Vascular Development

Mark W. Majesky

***Abstract*—**The vascular system forms as a branching network of endothelial cells that acquire identity as arterial, venous, hemogenic, or lymphatic. Endothelial specification depends on gene targets transcribed by Ets domain–containing factors, including Ets variant gene 2 (Etv2), together with the activity of chromatin-remodeling complexes containing Brahma-related gene-1 (Brg1). Once specified and assembled into vessels, mechanisms regulating lumen diameter and axial growth ensure that the structure of the branching vascular network matches the need for perfusion of target tissues. In addition, blood vessels provide important morphogenic cues that guide or direct the development of organs forming around them. As the embryo grows and lumen diameters increase, smooth muscle cells wrap around the nascent vessel walls to provide mechanical strength and vasomotor control of the circulation. Increasing mechanical stretch and wall strain promote smooth muscle cell differentiation via coupling of actin cytoskeletal remodeling to myocardin and serum response factor–dependent transcription. Remodeling of artery walls by developmental signaling pathways reappears in postnatal blood vessels during physiological and pathological adaptation to vessel wall injury, inflammation, or chronic hypoxia. Recent reports providing insights into major steps in vascular development are reviewed here with a particular emphasis on studies that have been recently published in *Arteriosclerosis, Thrombosis, and Vascular Biology.* **(*Arterioscler Thromb Vasc Biol*. 2018;38:e17-e24. DOI: 10.1161/ATVBAHA.118.310223.)**

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The vascular system first appears in the embryo as a highly branched network of structurally primitive vessels composed of endothelial cells and their basement membranes. Embryonic endothelial cells acquire an identify as arterial, venous, lymphatic, or hemogenic and then further specialize in an organotypic manner.[1–3] In addition, erythromyeloid progenitors in the yolk sac give rise to tissue-resident fetal macrophages that play important roles in morphogenesis and function of developing and angiogenic blood vessels.[4–7] Circulating within this embryonic vascular network are primitive erythroid cells that arise from hemogenic endothelial cells in the yolk sac blood islands around E7.5.[2,8,9] With time, these primitive erythroid cells are replaced by definitive erythroid cells.[9] Also circulating in this network are platelets whose function and hemostatic capacity in the fetal circulation are not well understood. Embryonic platelets have been reported to play an essential role in closure of the ductus arteriosus at the time of birth.[10] In a study of murine platelet function from E13.5 to E17.5, Margraf et al[11] report that platelet numbers and P-selectin contents are considerably lower at E13.5 than at E17.5. They also report that platelet integrin-activating proteins kindlin-3, talin-1, and rap-1 are reduced in abundance in fetal platelets, and these reduced levels are correlated with diminished spreading and hypoactive hemostatic functional capacity compared with adult platelets.[11] Yet by the time of birth, platelet function is sufficient to aggregate within the ductus arteriosus and produce an occlusive thrombus which, as indicated above, is required for permanent closure of the ductus.[10]

Transcriptional Control of Endothelial Differentiation

Two distinct mechanisms of blood vessel formation in the early embryo have been described.[12] Formation of endothelial cells de novo by differentiation from angioblasts followed by their self-assembly into vascular structures is called vasculogenesis. Vasculogenesis is dependent on specification of endothelial cell fate from progenitors in the mesoderm via activity of the E26 transformation-specific domain transcription factor Etv2 (also called Ets variant gene 2, or ETSRP71).[13–15] Angiogenesis is the formation of blood vessels from preexisting endothelial cells.[12] The question of whether Etv2 is also required for embryonic angiogenesis, either alone or in combination with other endothelial transcription factors, was addressed by Craig et al[16] using a zebrafish model of vascular development. This study examined functional interactions between Etv2 and the Ets domain–containing transcription factors fli1a and fli1b in early vascular development. Embryos double deficient for *Etv2* and *fli1b* failed to form angiogenic sprouts and exhibited greatly increased endothelial apoptosis throughout the developing vasculature.[16] RNA-Seq analysis and chromatin immunoprecipitation assays revealed that Etv2

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From the Center for Developmental Biology and Regenerative Medicine, Seattle Children's Research Institute, WA; and Departments of Pediatrics and Pathology, University of Washington, Seattle.

