Endothelial Differentiation
Molecular Mechanisms of Specification and Heterogeneity

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Abstract—A complex and diverse vascular system is requisite for the survival of higher organisms. The process of vascular development is highly regulated, involving the de novo formation of vessels (vasculogenesis), followed by expansion and remodeling of the primitive vasculature (angiogenesis), culminating in differentiation of endothelial phenotypes, as found in the mature vascular system. Over the last decade, significant advances have been made in understanding the molecular regulation of endothelial cell development and differentiation. Endothelial development, in particular the mechanisms in play during vasculogenesis and angiogenesis, is discussed in a sister review to this article. This review highlights the key pathways governing in endothelial differentiation, with a focus on the major molecular mechanisms of endothelial specification and heterogeneity. (Arterioscler Thromb Vasc Biol. 2011;31:1476-1484.)

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To distribute oxygen, nutrients, and paracrine factors to the far corners of a multicellular organism, a closed-loop circulatory system must be formed and connected to a pump very early in development. The afferent loop—the arterial system—must be able to endure high pressure and pulsatile blood flow and to accomplish tissue-specific delivery of circulating materials. Uninterrupted return of blood to the pumping chamber must be maintained under the low-pressure, low–shear stress, high-capacitance conditions of the venous system. The diverse functions of a continuous system requires specialization of components of the system, and the heterogeneity of endothelial cells (ECs) lining the lumen of the system plays a large part in creating this specialization.

Until the late 1990s, the initial driving force in creation of heterogeneous EC phenotypes was thought to be the exposure of those cells to flowing blood. We now have data demonstrating that EC heterogeneity, while retaining the plasticity to alter under a changing environment, has a genetic component that comes into play perhaps even before hemangioblasts differentiate into ECs and hematopoietic cells. Elegant experiments in zebrafish suggest that the arterial-venous cell fate decision of angioblasts is made even before migration. The major embryonic vessels are formed by the coalescence of angioblasts to form the dorsal aorta (DA), which lies ventral to the notochord, and the posterior cardinal vein, located just below and parallel to the DA. In addition to a genetic predestination of the angioblasts, the response to a vascular endothelial growth factor (VEGF) gradient initiates a hierarchy of signaling events that culminates in arterial versus venous EC differentiation (Figure 1).

Molecular Mechanisms of Specification
Vascular progenitor cells (angioblasts) originate in the lateral plate mesoderm and migrate to a midline position just ventral to the notochord, forming the inner cell mass. The inner cell mass gives rise to both blood and ECs. Cell-tracing experiments in zebrafish suggest that the arterial-venous cell fate decision of angioblasts is made even before migration. The major embryonic vessels are formed by the coalescence of angioblasts to form the dorsal aorta (DA), which lies ventral to the notochord, and the posterior cardinal vein, located just below and parallel to the DA. In addition to a genetic predestination of the angioblasts, the response to a vascular endothelial growth factor (VEGF) gradient initiates a hierarchy of signaling events that culminates in arterial versus venous EC differentiation (Figure 1).

Arterial Specification
At the top of the hierarchy is the secreted ligand sonic hedgehog (Shh). Shh is a pleiotropic molecule, with diverse roles in embryogenesis and patterning. It is expressed in the notochord and results in expression of VEGF by somites bordering the developing vessels. The exact mechanism of mediation of the Shh signal in this instance is unknown; classically, it acts
through its transmembrane receptor, patched-1, to increase VEGF activity and thus arterial specification. However, there is evidence that Shh also increases VEGF by regulating expression of the calcitonin receptor-like receptor expressed in somites and arterial progenitors.

