Published in final edited form as:
Wiley Interdiscip Rev Dev Biol. 2013 ; 2(3): 327–346. doi:10.1002/wdev.91.

Understanding vascular development:

WIRE Developmental Biology (2012)

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Abstract

The vasculature of an organism has the daunting task of connecting all the organ systems to nourish tissue and sustain life. This complex network of vessels and associated cells must maintain blood flow but constantly adapt to acute and chronic changes within tissues. While the vasculature has been studied for over a century, we are just beginning to understand the processes that regulate its formation and how genetic hierarchies are influenced by mechanical and metabolic cues to refine vessel structure and optimize efficiency. As we gain insights into the developmental mechanisms, it is clear that the processes that regulate blood vessel development can also enable the adult to adapt to changes in tissues that can be elicited by exercise, aging, injury, or pathology. Thus, research in vessel development has provided tremendous insights into therapies for vascular diseases and disorders, cancer interventions, wound repair and tissue engineering, and in turn, these models have clearly impacted our understanding of development. Here we provide an overview of the development of the vascular system, highlighting several areas of active investigation and key questions that remain to be answered.

INTRODUCTION

The cardiovascular system (CV) is the first functional organ system formed during vertebrate development. The major function of the CV system is to enable gas exchange, supply nutrients and remove waste from tissues in order to properly nourish cells within the body and sustain organism growth and viability. In addition to these obvious physiological roles, it is now appreciated that endothelial cells and vessels can also provide key regulatory and guidance cues to support the development of other systems, for instance in the pancreas or the nervous system[1–5]. As the embryo develops, the cardiovascular system also plays an important role in lymph regulation, systemic functions of the endocrine system, as well as immunological surveillance and inflammation.

In rodents and humans, the cardiovascular system is composed of a four-chambered heart connected to the rest of the vasculature through the pulmonary arteries and veins (to circulate blood to and from the lungs) and the dorsal aorta and sinus venosus (to circulate blood throughout the rest of the body). The vasculature can be subdivided into three main vessel systems. The arterial system carries blood away from the heart, with larger arteries such as the pulmonary artery or the dorsal aorta feeding into progressively smaller diameter arteries, arterioles, and capillaries. In contrast, the venous system transports blood back to the heart by collecting it from capillaries and transporting it through progressively larger

venules and veins. Finally, the lymphatic system of vessels transports interstitial fluid from tissues and organs and returns it to the circulation ultimately through drainage into the subclavian veins. Together, these three vascular beds form a closed system of vessels that comprise the circulatory system.

The vessels themselves are composed of several different cell types. The inner lining of vessels (the endothelium) is made up of endothelial cells arranged in a simple squamous epithelial layer that surrounds the internal lumen of the blood vessel. In small vessels and capillaries, the endothelium is also often supported by vascular smooth muscle cells and pericytes, collectively known as mural cells, that associate with the abluminal side of the vessel[6], although mural cell associations are sparse in many of the smallest vessels. Larger arteries and veins take on an even more complex structure, with the endothelium (also referred to as the tunica intima) surrounded by both a thick stabilizing layer of smooth muscle cells (tunica media) and an outermost layer of connective tissue, collagen, and elastic fibers (tunica adventitia)[7]. This structure confers stability to these vessels, while still allowing them to dynamically respond to changing metabolic demands by altering blood flow using both acute and chronic adaptations. Acute changes in vessel diameter and blood flow are regulated by contractile mural cells, which contract or relax depending on signals from the tissue. In contrast, chronic changes in the vasculature require the assembly, disassembly, or remodeling of vascular beds.