Correspondence to Mark W. Majesky, PhD, Center for Developmental Biology and Regenerative Medicine, Seattle Children's Research Institute, 1900 Ninth Ave, Room JMB-525, Seattle, WA 98101. E-mail mwm84@uw.edu

and fli1b share many of the same transcriptional targets during developmental angiogenesis. The authors conclude that Etv2 alone is required for vasculogenesis, while Etv2 and fli1b (but not fli1a) function redundantly during early embryonic angiogenesis.[16] One important target for Etv2 in endothelial cells is an Ets factor–binding intronic enhancer element in the gene encoding vascular endothelial growth factor receptor Flk1/VEGFR2.[17] This enhancer sequence also contains an essential Gata factor–binding motif that likely interacts with Gata2 in endothelial cells.[17] Etv2 is also required for angiogenesis in adult tissues. Park et al[18] used Etv2 conditional knockout mice and ischemic injury models in the eye and hindlimb to show that upregulation of Flk1/VEGFR2 and neovascularization responses requires de novo expression of Etv2 by adult endothelial cells. Overexpression of Etv2 has been used to direct vascular progenitor cells in the postnatal arterial adventitia toward an endothelial cell fate.[19] Etv2-transduced Sca1^{+} progenitor cells were shown to upregulate expression of endothelial-specific genes and inhibit intimal hyperplasia in wire-injured femoral arteries when grafted onto the adventitial side of the femoral artery wall. Thus, Etv2-directed progenitor cell differentiation may eventually become a useful clinical tool.[19,20] The chromatin-remodeling enzyme brahma-related gene 1 (BRG1) is important for early vascular development and primitive hematopoiesis. Menendez et al[21] sought to address the roles of BRG1 in vascular development and used Brg1$^{fl/fl}$;Cdh5Cre^{ERT2} to gain temporal control of *Brg1* deletion. They found that *Brg1* loss of function between E9.5 and E11.5 produced vessel dilation and lethal hemorrhage by E14.5. The defects observed strongly phenocopy those of endothelial deletion of serum response factor (*Srf*).[22] Following that lead, Menendez et al[21] showed that Brg1 directly binds to the promoters of the Srf coactivators myocardin-related transcription factors (*Mrtfa* and *Mrtfb*) and thereby controls the production of junctional protein ZO1 and cytoplasmic β-actin. In addition to ZO1, endothelial tight junctions also contain the ZO1-binding protein cingulin which plays a role in tight junction assembly and barrier function in brain endothelial cells.[23] In another study of endothelial junctions, Chrifi et al[24] showed that CMTM3 (CKLF-like marvel transmembrane domain 3) colocalizes with cytosolic VE-cadherin and early endosome markers EEA1 and clathrin in human umbilical vein endothelial cells. CMTM3 overexpression increases, whereas its knockdown reduces the internalization of cell surface VE-cadherin in vitro. The authors conclude that CMTM3 regulates the endothelial cell surface pool of VE-cadherin and thereby controls cell–cell adhesion at endothelial cell adherens junctions.[24] Although junctional adhesive interactions between endothelial cells have been intensively studied in vascular endothelial cells,[25,26] they remain poorly understood for lymphatic development. Yang et al[27] showed that lymphatic endothelial cell expression of VCAM (vascular cell adhesion molecule) is essential for lymphatic development in zebrafish embryos. VCAM binds to 2 cell surface receptors, integrins α-4 and α-9. Although VCAM binding to α-4 integrin is important for morphogenesis of nonvascular tissues, Yang et al[27] showed that integrin α-9 was the relevant VCAM receptor in zebrafish lymphatic development. Loss of α-9 integrin in mice slows, but does not disrupt, lymphatic development, suggesting that rapid tissue growth rates in embryonic tissues may shift the importance of cell–cell versus cell–matrix interactions for development of lymphatic vessels in vivo.[27] Lymphatic endothelial sprouting from cardinal vein endothelium initiates development of the lymphatic system and is dependent on miR-126a expression by lymphatic endothelium working in concert with VEGFC/Flt4 signaling to promote sprouting and extension of these early lymphatic vessels.[28]