Diffusion of VEGF from the somites to the developing vessels creates a gradient with higher levels of VEGF near the DA and lower levels at the posterior cardinal vein. In vascular plexuses, increased VEGF expression leads to an increase in the arterial:venous ratio. VEGF acts through 1 of several VEGFR tyrosine kinases to activate, among other targets, the phospholipase C-γ1-mitogen-activated protein kinase (MEK)/extracellular signal regulated kinase (ERK) pathway, solely in the dorsal angioblasts of zebrafish. In vitro experiments using mouse-derived EC suggest that ERK can contribute to activation of the Foxc1 and Foxc2 transcription receptors that subsequently upregulate expression of members of the Notch pathway in vivo.

Activation of the Notch pathway is a defining characteristic of arterial EC. The Notch family is composed of 4 receptors (Notch1 to Notch4) and 5 ligands (Jagged-1 and -2 and Delta-like ligands [Dll] 1 to 4). In mice, Notch1, Notch4, Jag1, Jag2, and Dll4 are all expressed in arterial but not venous ECs. When Notch is activated, the intracellular domain is cleaved and translocates to the nucleus, where it acts as a cofactor to upregulate transcription. Integrated expression of Notch genes during vessel development is required for appropriate vessel identity. Hemizygous deficiency of Dll4 is embryonic lethal in mice because of abnormal arterial development. Whereas Dll4 is essential for initiation of the arterial development program, Dll1 is required for maintenance. Alterations in levels of downstream targets of Notch lead to loss of arterial markers and arterial/venous fusion (compound Hey1/Hey2 mutant in mice19,20) and localized defects in the DA (gridlock [grl] deficiency in zebrafish21). The Sox7 and Sox18 gene products may regulate arterial-venous specification by acting upstream of grl. In fact, arteriovenous malformations develop when Notch signaling is either reduced or constitutively active.

The ephrinB2 ligand and its cognate receptor, EphB4, are differentially expressed in the arterial and venous ECs, respectively, of the mouse primary vascular plexus before the
onset of circulation.25-26 This seminal discovery, made in 1998, provided the first evidence that arterial-venous identity is genetically predetermined. Although the members of this ligand-receptor pair have distinct cellular locations, interactions between the 2 are required for proper vascular development/remodeling. The ephrin-Eph subclass of receptor tyrosine kinases can participate in an unusual bidirectional signaling process. Forward signaling (ephrin ligand to Eph receptor) is initiated by ephrin ligand engagement and activates the receptor kinase domain. Reverse signaling (Eph receptor to ephrin subclass B ligand) leads to phosphorylation of tyrosine residues in the cytoplasmic domain of the ligand. This large subfamily of signaling molecules regulates a variety of morphogenetic processes in different tissues (reviewed in1). The lack of ephrin B2 or EphB4 does not impair the initial specification of arteries and veins, but ephrinB2-EphB4 signaling is required for maintenance of the arterial-venous interaction. Mouse mutants defective for the pair lose the differentiation of blood vessels into morphologically distinguishable arteries and veins.25-27 This limited role leads to the subsequent identification of upstream factors, such as Notch, in the process of arterial/venous EC differentiation.

Expression of the neuropilins (NRP) is temporally related to the expression of the ephrins and also displays a restricted pattern of expression. Nrp-1 is found exclusively in cells fated to be arterial and Nrp-2 in those that will be venous.28 In mice with a CD1 background, Nrp-1 deficiency leads to abnormal vascular network formation and embryonic lethality at embryonic day 13.5. In the C57Bl/6 background, the vascular defects are not apparent until after the onset of blood flow. In these mice, deficiency abrogates the normal vascular remodeling that occurs coincident with the initiation of flowing blood (embryonic day 10.5).29-31 The NRPs were previously identified as cell surface receptors for semaphorins. In this system, Nrp-1 functions at least in part by acting as a coreceptor for VEGF and facilitating VEGF signaling in concert with VEGFR-2.32