Development of the circulatory system involves the orchestration of several overlapping events to build then remodel the vasculature into mature vessels. Endothelial cells must be specified and assembled or added into growing vessels either through *vasculogenesis*, the *de novo* formation of vessels from aggregated endothelial precursors, or *angiogenesis*, the formation of neovessels from existing vessels. Vessels are then remodeled to refine the structure based on metabolic need and structural constraints of the tissue (Figure 1). Remodeling can also facilitate vessel regression to eliminate transient or unnecessary vessels during morphogenesis. Interestingly, although these events are required to establish the functional circulatory system in the embryo, many of these processes continue to be active throughout the life of the organism. The CV system must remain constantly adaptive to changes in the body triggered by tissue growth (including adipose expansion or loss), muscle addition/atrophy, cyclic remodeling of the reproductive system, and wound repair, as well as during pathological conditions, such as diabetes or cancer[8–13]. The same principle processes that regulate development thus continue throughout life to maintain one of the most dynamic organ systems within the body.

Though many informative reviews have been written about vasculogenesis and angiogenesis in vertebrates[7, 14–17], utilization of new markers and experimental strategies have revealed novel insights into the mechanisms of blood vessel formation. Thus, the purpose here is to highlight the latest advances in research on vasculogenesis and angiogenesis. Examples from several vertebrate model systems, including avians, teleosts and mammals are discussed, with an emphasis on data from mouse studies.

ENDOTHELIAL CELLS: THE BUILDING BLOCKS OF VESSELS

The first step in building blood vessels is to specify endothelial cells. Though it is currently unclear precisely how endothelial cells are specified, early endothelial cells (also referred to as angioblasts) can be identified in avian embryos using MB1 and QH1 immunochemical markers[18, 19]. Classical approaches in avians, including surgical ablation and chick-quail transplants, have revealed that extraembryonic angioblasts arise from extraembryonic mesoderm that exits the posterior primitive streak[14, 15, 20–23]. In contrast, intraembryonic angioblasts form from both somatic and paraxial mesoderm[14, 24]. In mice, angioblasts have been identified as non-aggregated TAL1+(t-cell acute lymphocytic leukemia protein 1)/KDR+(kinase insert domain protein receptor, also known as vascular endothelial growth factor receptor 2 or fetal liver kinase 1) mesodermal cells of the E6.5 extraembryonic yolk sac, and after E7.5, endothelial cell differentiation is marked by the upregulation of *Pecam1* (platelet endothelial cell adhesion molecule 1), *Tek* (endothelial-specific receptor tyrosine kinase, also known as tyrosine kinase with immunoglobin-like and egf-like domains 2 [Tie2]) and *Laminin*, followed by the downregulation of *Tal1*[14, 25].

In both mice and avians, extraembryonic angioblasts are found in the yolk sac clustered with hematopoietic cells in blood islands, whereas in zebrafish, blood islands are found intraembryonically in the lateral mesoderm[14, 26–29]. Due to this close association between these early endothelial and hematopoietic cells, it has long been postulated that the two cell types arise from a common bipotent progenitor termed a hemangioblast[28, 29]. However, the exact nature of this lineage relationship is controversial, particularly because different experimental models for exploring their lineage have often yielded multiple outcomes. For example, recent fate mapping and live imaging experiments have shown that at least a subset of all hematopoietic cells are derived from an endothelial cell intermediate, known as the hemogenic endothelium[30–36]. Further complicating the picture is the fact that in some cases, endothelial and hematopoietic precursors arise independently[14, 37–39,40], most notably during intraembryonic vasculogenesis, where angioblasts arise alone[14, 37, 38, 40]. Future work will hopefully reconcile some of the opposing results observed using these different systems and further illuminate the subtleties of this relationship between vasculogenesis and hematopoiesis.

At present, the regulatory mechanisms that drive angioblast differentiation are not completely clear. Many factors that are required for the normal specification of endothelial cells are also required for hematopoietic development[16, 41] so it is not clear how these two different cell types emerge in mammals. Although specific transcription factors expressed exclusively in hematopoietic cells have been identified, there is no known single transcription factor expressed in endothelial cells that could act to control endothelial specific transcription[41]. That said, it is clear that the regulatory regions of endothelial-specific genes share similarities in binding site motifs, and it has been proposed that a specific combination of factors may be required for endothelial specification[41]. Moreover, since there is a diversity of endothelial cell sub-types (arterial, venous and hemogenic endothelial cells), some of which arise in very early development, it is possible that there may be distinct pathways that regulate these different sub-types. More studies need to be performed to determine if all endothelial cells are specified via the same pathway, or if

endothelial cell sub-types arise from lineage-restricted populations under the control of distinct factors.