Endothelial Control of Vascular Lumen Diameter

Recent studies published or discussed in the pages of *ATVB* extend our understanding of how the vascular system develops to include fundamental mechanisms of control of lumen diameter, network branching complexity, and stabilization of newly formed vessels.[29–32] In small arteries and arterioles, lumen diameter is controlled mostly by myogenic and neurogenic control of vasomotor activity in circumferentially arranged vascular smooth muscle cells (SMCs).[33] For example, myogenic tone in mouse mesenteric resistance arteries is mediated, in part, through uridine-activated P2Y6 receptor signaling.[33] Such diameter changes can be very rapid and responsive to short-lived signals such as nitric oxide. In larger conduit arteries, lumen diameter is primarily controlled by wall remodeling and is blood flow responsive and endothelial dependent.[34,35] In developing vessels that have yet to form an organized medial layer, increases in lumen diameter can, in principle, be produced by increases in endothelial cell size, cell migration from downstream vessels undergoing regression, or by increases in cell proliferation. Wang et al[36] addressed this question using intermedin-null mice. Intermedin, also called adrenomedullin-2, is a member of calcitonin gene–related peptide family that includes calcitonin, adrenomedullin, and amylin.[37] These peptide hormones initiate signaling via binding to calcitonin receptor–like receptors, and signal propagation is controlled by 3 receptor activity–modifying proteins called RAMPs.[38] Previous work from the Zhang et al[39] group had shown that intermedin promotes increases in blood flow through a neovascular network by increasing the size of the vascular lumen and reducing the number of excessive vascular sprouts. Using CRISPR/Cas9 to generate intermedin-deficient mice, together with experiments performed in vitro, Wang et al[36] were able to show that intermedin promotes increased vascular lumen size by stimulating the proliferation of confluent endothelial cells while maintaining organized cell–cell contacts with no significant changes in overall cell shape.

Coordination of Radial and Axial Growth

A more dramatic example of lumen control regulation is found in the Eln (elastin)-null mouse where complete lack of Eln results in uncontrolled subendothelial SMC proliferation leading to perinatal lethal obstruction of the lumen in large elastic arteries.[40] By contrast, *Eln*$^{+/-}$ mice do not develop obstructive phenotypes, but rather exhibit lumen diameters that are only slightly smaller than wild type.[41] These animals develop moderate hypertension (25–30 mm Hg) and live a normal life span.[41] The lethal phenotype of *Eln*$^{-/-}$ mice can be rescued

by the replacement of the mouse *Eln* gene with the human *ELN* gene delivered in a bacterial artificial chromosome.[42] The human genomic elements thus transferred into the mouse are sufficient to closely phenocopy the correct temporal and spatial expression patterns and alternative splicing profiles found in the mouse. However, $Eln^{-/-}$ mice rescued with a human *ELN* gene delivered in a bacterial artificial chromosome transgene had reduced aortic internal and external diameters while being significantly longer than $Eln^{+/+}$ mice. In an elegant analysis of the development of aortic wall structure in $Eln^{-/-}$ mice rescued with a human *ELN* gene delivered in a bacterial artificial chromosome transgene, Jiao et at[43] showed that reduced circumferential growth was accompanied by increased axial growth and formation of a thicker media. These morphogenetic changes were reminiscent of those seen in $Eln^{+/-}$ mice and are likely the consequence of a reduced content of Eln protein present in the artery wall. The authors concluded that reductions in lumen diameter of Eln-deficient arteries were not because of excess SMC proliferation and intimal thickening, but rather were a consequence of reduced circumferential growth in the perinatal period.[43,44] On the face of it, it seems unlikely that reduced circumferential growth of the aorta in this model is because of reductions in chronic blood flow, although this should be tested. Because these Eln-deficient arteries also exhibit excessive axial growth, the results suggest that the 2 morphogenic parameters are somehow interdependent and possibly linked mechanistically. What the nature of the feedback mechanisms might be between radial growth of endothelial cells and axial growth of SMCs is not clear but is an important direction for future research.