Venous Specification
Unlike arterial specification, details of the molecular mechanisms controlling venous specification are still largely unknown. Exposure to lower VEGF concentrations is likely to be important as a negative regulator of arterial specification. In vitro experiments have shown that higher concentrations of VEGF (50 ng/mL) induces expression of arterial markers such as ephrinB2 and Nrp-1 in embryonic stem cells, whereas low VEGF concentrations (2 ng/mL) lead to expression of venous endothelial markers.33 In zebrafish, loss of Shh signaling leads to loss of the arterial marker Ephrin-B2a and greater amounts of the venous marker Flt4.34 Thus, the greater distance of the posterior cardinal vein from the VEGF-spewing somites (as compared with the DA) may contribute to its differentiation into a venous structure (reviewed in3). In vitro studies have shown that VEGF activates a plethora of signaling pathways, including the phosphoinositide-3-kinase/Akt pathway, which can act in concert with VEGF-activated ERK; however, in regard to arterial EC specification, in vitro and in vivo experiments suggest that the MEK/ERK and phosphatidylinositol 3-kinase/AKT pathways may have competing roles.35 Specifically, activation of AKT can inhibit the activity of MEK/ERK in zebrafish, thus steering EC away from arterial specification.14 (Replication of Notch inhibition by phosphatidylinositol 3-kinase in in vitro experiments has not been accomplished; loss of the complexities of the in vivo context is considered a potentially important factor.36,37) AKT is also hypothesized to induce chicken ovalbumin upstream promoter-transcription factor (COUP-TFII) expression.2 COUP-TFII is specifically expressed in venous ECs and is a genetic determinant of venous specification. Although compound homozygous mutants for Foxc1 and Foxc2 lack arterial specific genes, they do express the venous marker COUP-TFII.16 As a nuclear orphan receptor, the natural ligand for COUP-TFII is retinoic acid. Activation of COUP-TFII by retinoic acid suppresses Notch signaling, potentially at the level of NRP-1 expression, thus releasing factors such as EphB4 from Notch-mediated repression and conferring a venous EC phenotype.38 COUP-TFII directly regulates expression of Nrp-2, both leading to its restricted expression in venous (versus arterial) ECs and preparing cells for differentiation into lymphatic EC (LEC).39

Lymphatic Specification
LEC are derived from lymphatic vessel hyaluronan-1 receptor-1–positive subpopulations of cells in the cardinal vein, which bud off to form primary lymphatic sacs. Lymphatic vessel hyaluronan-1 receptor-1 is a specific marker for cells capable of becoming LEC but is not required for normal lymphatic development.50,41 By molecular mechanisms not yet elucidated, the transcription factor SRY box 18 (Sox18) is induced in murine lymphatic vessel hyaluronan-1 receptor-1–positive venous cells that will commit to the LEC lineage. Sox18 directly induces expression of Prox1, the master regulator of LEC identity.42 Recent data suggest that Prox1 is required for both initiation and maintenance of the LEC phenotype. Prox1-deficient EC can bud from the cardinal vein, but they display abnormal VEGF-induced migration and do not express LEC markers.43,44 Recent data shows that lymphatic sprouting, specifically (versus angiogenic), is regulated in zebrafish by the PDZ domain–containing scaffold protein synectin; in vitro experiments with human dermal LECs suggest that synectin functions, in part, by regulating Sox18-mediated induction of Prox1.45 Pedrioli et al used microarray analysis to identify human blood EC- or LEC-specific microRNAs and found that miR31 suppresses lymphatic differentiation.46 Mechanistic analysis demonstrated that the inhibitory effect is partially mediated via direct repression of Prox1.

Prox1 mediates upregulation of important regulators/markers of lymphatic differentiation, including VEGF receptor 3 (VEGFR-3). VEGFR-3 is expressed in all EC during early development; however, its expression is enhanced in cells committed to the LEC lineage, and as the lymphatic system develops, it becomes largely restricted to LECs.47 Activation of VEGFR-3, in particular, by VEGF-C is required for lymphatic development.48 It is proposed that the VEGFR-3 coreceptor Nrp-2 increases the response of LEC to VEGF-C by a mechanism analogous to the interaction of Nrp-1 and VEGFR-2 in arterial EC (as reviewed in6).
The presence of COUP-TFII in EC may well be a determinant factor for the venous origin of cells destined to become LEC. Sox18 cooperates with COUP-TFII to promote expression of Prox1. Prox1 appears to act in concert with COUP-TFII in enhancing expression of VEGFR-3, and by upregulating the VEGFR-3 coreceptor Nrp-2, COUP-TFII enhances VEGFR-3 activity.

