xenopus* and quail as model systems, Vokes and Krieg [12] demonstrated that endoderm is vital for the formation of vascular tubes but not for prior angioblast specification, and that bFGF (basic fibroblast growth factor) stimulates angioblast development in animal cap assays in the absence of endoderm. Therefore, the endoderm is probably the source of several factors that influence vascular development, either directly or via the induction of VEGF expression. The combinatorial action of many of these factors, including some yet to be discovered, is likely to be important for the specification of endothelial cell fate.

The choice between arterial and venous cell fate

The loss of arterial or venous endothelial cell identity can have drastic consequences for the development and function of the circulatory system. In zebrafish and mammals, members of the Notch family mediate the choice of fate between arterial and venous endothelial cells [14–16,17•]; this choice is made soon after angioblast specification [16] (Figure 2). Notch activity promotes arterial cell fate, at least partially, via the activity of 'gridlock', a transcriptional repressor that negatively regulates venous cell identity [14–16]. Lawson and colleagues [15] demonstrated that the sonic hedgehog (Shh) and VEGF signaling pathways act upstream of Notch to determine arterial cell fate; Shh induced VEGF expression in the somites of zebrafish embryos, a signal that is vital for the induction of arterial identity (Figure 2). Thus, VEGF is important, not only for the guided migration and proliferation of vascular endothelial cells, but also, at least in zebrafish, for the specification of arterial cell fate. Gradients of VEGF expression within the embryo are likely to affect the cellular response achieved in competent cells.

Figure 2



A model of vascular development in the zebrafish embryo. Notochord-derived Shh is responsible for inducing VEGF expression in the somites, a signal that is essential for Notch-mediated arterial specification [14–16]. Whether or not the induction of arterial identity is directly dependent on gridlock is still controversial [14]. The pathway is likely to be more complex in amniote embryos, but several aspects are conserved. Members of the VEGF, hedgehog and FGF families are important for angioblast specification and migration [2–4,11,12]. In a parallel to zebrafish, Notch and its downstream targets Hey1 and Hey2 (the mammalian gridlock orthologue) are important for the specification of arterial cell fate in mouse [17]. DA, dorsal aorta; CV, cardinal vein.

EphrinB2 and EphB4, acting via a ligand–receptor relationship, have also been implicated in determining arterial and venous cell identities, probably via mediating a repulsive signal that separates arterial and venous endothelial cells [18–20]. In the cardiovascular system, ephrinB2 expression is restricted to the arteries [18–20], smooth muscle cells, pericytes and mesenchyme that surrounds sites of vascular remodeling [21,22]. EphB4 is expressed predominantly on venous and lymphatic endothelial cells [18–20; NLH and GO, unpublished observations]. Targeted inactivation of *Alk-1*, a receptor for members of the TGF-β superfamily, revealed a role for Alk-1 in mediating the separation of arteries and veins during embryonic development. In the absence of signaling via this pathway, arterial ephrinB2 was not induced [23]. Together, these results suggest that venous identity is the default status of endothelial cells, and that upon VEGF and Notch signaling events, arterial fate is gained, resulting in the separation of arterial and venous endothelial cells via ephrinB2/EphB4 signaling.

The formation of endothelial tubes: factors involved in the proliferation, guidance, migration, coalescence and lumen formation of vessels

As discussed previously, members of the VEGF, angiopoietin, Eph/ephrin and hedgehog families mediate the migration and association of endothelial cells, two processes that are essential for normal vasculogenesis and angiogenesis. The most recently identified genes that contribute to these processes include *retinaldehyde dehydrogenase 2* (*Raldh2*), *Norrin*, *Frizzled-4 (Fz4) and Nogo-B*. Generation of *Raldh2*-null mice revealed a vital role for retinoic acid (RA) in regulating endothelial cell proliferation during vasculogenesis [24]. *Raldh2*$^{-/-}$ embryos do not produce active RA, fail to remodel the primary vascular plexus and do not recruit supporting mural cells to developing vessels. The failure of vascular remodeling in these early embryos is caused by the continued, uncontrolled proliferation of endothelial cells and demonstrates that RA suppresses cell cycle progression during vascular development. *Norrin* and *Fz4* form a high-affinity ligand–receptor partnership; these genes are important for vascular development, at least in the eye and ear, and are mutated in at least two human vascular diseases [25••]. The identification of Fz4-mediated signaling via Norrin binding is the first example of Fz4 receptor activation by a ligand other than a Wnt. Fz4-mediated signals may mediate endothelial cell migration and vascular arborization during retinal development [25••]. The requirement of cytoskeletal reorganization for the response to angiogenic factors, including VEGF, has been demonstrated recently by knocking out WAVE2 [WASP (Wiskott-Aldrich syndrome protein) family verprolin homologous protein], a protein that is crucial to Rac-induced membrane ruffling in mice [26]. In WAVE2 knockout mice, the formation of migration-facilitating lamellipodia in response to VEGF was reduced, resulting in decreased angiogenic sprouting and remodeling. Although, initially, Nogo-A was identified as an inhibitor of axonal growth and repair, recently, Nogo-B was shown to be expressed in vascular endothelial and smooth muscle cells, and to have an important role in regulating vascular remodeling processes, particularly the recruitment of smooth muscle to injured vascular endothelium [27•].