Mechanical Stretch, Wall Strain, and SMC Phenotypes

As the embryo continues to develop, arteries throughout the embryo acquire a mural cell coating to provide mechanical strength and vasomotor control. Investment with SMCs requires migration to and spreading over endothelial cells, a process recently shown to be dependent on expression of endoglin by SMCs.[45] Increases in lumen diameter and blood pressure place a circumferential stretch on mural cells that is thought to promote their differentiation to mature SMCs. SMC differentiation is mediated by RhoA-dependent polymerization of cytoskeletal actin, translocation of myocardin-related transcription factors to the nucleus, and Srf/myocardin-mediated transcription of smooth muscle marker genes together with long noncoding RNAs that amplify SMC differentiation.[46–50] Polymerization of cytoskeletal actin is catalyzed by profilin and associated mDia1 and mDia2 (diaphanous-related formins).[51] To test the role of mDia1 in development and differentiation of vascular SMCs, Weise-Cross et al[52] used a cell-specific overexpression approach using a well-characterized DN-mDia1 (dominant-negative form of mDia1). In these experiments, DN-mDia1, behind a floxed-STOP cassette, was conditionally expressed in SMCs by crossing to SM22Cre mice. Analysis of mid- to late-gestation embryos indicated that hemorrhage was found in 6 of 17 embryos between E15.5 and E18.5 particularly in superficial blood vessels in the head, limbs, and body wall. Smaller vessels in these tissues were found to be poorly invested with vascular SMCs and severely dilated.[52] These findings are consistent with an important role for mDia1 in vascular SMC differentiation, survival, and extracellular matrix production. In addition to cytoskeletal control of SMC differentiation, myocardin expression is also controlled by DNA methylation. Zhuang et al[53] showed that DNA methyltransferase 1 knockdown or 5-aza-2′-deoxycytidine treatment decreased 5-methylcytosine content of the myocardin promoter and increased myocardin expression levels in SMCs in vitro. These treatments increased SMC differentiation, inhibited PDGFBB-induced cell proliferation, cell migration, and suggest a mechanism for inhibition of neointimal formation in vivo.[53] In a related study, Niu et al[54] showed that inhibition of cofilin phosphorylation via activation of the P2Y12 receptor on SMCs activated cofilin's F-actin severing activity, increased SMC motility and migration, and promoted atherosclerosis. Likewise the orphan receptor CD248/endosialin promotes SMC dedifferentiation and increases the extent of atherosclerosis in $ApoE^{-/-}$ mice.[55] CD248/endosialin also promotes the expression of a proinflammatory secretory phenotype in SMCs.[55] Vascular SMC responsiveness to mechanical stretch and wall strain is a major morphogenic pathway in vascular development, an important adaptive pathway in hypertension, and a pathogenic pathway in aneurysm formation.[56–59] However, maladaptive responses to sustained or aggravated stretch can also occur. A study by Rodríguez et al[60] investigated the role of Nox1 (NADPH oxidase isoform-1) in maladaptive stretch–induced SMC phenotypes. These authors found that cyclic stretch (10% at 1 Hz) increased Nox1 expression in a Mef2B-dependent manner. Increases in Nox1 activity increased osteopontin expression and downregulated contractile phenotype markers calponin1, smoothelin-B, and total actin fiber density.[60] Nox1 is also proposed to play a role in pulmonary arterial hypertension as pulmonary artery SMCs from pulmonary arterial hypertension patients or from $Nox1^{-/-}$ mice exhibited increases in cell proliferation and extracellular matrix production, in part, through a 5HT1b serotonin receptor–mediated signaling pathway.[61] Moreover, NoxO1 (NOX organizer-1), a cytosolic protein that promotes the assembly of active NOX complexes, inhibits angiogenesis in the retina and hindlimb models.[62] NoxO1 promotes a switch from a tip cell to a stalk cell phenotype in lung endothelial cells via increased oxidation of ADAM17 and enhanced ADAM17-mediated notch signaling in endothelial cells.[62]