Plasticity of Vascular Specification

Although the initial molecular identity of ECs is genetically predetermined, there is significant plasticity in subsequent arteriovenous differentiation. Physiological requirements and hemodynamic influences can reverse the phenotype of an apparently committed cell. ECs in transplanted arterial and venous vessel grafts can change identity, completely switching their expression profile to match the host vessel. Forced overexpression or loss-of-function of critical molecular determinants of specification can also reprogram a differentiated EC. For example, Notch overexpression in the venous compartment results in arterialisation with upregulation of arterial EC markers, whereas inhibition of Notch signaling in the arterial compartment results in loss of arterial fate and upregulation of venous markers. Ablation of EC COUP-TFII allows veins to acquire arterial characteristics and express arterial markers. The mature LEC phenotype is highly plastic. As discussed above, constant expression of Prox1 maintains the mature differentiated LEC state. Conditional embryonic, postnatal, or adult deletion of Prox1 in mice results in the appearance of blood-filled lymphatic vessels and down regulation of LEC markers with the concomitant ectopic expression of blood EC markers. Conversely, ectopic overexpression of Prox1 in cultured blood ECs leads to the acquisition of LEC identity. An exquisite feedback regulatory equilibrium exists among the 3 major EC fate regulators (Notch, COUP-TFII, and Prox1) that directs the plasticity in arteriovenous-lymphatic cell fate.

Molecular Mechanisms of Heterogeneity

Beyond the specification to arterial, venous, or lymphatic fate, it is currently recognized that ECs undergo further differentiation specific to the vascular bed or organ in which they reside. This phenotypic heterogeneity is the primary mechanism by which the endothelium carries out myriad vital functions, including control of microvascular permeability, vessel wall tone, coagulation and anticoagulation, inflammation, and angiogenesis. Endothelial heterogeneity is also responsible for the varied and diverse responses across differing vascular beds to pathological stimuli and disease states.

Structural Classifications

Based on structural content, the endothelium has been classically characterized into 3 main structural types: continuous, which is further subdivided into fenestrated or nonfenestrated, and discontinuous (or sinusoidal) (Figure 2A).

Continuous Nonfenestrated

Continuous nonfenestrated endothelium is found predominantly in arteries, veins, and capillaries of the brain, skin, muscle, heart, and lung. Tight junctions and adherens junctions are the 2 main types of barrier forming intracellular junctions found in this type of endothelium. Their expression is variable across the endothelial tree, with higher expression in the continuous endothelium of arteries compared with capillaries and venules. Molecules cross this endothelium by the active process of transcytosis, which is mediated by specialized structures including caveolae and vesiculo-vacuolar organelles. Caveolae, flask-shaped membrane bound vesicles (∼70 nm in diameter) that usually open to the luminal or abluminal side, are present in all types of endothelium but are highest in capillaries that contain continuous nonfenestrated endothelium. Caveolin-1 is the main structural component of caveolae and is regulated by distinct transcriptional mechanisms in ECs. Vesiculo-vacuolar organelles also contain caveolin-1 and are focal collections of membrane bound vesicles of variable size that span the cytoplasm of the ECs and are mostly found in venules with continuous nonfenestrated endothelium.