While initial endothelial cell specification is established during gastrulation in the embryo, it is less clear how endothelial cell differentiation is regulated in the adult. Studies over the past 15 years have suggested that circulating endothelial progenitor cells (EPCs) that derive from hematopoietic and mesenchymal stem cells in the bone marrow[42, 43] can contribute to new blood vessel formation or blood vessel repair in adult tissues[44–52]. However, conflicting studies make it difficult to ascertain whether EPCs are directly integrated into vessels, or if they contribute indirectly by releasing localized paracrine factors that promote proliferation of endogenous endothelial cells[53–58].

VASCULOGENESIS: ASSEMBLY OF VESSELS FROM INDIVIDUAL CELLS

New vessels can be generated via two different, well-recognized processes. Vasculogenesis describes the process by which new vessels form from the coalescence and assembly of endothelial cells into functional vessels. Later, we will also discuss angiogenesis, which is the sprouting of new vessels from existing vessels. In some cases vasculogenesis and angiogenesis can occur together, but in the very early embryo, the first vessels including the presumptive dorsal aortae and aortic arches, the internal carotid arteries, and the anterior and posterior cardinal veins[59], are assembled *de novo* from individual endothelial cells. For some other vessel networks, vasculogenesis results in the formation of an intermediate network of similar sized vessels with polygonally-shaped avascular spaces – known as a vascular or capillary plexus (Figure 1). These networks are often remodeled into branched tree-like hierarchies as the surrounding tissues mature, demanding more perfusion. Examples include the perineural plexus that surrounds the developing neural tube, the capillary plexus in the allantois that is remodeled into the umbilical vein and artery, and one of the first vessel structures in the embryo, the primitive capillary plexus of the visceral yolk sac in mammals and avians.

Surprisingly, little is known about the precise mechanisms that drive vasculogenesis *in vivo*. In the extraembryonic yolk sac of the quail, blood island clusters are composed of an outer layer of angioblasts that differentiate into flattened endothelial cells and surround an inner group of rounded primitive blood cells. Here, differentiating endothelial cells in the blood islands form sprouts and anastomose (interconnect) to other blood islands, ultimately forming the primitive yolk sac vascular plexus[19, 23, 60–62]. In this way, the yolk sac plexus serves the important function of connecting the first site of blood development, the blood islands, to the embryo proper. Similarly, in mice, primitive blood cells form one large band around the proximal yolk sac that is apparent by E7.5-E7.75, but here they only loosely associate with angioblasts, until eventually the differentiating endothelial cells ensheathe the blood cells through an unknown mechanism[63]. In contrast, intraembryonic vessels initially form independently of such an association with primitive blood cells by the *de novo* formation and coalescence of angioblasts into the major intraembryonic vessels and plexuses. It should be noted that though the coalescence of angioblasts is critical to the formation of the founding extraembryonic and intraembryonic vessel plexuses, it is unknown what extent angiogenesis plays in the formation of these early plexuses. Further

studies need to be performed at the appropriate resolution to more clearly distinguish when these different mechanisms are enacted. It is clear, however, that as development proceeds angiogenesis becomes an increasingly important means of vessel formation.

Due to the complexity of studying vasculogenesis in a live animal, *in vitro* models using two-dimensional (2D) cultures or three-dimensional (3D) extracellular matrices have been used to enhance our understanding of the mechanisms of vasculogenesis[64, 65]. Such cultures have been implemented using human or murine embryonic stem cells that, under the appropriate culturing conditions, differentiate into a vasculature[64]. While many *in vitro* systems have been used to study endothelial cell biology, assays in 2D and 3D cultures have been able to recapitulate many fundamental aspects of early vasculogenic processes such as the dynamic migration of endothelial cells and pericytes, cord formation, as well as the formation of lumenized vessels. Using a combination of time-lapse microscopy, molecular genetic approaches and biochemical analysis, these studies have revealed that key interactions of isolated endothelial cells with various extracellular matrix (ECM) components, such as collagen and fibrin, can induce changes in endothelial morphogenesis that promote vascular assembly[65].