Also 'borrowing' from the axon navigation system, the semaphorin SEMA3A mediates angiogenic vascular remodeling by inhibiting integrin-mediated adhesion of endothelial cells to the extracellular matrix [28]. Analysis of Sema3a1 function in zebrafish indicates an early role in regulating the migration of angioblasts expressing Nrp-1 (a component of the Sema3a receptor) during formation

of the dorsal aorta [29]. The nervous system itself guides the patterning and differentiation of arteries in the skin, a process that is mediated by VEGF, which is produced by sensory nerves, associated Schwann cells or both [30]. The necessity of negative regulation of vascular patterning for normal vascular development was recently demonstrated in quail; the quail notochord is the source of signals that repress the formation of vessels at the embryonic midline [31•]. The BMP antagonists Chordin and Noggin are strong candidates for the notochord-derived factors that inhibit endothelial cell migration. BMP4 activates the migration of endothelial cells and induces the formation of a midline vascular plexus when ectopically expressed. Notochord-derived signals might participate in formation of the paired dorsal aortae that are characteristic of amniotes. Not only do endothelial cells rely on the provision of local tissue-specific cues for their embryonic patterning and development, but they also provide signals that induce formation of organs such as the pancreas [32,33] and liver [34].

A vital event in the formation of functional vessels is the opening of a lumen. Recently, Egfl7, an endothelial cell-derived factor that is important for tube formation was identified [35]. Egfl7 is a secreted protein that is expressed in endothelial cell progenitors and in the endothelium of vessels, and it is thought to maintain an association between the extracellular matrix and endothelial cells. Knockdown of *Egfl7* in zebrafish embryos prevented lumen formation in the major axial blood vessels, although the endothelial cell number was unchanged [35]. This lack of lumen formation might be caused by the abnormal retention of extensive junctions between endothelial cells, resulting in the failure of endothelial cells to change shape and be reorganized to form lumenised vessels.

The lymphatic vasculature: a venous-derived, specialized vascular network

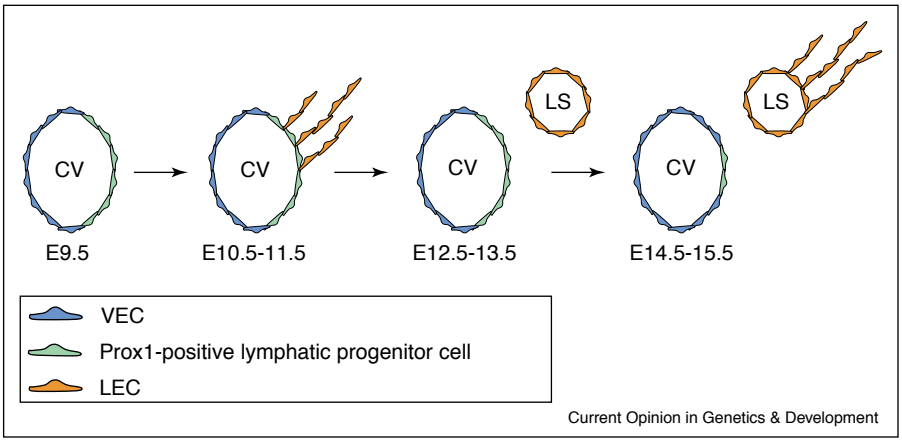
Despite their first description centuries ago [36], the lymphatic vasculature remained poorly characterized until the recent identification of several lymphatic-specific molecular markers. The lymphatic vasculature was previously identified on the basis of morphologic differences between blood and lymphatic vessels. Targeted gene inactivation in mice has identified many molecules that are vital for lymphatic development. The homeobox gene *Prox1* [37] is essential for specification of the fate of lymphatic endothelial cells. *Prox1*$^{-/-}$ embryos display multiple phenotypic abnormalities, including the complete absence of lymphatic vessels, and die around midgestation. In wild-type embryos, Prox1 expression is initiated at approximately E9.5 in a subpopulation of cardinal vein endothelial cells; these lymphatic progenitor cells subsequently bud, proliferate and migrate to form the embryonic lymph sacs and vascular network [37,38] (Figure 3). The signal that initiates Prox1 expression in those progenitors remains elusive. Prox1 activity is necessary and sufficient to promote lymphatic endothelial fate in otherwise blood vascular endothelial cells [39]. VEGF-C, which regulates lymphatic endothelial cell proliferation via its association with VEGFR-3 [40–42], is vital for the migration and proliferation of Prox1-positive lymphatic endothelial cells from the cardinal veins [43•]. In the absence of VEGF-C, lymphatic development is arrested, although Prox1 expression is still initiated in cardinal vein endothelial cells [43•]. These studies confirm Sabin's hypothesis that lymphatic vessels originate from embryonic veins via sprouting and migration [44,45]. Whether there are additional sources of lymphatic progenitor cells in murine embryos, as there appear to be in avian embryos [46], remains to be established.