Continuous Fenestrated

Fenestrae are transcellular pores (∼70 nm in diameter) that extend through the full thickness of the EC and are thought to allow rapid exchange of molecules between the circulation and the surrounding tissue. The majority of fenestrae contain a thin diaphragm across their opening that acts as a molecular filter. The type II membrane glycoprotein plasmalemmal vesicle-associated protein-1 is currently the only molecular protein localized to the diaphragm, and it has been discovered to be both necessary and sufficient for diaphragm formation in cultured ECs. Other diaphragm-containing components of continuous endothelium include caveolae and transendothelial channels, which are patent pores spanning the EC from the luminal to abluminal side. Compared with nonfenestrated endothelium, continuous fenestrated endothelium is more permeable to water and small solutes. This endothelium typically occurs in locations that are characterized by increased filtration or increased transendothelial transport and is found in capillaries of all exocrine and endocrine glands, digestive tract mucosa, and kidney (eg, glomeruli and a subpopulation of renal tubules).

Discontinuous Fenestrated

Discontinuous fenestrated endothelium is characterized by large heterogeneous fenestrae (100 to 200 nm in diameter) without diaphragms. It has few caveolae and contains clathrin-coated pits and vesicles, which play an important role in receptor-mediated endocytosis. This endothelium is found in certain sinusoidal vascular beds, most notably the liver and bone marrow, which lack a well-formed basement membrane.

The phenotypic heterogeneity of the endothelium of the various organ and vascular beds has been highly studied. Remarkably, despite these detailed observations, the molecular mechanisms of endothelial heterogeneity remain largely unknown. In recent years, the study of ECs in several vascular beds have made significant strides in elucidating some of these molecular mechanisms.

Kidney Endothelium

The kidney is a highly vascular organ that contains a large degree of endothelial heterogeneity. Blood from the renal
arteries reaches the kidney and branches into the afferent arterioles that enter into the glomerular tufts. The glomerular endothelium, in conjunction with the basement membrane and underlying podocytes, helps to form the glomerular filtration barrier, which serves as both a size- and charge-selective filter. The glomerular endothelium actively synthesizes a 60- to 300-nm-thick gelatinous surface coat called glycocalyx (composed of proteoglycans, glycosaminoglycans, glycoproteins, glycolipids, and associated plasma proteins) that covers the luminal side and facilitates charge selectivity. The glomerular endothelium actively synthesizes a 60- to 300-nm-thick gelatinous surface coat called glycocalyx (composed of proteoglycans, glycosaminoglycans, glycoproteins, glycolipids, and associated plasma proteins) that covers the luminal side and facilitates charge selectivity. The glomerular capillaries consist of a continuous fenestrated endothelium with large fenestrae that cover 20% to 50% of the entire endothelial surface. A unique feature of the glomerular endothelium is that most fenestrae do not contain diaphragms. The majority of evidence suggests that the glomerular ECs lose their expression of plasmalemmal vesicle–associated protein-1 and fenestral diaphragms during the development and maturation of the glomerulus. Importantly, recent studies of the glomerular endothelium have confirmed the initial observations that VEGF plays a role in the formation of EC fenestrations. Cross-talk between podocytes, expressing VEGF-A, and the glomerular endothelium, expressing VEGFRs, has been shown to be important for the development and maintenance of fenestrae and barrier function. Studies are ongoing investigating the detailed molecular pathways, but initial findings implicate activation of the small GTPases (ie, Rho/Rac) and rearrangement of the actin cytoskeleton.

Efferent arterioles exit the renal glomeruli and terminate in the vasa recta. Endothelial heterogeneity of the vasa recta is critical for the countercurrent exchange that occurs in the medulla of the kidney. The descending vasa recta (ie, an arteriole entering the medulla) is lined by continuous nonfenestrated endothelium and contains large concentrations of urea transporters and aquaporin-1 water channels. In contrast, the ascending vasa recta (ie, a vein exiting the medulla) is lined with fenestrated endothelium. This heterogeneity allows for shuttling of osmotically active solutes between descending and ascending capillaries, thereby maintaining the hypertonicity of the medulla that is essential for gradient translocation of solutes.
mediated filtration in the kidneys. In addition, this process helps to deliver nutrients and oxygen to the medullary tissue.91