ANGIOGENESIS: THE GROWTH OF NEW BLOOD VESSELS FROM A PREEXISTING VASCULATURE

Sprouting angiogenesis

In addition to vasculogenesis, new vessels can form by sprouting from the existing vasculature. This process underlies the formation of many vessel structures during development, including but not limited to formation of the intersegmental vessels, and vessels of the developing retina, limbs, yolk sac, heart and CNS[15, 66–72]. Similar processes are also utilized in response to hypoxia, tumor growth and wound healing in the adult organism[11, 12, 73].

Vessel sprouting was first recognized *in vivo* in the late 1800s using light microscopy. Vessel sprouts were observed in tadpole tails and in the newt by Platner and Meyer, as characterized by the presence of thin fibers which expanded in diameter and later became hollow[74–76]. Such observations were also described during wound healing and in tumor angiogenesis in early studies[77].

Despite these very early observations, the cellular and molecular mechanisms that regulate sprouting angiogenesis have only recently begun to be elucidated. Moreover, advances in genetic models and tools to analyze cell-signaling have revealed five key events associated with sprouting angiogenesis. First, cell-cell signaling initiates new vessel formation. Second, a subset of endothelial cells takes on the role of endothelial tip cells, which make up the distal-most cells of a temporarily close-ended vessel and extend lamellipodia and filopodia into the avascular space. Third, juxtaposed endothelial cells form endothelial stalk cells whose main function is to proliferate and provide more cells to extend the growing vessel[73, 78, 79]. Fourth, vessel outgrowth propels the sprout into the tissue perpendicular to the axis of the parent vessel. Last, individual sprouts anastomose with other vessels. These events (1–5) are illustrated in Figure 2, and are further described below. Though these

events are demonstrated in a linear manner, changes in the vasculature and the extracellular environment may elicit a reprogramming of the angiogenic response; thus, there is some fluidity in endothelial tip versus stalk cell identity during vessel outgrowth and fusion.

1) Signaling to induce new vessel formation—The induction of new vessels can be initiated by several known pro-angiogenic factors that are used in development, repair, homeostasis, and tumor angiogenesis[80]. Perhaps the most well-studied factors are those of the vascular endothelial growth factor (*Vegf*) family, which together with NOTCH signaling, are known to regulate new vessel outgrowth in a variety of different sites throughout embryogenesis[80]. There are several members of the *Vegf* family (*Vegfa*, *Vegfb*, *Vegfc*, *Figf* and *Pgf*), and multiple isoforms of VEGFA protein with different molecular weights that can each have different activities[6, 81]. VEGF ligands signal primarily through VEGF receptors (transmembrane receptor tyrosine kinases) and have diverse roles in endothelial cell differentiation, survival, vessel diameter regulation, vessel barrier integrity and endothelial cell chemotaxis[82–88]. In addition to these roles, VEGF also functions in mediating sprout induction and vessel outgrowth. Differences in the response of endothelial cells to VEGFA are likely to relate to signaling through different receptors and the activation of different intracellular pathways, including importantly the NOTCH pathway (discussed further below)[16].

It is thought that the establishment of a VEGF gradient, initiated by hypoxia or cell-cell signaling, directs growing vessels[89] (Figure 2). Though VEGF is a widely known mediator of sprouting angiogenesis, other factors have also been shown to direct vessel outgrowth. For instance, FGF2 (basic fibroblast growth factor) is a potent angiogenic factor in addition to playing diverse roles in many different tissues[90, 91]. Recently, it has been shown that FGF2 induces *Kdr* expression, adding to our understanding of how these factors may interact during angiogenesis[92].