Although derived from the embryonic veins, the lymphatic and blood networks remain separated at all but one connection point: the junction between the thoracic duct and the left subclavian vein. A signaling pathway that involves the tyrosine kinase Syk and the adaptor signaling proteins SLP-76 [Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa] and PLCγ2 (phospholipase Cγ2) is required to maintain the separation of the two vascular networks [47•]. In mice deficient for Syk or SLP-76, abnormal connections persist between the blood and lymphatic vasculature, resulting in embryonic hemorrhage and cardiac hypertrophy. In these mice, blood is present in the lymphatic vessels, a defect that is conferred to wild-type mice after the transplantation of Syk- or SLP-76-deficient bone marrow [47]. To explain these results, two possible hypotheses have been proposed: (i) a resident lymphatic progenitor exists in the hemopoietic compartment, and (ii), in Syk-, or SLP-76-deficient bone marrow, this lymphatic progenitor cell is defective in the signaling pathway that is required for separation of the blood and lymphatic vascular systems.

Maturation of the lymphatic vasculature occurs in a stepwise process [38] (Figure 3). Many genes that regulate lymphatic development have recently been identified via gene inactivation studies. The generation of mice with targeted inactivation of *Angiopoietin-2* (*Ang2*) revealed that this gene is vital for the patterning and development of the lymphatic vasculature and for postnatal vascular remodeling in the eye [48]. *Ang2*-null mice die within two weeks of birth; these animals exhibit chylous ascites and subcutaneous edema, conditions that indicate lymphatic dysfunction. Interestingly, the lymphatic defects of *Ang2*-null mice were rescued by *Ang1*, suggesting that Ang2 is an agonist of the Tie2 receptor in the developing lymphatic network and that it stabilizes lymphatic vascular structure and integrity [48].

Nrp2 appears to play a selective role in the development of small lymphatic vessels [49]. Surviving *Nrp2*-null mice

Figure 3



Murine lymphatic development occurs in a stepwise fashion [38]. Prox1 expression is initiated at approximately E9.5 in the endothelial cells located on one side of the anterior CVs. Prox1-positive lymphatic endothelial cell progenitors start to express additional lymphatic markers and progressively bud and migrate from the veins as development proceeds. These LECs associate to form the primitive lymph sacs (LS) at discrete locations within the embryo, from where further sprouting and migration lead to the development of the entire lymphatic vasculature.

exhibit a transient defect in the formation of lymphatic capillaries in various tissues. *Nrp2*-null lymphatic endothelial cells exhibit a much lower rate of proliferation than do their $Nrp2^{+/-}$ or wild-type counterparts [49], suggesting that the *Nrp2*-null phenotype is caused by decreased, VEGF-C-induced, proliferation of lymphatic endothelial cells. Nrp2 may contribute to this process by acting as a receptor for VEGF-C [50]. The lymphatic vessels of Nrp2-mutant mice regenerate postnatally, suggesting that they overcome dependence on Nrp2-mediated signaling. The development of large, collecting lymphatic vessels is not affected in *Nrp2*-null mice, a finding that indicates differential control of the formation of large and small-caliber lymphatic networks [49].

The transmembrane glycoprotein podoplanin may contribute to lymphatic endothelial cell adhesion and migration and to the formation of connecting lymphatics between superficial and deep lymphatic plexi [51]. *Podoplanin*-null mice display defects in lymphatic vessel structure and function, and obvious dilations of the cutaneous and submucosal intestinal lymphatic vasculature, which lead to lymphedema [51].

Mutations in *VEGFR-3* [52,53], *FoxC2* [54–56] and *Sox18* [57] have been identified in several hereditary human lymphoedema syndromes; therefore, these proteins also regulate lymphatic development and function. Although the mechanism of VEGFR-3 activity has been well characterized [42,58], those of FoxC2 and Sox18 currently remain unknown.

Conclusions

A large body of genetic evidence illustrates the roles of many molecules that affect vasculogenesis, angiogenesis and lymphangiogenesis in a complex and tightly regulated manner. However, many questions remain unanswered. What is the origin and identity of the initial signal that specifies endothelial cell fate? What signals to a hemangioblast to choose an endothelial or hemopoietic cell fate? Is venous cell identity the basis from which the expression of genes are activated (or repressed) to specify arterial or lymphatic cell fate? Do lymphatic progenitor cells arise from other sources in the developing embryo besides the veins? Are the veins a constant source of lymphatic progenitors at later stages of embryonic development or in the adult? Many signals must be integrated for the correct specification, development and function of arteries, veins and lymphatic vessels. Although they all share the same endothelial basis, each cell type has acquired unique characteristics that are vital to cardiovascular system function.

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- •• of outstanding interest

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