**Brain Endothelium**

The brain endothelium has an extremely specialized characteristic that allows it to selectively control permeability between blood and the central nervous system, thereby forming the blood-brain barrier (BBB).92,93 This barrier function is primarily mediated by both a physical barrier due to tight interendothelial junctions and a highly selective transporter system. The hallmark feature of the endothelium of the BBB is a continuous nonfenestrated endothelium with few caveolae and high expression of tight junction proteins, namely occludin and members of the claudin family (claudin 1, 3, 5, and 12). As tight junctions form, the brain ECs also begin to express selective membrane transporter proteins such as glucose transporter type 1 (Glut-1) and members of the ATP binding cassette (ABC) transporter family (ABCB1/P-glycoprotein, MDR1, and BCRP/ABCG2). This differentiation and maturation process of acquiring the unique properties of the BBB is frequently called barrierogenesis.94,95

The brain endothelium is surrounded by several other cell types, including pericytes, astrocytes, and neurons, forming the neurovascular unit,96 and interaction between multiple components of the neurovascular unit has been found to be necessary for proper formation and function of the BBB. Recent studies demonstrate that pericyte-EC interactions are necessary for BBB development by regulating the formation of tight junctions, as well as controlling pinocytic transport vesicles in ECs.97,98 In addition, the astrocyte-derived factor Src-suppressed C kinase substrate strengthens the BBB by decreasing VEGF expression and inducing angiopoietin-1, resulting in increased tight junction expression in brain ECs99 (Figure 2B).

Recently, the canonical Wnt pathway has been discovered to play a major role in barrierogenesis.100 In these studies, the expression of Glut1 was induced by Wnt7a and Wnt7b.101,102 In addition, Wnt signaling in ECs was necessary and sufficient for the induction and maintenance of BBB characteristics by increasing claudin 3 expression and inhibiting plasmalemmal vesicle–associated protein-1103 (Figure 2B). These recent findings in barrierogenesis predict that the Wnt signaling pathway, which has been shown to play important roles in vascular morphogenesis, is likely to be involved in the mechanism of endothelial heterogeneity in other vascular beds and organs.104

**Aortic Endothelium**

Endothelial heterogeneity exists not only between various organs and vascular beds but also within a single vascular bed. One of the most striking examples of this type of heterogeneity is the aortic endothelium, where variable blood flow dynamics results in the nonuniform distribution of atherosclerosis,105,106 Laminar blood flow in straight segments of the aorta induces factors such as endothelial nitric oxide synthase and thrombomodulin, thereby conferring potent antithrombotic, antiadhesive, and antiinflammatory properties to the endothelium.107,108 Conversely, nonlaminar or turbulent blood flow at areas where arteries branch or turn sharply reduces endothelial nitric oxide synthase expression and induces adhesion molecules such as vascular cell adhesion molecule-1, resulting in an inflammatory endothelial phenotype.109,110 Two members of the Krüppel-like factor transcription family (Krüppel-like factor-2 and Krüppel-like factor-4) are strongly induced by laminar flow, via the MEK/ERKs/myocyte enhancer factor-2 pathway, and have been shown to be key molecular mediators of flow-mediated endothelial heterogeneity111 (Figure 2C).

**Summary and Future Directions**

As we continue to unravel the molecular and physiological mechanisms that create the diversity of ECs, we hope to become increasingly able to harness the plasticity of the EC phenotype to modulate the pathophysiological events specific to various vascular beds. Such molecular insights may allow one to manipulate EC phenotype in the treatment of vascular-centric disease states that are major sources of morbidity and mortality including atherothrombosis, sepsis, or tumorigenesis. Finally, advances in this area of biology may be facilitated through establishment of improved models of vascular development and plasticity. Although much of the work to date has relied on studies in zebrafish and mice, a recent study found that the mechanism of cell commitment in early embryos differs significantly between mice and cows.112 In this regard, use of human induced pluripotent stem cell technology may allow for novel insights into human endothelial differentiation, specification, and heterogeneity.

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None.

**References**