2) Induction of a tip cell—Tip cells are induced by high concentrations of VEGFA signaling through KDR to specify a single endothelial cell to lead the vessel sprout [93] (Figure 2). Endothelial tip cells are so-named because they are located at the distal end of a close-ended outgrowing vessel. Morphologically, they are characterized by the presence of lamellipodia and filopodia extensions, and they rarely proliferate. Molecularly, they express high levels of *Dll4* (delta-like 4) mRNA[93] and secrete proteinases which break down the basement membrane[94].

3) Formation of stalk cells—Tip cells are flanked by endothelial stalk cells. In contrast to tip cells, stalk cells maintain adherens junctions and do not extend processes. These cells proliferate in response to VEGFA, and unlike tip cells, do have lumens[93]. Since only one endothelial tip cell is formed at the leading edge of a sprout, a mechanism must exist to prevent neighboring endothelial cells from also forming tip cells. Competition for tip cell versus stalk cell specification occurs via a NOTCH-mediated lateral inhibition mechanism, whereby the cell exposed to the highest amount of VEGF signaling upregulates *Dll4* expression to become the NOTCH signal-sending cell and the tip cell (Figure 2). Then, neighboring NOTCH signal-receiving cells downregulate *Kdr* expression, reducing their ability to respond to VEGFA; these cells form endothelial stalk cells[66, 79, 95].

4) Vessel outgrowth—After establishment of tip and stalk cells, vessel outgrowth begins. Recent studies in mouse embryoid bodies and mouse retinal vessels have shown that perpendicular vessel outgrowth occurs due to the production of soluble FLT1 (a soluble isoform of FMS-like tyrosine kinase 1, a VEGF receptor) in endothelial cells that neighbor the tip cell (Figure 2). Upregulation of soluble FLT1 in neighboring stalk cells provides a sink for VEGF ligand to increase the sharpness of the VEGF gradient and direct outgrowth perpendicular to the existing vessel[96].

5) Vessel fusion to other vessels—Finally, once vessel outgrowth occurs, the sprouting vessels eventually undergo anastomosis to fuse to neighboring vessels. Though our understanding of the mechanisms that regulate anastomosis is incomplete, studies in the mouse hindbrain and retina, as well as in intersomitic vessels of zebrafish, show that macrophages can act as cell chaperones for vessel anastomosis (Figure 2)[97–99]. For example, the release of VEGFC by macrophages can activate FLT4 in endothelial tip cells to promote the rapid conversion of tip cells to stalks cells at vessel fusion points[98]. Though macrophages have also been implicated in aiding angiogenesis during repair[100, 101] and facilitating interactions between tumor cells and vessels,[100, 101] it is unknown whether macrophages also play a physical role in chaperoning vessel anastamosis.

Intussusceptive (splitting) angiogenesis

Another type of angiogenesis is called intussusception. Prior to the discovery of intussusception, it was thought that all vessel angiogenesis occurs via a sprouting angiogenesis mechanism. Though the process of intussusception was initially seen with longitudinal observations of the rabbit ear vasculature[102], intussusception has now been characterized in different species (humans, rats, and birds), and in many different tissues and organs (lung, chorioallantoic membrane, kidney, heart, endometrium, ovary, retina, brain), as well as in disease (tumor induced angiogenesis)[37, 103–114]. In its simplest form, intussusception (also known as splitting angiogenesis) occurs in a vessel by the formation of an endothelial-lined pillar that extends into the middle of a vessel, followed by the expansion of pillar diameter, resulting in the splitting of the vessel (Figure 1). Studies within the past 25 years have provided the spatial resolution required to discern the cellular mechanisms of intussusception[104, 105, 109–111, 115–120] (see below), but little is known about the mechanisms of transcapillary pillar induction. Analysis of computational models reveals that pillars form in areas of either low shear stress or oscillatory blood flow[106, 121].