Marfan syndrome is associated with mutations in FBN1 (fibrillin1), an extracellular matrix protein that plays important roles as a component of microfibrils that help assemble Eln filaments in the walls of elastic arteries.[59,63] FBN1 also directly binds the large latent TGF (transforming growth factor)-β complex and BMPs (bone morphogenetic proteins) and sequesters the growth factors in an inactive form.[63,64] Thus, FBN1 mutations can produce gain-of-function phenotypes for TGF-β and BMP signaling. Tissue samples from dilated aortas of Marfan patients showed overexpression of differentiated SMC marker proteins including smoothelin, calponin, and SM22α as well as collagen I.[65] This phenotype was cell autonomous in that explant-derived aortic SMCs

from Marfan patients retained overexpression of these SMC differentiation markers, together with myocardin, RhoA-GTP levels, actin stress fibers, and focal adhesion complexes, even when removed from the aortic wall. Overexpression of these differentiated SMC markers was normalized by antagonists of TGF-β signaling.[65] Further, atomic force microscopy showed that Marfan aortic SMCs and their extracellular matrix were both stiffer than FBN1 wild-type controls. Although these changes in aortic SMC phenotypes are consistent with TGF-β gain-of-function responses, it remains somewhat difficult to reconcile with the ultimate aortic aneurysm clinical outcome. Perhaps the study by Cook et al[66] provides an explanation. These authors found that the effects of treatment with anti–TGF-β–neutralizing antibody either worsened or mitigated formation of thoracic aortic aneurysms depending on whether treatment was started before or after aneurysms appeared, respectively. One interpretation of these findings is that elevated TGF-β signaling observed after aneurysms were present may be a reaction to wall pathology in attempts to repair the aneurysm rather than a cause of it. In pulmonary arterial hypertension, the role of BMPR2 (BMP receptor 2) in control of elastic fiber assembly was examined by Tojais et al.[64] In pulmonary artery SMCs and adventitial fibroblasts, TGF-β1–mediated increases in ELN and FBN1 production were dependent on BMPR2. In *Bmpr2/1a* heterozygous mice, reduced Fbn1 levels in pulmonary artery walls impairs elastic fiber assembly, increases susceptibility of elastic fibers to degradation, and promotes the development of pulmonary arterial hypertension.[64] BMP signaling in endothelial cells also plays an important role in vascular development[67] and postnatal angiogenesis.[68]

Surprising Developmental Complexity of Ascending Aortic SMCs

Genetic fate–mapping approaches have shown that arterial SMCs arise from multiple embryonic origins in vertebrate development.[69–71] Different SMC origins generally map onto different axial domains that correspond spatially to the anterior–posterior organization of the early embryo. The aortic root and ascending aorta are composed of SMCs that originate either from cardiac neural crest SMCs or from the second heart field (SHF-SMCs).[69,72,73] Sawada et al[74] used a well-characterized Mef2c SHF enhancer-Cre mouse[75] to reexamine the distribution of SHF-SMCs in the outflow tract and ascending aorta from 3 to 25 weeks of age. Previous reports suggested the SHF contribution was limited to the aortic root in chick[72] and mouse[73] embryos using chick-quail chimeras and Nkx2.5cre lineage-tracking approaches, respectively. Sawada et al[74] confirmed the previous findings and extended them to show that the SHF-derived SMCs actually distribute from the aortic root along the outside of the developing aortic wall to form the outer medial layers of the ascending aorta. Using Wnt1-cre mice to track neural crest–derived SMCs, these authors found that there was little or no mixing of the 2 types of aortic SMCs in most of the ascending aorta except, curiously, for a restricted region in the lesser curvature of the aortic arch.[74] The new picture that emerges is that the ascending aortic media is actually composed of 2 compartments with inner medial layers composed of neural crest SMCs and outer medial layers made up of SHF-SMCs.[74] By contrast, abdominal aortic SMCs originate from paraxial mesoderm via the somites.[69,70,76] Angelov et al[77] studied the role of TGF-β signaling during aortic aneurysm formation in the angiotensin II–infused mouse model. These authors compared systemic inhibition of TGF-β signaling obtained with neutralizing anti–TGF-β antibody to local inhibition via SMC-specific deletion of TGF-β receptor II for their effects on aortic wall pathology. They concluded that TGF-β signaling normally protects against aortic aneurysms but does so by different mechanisms in the thoracic compared with the abdominal aorta.[77] The embryonic origin and lineage history of a cell are important determinants of the overall organization and function of its epigenome.[78] Patterns of epigenetic marks form an epigenetic landscape, a molecular code that specifies patterns of gene expression and cell identity.[79,80] These findings, therefore, raise the intriguing possibility that the distribution of aortic dissecting aneurysms, which most frequently arise in the outer media of the ascending aorta in mice[81] and humans,[82] may have a developmental basis. Such an interpretation has been proposed for homeobox gene–dependent regulation of NF-κB activity in aortic SMCs[83] and the distribution of aortic calcification.[84]