There are three major forms of intussusceptive angiogenesis – intussusceptive microvascular growth, intussusceptive arborization and intussusceptive branching remodeling. Intussusceptive microvascular growth is a mechanism for capillary expansion that was first identified in EM studies of pulmonary capillaries, but has been found in many tissue types[116, 117]. Observations in chick chorioallantoic membrane (CAM) vessels have directly documented pillar formation and confirmed the impact of the mechanism on microvascular growth[104, 105, 109, 110]. Other studies have shown that intussusceptive microvascular growth can allow for rapid expansion (on the order of hours) in the number of capillaries in a vessel bed with little contribution from cell proliferation or apoptosis, thus minimizing the amount of energy required for vessel expansion[115].

Intussusception is utilized not only for the expansion of capillaries, but also for shaping vessel morphology. In intussusceptive arborization, vessel hierarchies are established by the formation and fusion of transcapillary pillars from unremodeled capillaries[105]. Thus, initially large capillaries are parsed into proximal feeding vessels that extend into progressively smaller distal capillaries. In intussusceptive branching remodeling, transcapillary pillars are formed at vessel bifurcations which eventually fuse to the connective tissue to decrease branch angles[106].

LUMEN FORMATION IN DEVELOPING BLOOD VESSELS

Regardless of whether new vessels form by vasculogenesis or angiogenesis, they must eventually support blood flow, which requires that a lumen forms *de novo* or by extension from the parent vessel. Models of lumen formation in blood vessels date back to the early 1900s. Studies by Clark and Clark in the 1930s showed that newly sprouted vessels contained discontinuous vacuolation in rabbit ear vessels[122–124], raising the idea that vacuole formation was a key mechanism to generating vessel lumens. Time-lapse microscopy studies in 3D *in vitro* cultures have beautifully shown that indeed, isolated endothelial cells undergo simultaneous sprouting and intracellular vacuolation. Interestingly, anastomosing endothelial cells undergo exocytosis of intracellular vacuoles to produce an internal lumen[65]. Here intracellular vacuoles ultimately coalesce, span the length of the cell and subsequently fuse to the plasma membrane and connect with the newly formed lumens in neighboring cells[125]. Work in 3D cultures has clearly shown that this process mechanistically depends on INTEGRIN/ECM interactions which signal through RAC1, RHOA and CDC42 GTPases[65].

Though *in vivo* studies using time-lapse microscopy initially suggested that lumen formation in zebrafish occurs via the coalescence of intracellular vacuoles within endothelial cells[126], follow up studies showed that these intersegmental vessels actually form by intercellular lumenogenesis[127]. Close inspection of intersegmental vessels reveals that they are comprised of more than one cell, and lumen formation occurs at cell-cell contacts. Still, intracellular vacuoles may undergo exocytosis with this extracellular space to at least partially contribute to lumen formation [127]. Another model for intercellular lumen formation has recently been demonstrated in the developing mouse dorsal aortae. Here the dorsal aortae lumenize by the aggregation of angioblasts due to the establishment of specific extracellular interactions, followed by the reorganization of junctions at lateral regions between the cells, repulsion of the apical surface of adjacent cells, and the subsequent change in EC shape established by the apical actomyosin complexes[128]. Taken together, it appears as if an intercellular lumenogenesis mechanism is adopted *in vivo*, but whether intracellular lumenogenesis occurs in other vascular beds remains to be determined.

ESTABLISHING ARTERIAL, VENOUS AND LYMPHATIC IDENTITIES

Three distinct types of vessels are formed during development: the arterial network that carries blood away from the heart, the venous network that carries blood to the heart, and the lymphatic network that regulates lymph drainage by carrying small molecules and solutes from the tissues. As soon as the first vessels are established within the embryo, and in some