Development and Composition of the Adventitia

Completion of aortic media formation around E15.5 marks the initiation of development of the adventitia.[57] The adventitia is the most complex layer of artery wall and is composed of different types of fibroblasts, inflammatory cells, nerves, microvessels, and resident progenitor cells.[85–91] The adventitia is a sensor of biomechanical wall strain, contains a microvascular network called the vasa vasorum, plays a critical role in immune cell trafficking,[92] and provides a progenitor niche environment for the artery wall.[86–91] Lineage-tracking studies using Wnt1-cre mice showed that aortic adventitial cells in the ascending aorta were not derived from progenitors in the cardiac neural crest.[93] Given the recent evidence from Sawada et al,[74] an origin of at least some of these adventitial cells in the ascending aorta from the SHF seems likely. Adventitial fibroblasts, like fibroblasts in all tissues, are a diverse group of cells with multiple functions.[94] For example, fibroblast transcriptional profiling experiments show that fibroblasts cluster into groups that are best defined by their anatomic site of origin.[95] Later work showed that dermal fibroblasts can be sorted into 2 groups based on their origins from Engrailed1-positive progenitors in the dermatome of the somite.[96] Engrailed1-derived fibroblasts were responsible for the bulk of fibrotic tissue produced in response to injuries to cutaneous tissue when compared with dermal fibroblasts in the same tissue that were not derived from Engrailed1-positive precursors.[96] Adventitial fibroblasts also are active innate immune regulators producing proinflammatory cytokine IL (interleukin)-6 and monocyte chemoattractants such as MCP1 in response to an inflammation-provoking stimulus.[97] A critical mediator of angiotensin II–induced IL-6 and IL-1β production in abdominal aortic adventitial cells is NF-κB/RelA.[98] In addition to innate immune functions, adventitial cells are also major mediators

of artery wall fibrosis that accompanies vascular remodeling in atherosclerosis, restenosis, and aortic aneurysm formation. TGF-β, an important mediator of tissue fibrosis, requires the activity of CYLD (cylindromatosis), a deubiquitinase enzyme to stimulate the production of IL-6 and MCP1 by adventitial fibroblasts.[99] Deubiquitination of Nox4, which directly interacts with CYLD, increases the half-life of Nox4 in adventitial cells and plays an important role in the fibroblast to myofibroblast transition leading to artery wall fibrosis.[99] The aortic adventitia lies in close proximity to perivascular adipose tissue. Leptin is an adipokine with important metabolic regulatory functions mediated via LepR (leptin receptor) signaling. Leptin also increases the migratory activity of adventitial Sca1$^+$ progenitor cells in vitro, and LepR antagonists inhibit neointima formation in a femoral wire injury model.[100] The development of the adventitia with respect to the timing and order of appearance of the different resident cell types described above is not known. Also not yet clear is whether resident progenitor cells in the adventitia contribute to the formation of the adventitia itself during postnatal development or are strictly present for repair and remodeling.

Summary

The studies reviewed above suggest that vascular development is a sequential and highly orchestrated process molded by cell–cell interactions and biophysical forces. By necessity, blood vessels rapidly extend to all parts of the growing embryo and play multiple roles in the patterning and organogenesis of diverse tissues. Blood vessels are developmental mosaics whose walls are assembled from progenitor cells that arise from many different embryonic origins and thus confer origin-specific properties on the final structures produced. At the same time, blood vessels, and the cells within their walls, are highly responsive to changes in the local environment and exhibit remarkable and unexpected plasticity. Focused study of the mechanisms of vascular development can produce surprising clues about adult vascular disease as cells respond to tissue damage and reexpress developmental programs to repair injury and remodel vessel walls.[101–106]

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Disclosures

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