cases even before blood flow begins, heterogeneity among endothelial cells is initiated by the specification of arterial-venous fates, although these fates are not irreversibly determined. In many experiments, arterial-venous fates have been assessed by the analysis of marker gene expression. In the avian yolk sac plexus, NRP1 (neuropilin 1, a KDR coreceptor expressed in arteries) and NRP2 (neuropilin 2, a KDR coreceptor expressed in veins) are found to be segregated in some blood islands and later in the presumptive arterial and venous regions before blood flow begins[129]. Separate arterial and venous expression in the yolk sac plexus is similarly observed in the mouse yolk sac with *Efnb2* (ephrinB2) and *EphB4* (eph receptor B4), respectively[130]. However in intraembryonic vessels of the mouse, expression of arterial markers (for example, *Efnb2*, *Gja4/5*, *Hey1/2*, *Nrp1*, *Notch1/4*, *Dll4*) precedes that of venous markers, and is found both in endothelial cells of the arteries (which form first) as well as endothelial cells that give rise to veins[131]. At a slightly later stage, the endothelial cells that give rise to veins start to coalesce to form the future cardinal veins, and these endothelial cells begin to express venous markers (for example, *Nrp2*, *Aplnr* [apelin receptor], and *Flt4*)[131]. Thus, within the mouse embryo, a specific spatiotemporal sequence is beginning to emerge for how arteries and veins are specified.

Recent studies have further illuminated the underlying molecular mechanisms of arterial/venous (A/V) specification. Studies in zebrafish have revealed that Shha (sonic hedgehog a), Vegfaa and Notch signaling may specify arterial and venous fates of the presumptive dorsal aorta and posterior cardinal vein[132, 133]. First, Shha released from the notochord stimulates the expression and release of Vegfaa from the paraxial mesoderm[133]. Second, exposure of the adjacent presumptive dorsal aortae to high levels of Vegfaa induces arterial differentiation and *efnb2a* expression through Notch activation. In contrast, the more distal presumptive cardinal vein is exposed to a lower amount of Vegfaa, Notch is not activated, and a program of venous differentiation begins[132, 133]. Supporting the zebrafish studies, mouse embryonic stem cells exposed to high or low concentrations of VEGFA begin expressing arterial (*Efnb2*, *Nrp1*) or venous (*Nr2f2*, also known as *CoupTFII*, and *Nrp2*) specific markers, respectively[134]. Another recent study has shown that the presumptive dorsal aorta and cardinal vein first form as a single cord, where the dorsal domain of the vessel is specified by NOTCH signaling to become arterial, and the ventral domain becomes venous [135]. Then, via *Efnb2*/*Ephb4* bidirectional signaling, venous-fated angioblasts sprout ventrally and segregate from the developing dorsal aorta to form the posterior cardinal vein[135]. Other studies in mice and zebrafish have further characterized NOTCH as a mediator of arterial specification, as downstream effectors of NOTCH (*Hey1*, *Hey2* and *gridlock*) are required for proper arterial specification, while NOTCH activation and effector expression are absent in veins[136, 137]. Despite this genetic control in A/V specification, however, Moyon et al. have demonstrated plasticity in cell fates, as A/V grafts from the quail can incorporate into both arteries and veins of the chick[138]. Also, Le Noble et al. have revealed that the initiation of artery specification is flow independent, but that flow is important in maintaining A/V cell fates, as ligated (flow reduced) arteries differentiate into and connect with veins[139]. Similarly, in the mouse dorsal aortae, flow is required for expression of a select group of arterial markers[131, 140]. Thus, to some extent, hemodynamics and interactions with neighboring endothelial cells can influence the plasticity of endothelial cell fates.

A third endothelial cell subtype that arises after A/V specification is that of the lymphatic endothelial cell (LEC). Lymphatic vessels make up a tree-like vascular system that functions to return extravasated fluid from the interstitial environment back to the blood circulation, to aid in immune surveillance by transporting white blood cells, and to absorb lipids in the digestive tract[141, 142] (Figure 3). LEC fate is specified in mice around E9.75 (embryonic day) in a subset of venous endothelial cells that make up the walls of the anterior cardinal vein (Figure 3). This process is controlled by *Prox1* (prospero related homeobox 1), which has been shown to be both necessary and sufficient for inducing lymphatic differentiation of endothelial cells[143–145]. Transcriptional control of *Prox1* expression is regulated by SOX18 (sry-related hmg-box transcription factor) and NR2F2. Though transient expression of *Sox18* is found in the dorsal aorta, blood islands, cranial surface vessels, and microvasculature of the caudal trunk and hind limb[146, 147], the convergent expression of *Sox18* in the dorsal lateral region of the anterior cardinal vein together with venous expression of *Nr2f2* is necessary for *Prox1* expression in this region[148–150]. Following induction of *Prox1* expression in venous endothelial cells of the cardinal vein, they begin to downregulate genes specific to blood endothelial cells and upregulate genes specific to LECs, including *Flt4*, *Pdpn* (podoplanin), and *Foxc2* (forkhead box c2) transcription factor[151–155]. Importantly, *Flt4* expression in these newly specified LECs makes them competent to respond to VEGFC signaling from the nearby mesenchyme, and at E10.5 they begin to bud off from the cardinal vein in response. During this process, clot formation by platelets is essential for closing off the connection between the cardinal vein and the nascent lymphatic vasculature[151, 156]. Once this process is complete, the LECs coalesce to form the lymphatic sacs, which will eventually give rise to the rest of the lymphatic vasculature via sprouting angiogenesis (see above)[157–159]. Studies in zebrafish and *Xenopus* have shown that many of these pathways regulating lymphatic development are highly conserved[160–163].

VESSEL REMODELING AND MATURATION: CREATING STABILIZED VESSEL HIERARCHIES

Vascular remodeling

Simultaneous to embryonic vasculogenesis in the mouse, the heart tube also forms, connects to the dorsal aortae by 0-3 somites, and becomes functional by 3 somites, where functionality is first determined by the onset of plasma flow[164]. Then, as blood cells enter the circulation at 5–6 somites[165, 166], hemodynamic action marks the onset of an aspect of vascular development that is important for the maturation and stabilization of vessels—vascular remodeling. In the adult, vessel remodeling is usually a term used to describe a change in the size of a vessel (inward remodeling, which is a reduction in vessel diameter, and outward remodeling, which is an enlargement of vessel diameter) to enable vessels to cope with changes in blood flow[167, 168]. However, during early embryonic development, vascular remodeling mainly serves in the maturation of blood vessels from a primitive vasculature to a hierarchical network of large-diameter proximal arteries and veins that feeds into the more extensive network of small-diameter distal capillaries (Figure 1).

For over one hundred years it has been posited that physical forces imparted by blood flow directly influence changes in the endothelium that are responsible for vascular

remodeling[169–171]. Recently, it has been shown that viscous forces imparted by blood flow are necessary and sufficient for normal vascular remodeling of the mouse yolk sac vascular plexus, independently of changes in oxygenation or a circulating signal[164, 172]. Other studies also implicate mechanical forces imparted by blood flow in regulating the remodeling of the heart, aortic arches, and other vessels in a variety of animal models[169, 172–177]. In all of these cases, it is still unclear how mechanical forces are interpreted by cells at the molecular level *in vivo*. A large body of work shows that exposure of endothelial cells in cell culture to fluid shear stress can activate a large number of mechanotransduction pathways and induce changes in several EC behaviors that could influence remodeling, including endothelial cell migration, alignment, proliferation and apoptosis[178–183]. However, it remains to be determined which mechanisms are utilized *in vivo* during development. This has proven to be difficult for several reasons. First, it is highly likely that signaling pathways that regulate mechanotransduction *in vivo* are also used to regulate other events such as growth factor signaling, interactions with the ECM, survival, and proliferation. Thus, the role in mechanotransduction is not likely to be revealed by simple genetic analysis or through the use of inhibitors unless sophisticated assays are used. Second, we have only begun to understand exactly which cellular behaviors are altered by mechanical signals *in vivo*. Understanding these questions would be a tremendous benefit to the design of more specific experiments to test this hypothesis.

Vessel Maturation

Once vessel formation and remodeling are complete, vessel stability and tissue homeostasis becomes crucial for a new vessel. This is partially achieved through the recruitment of smooth muscle cells and pericytes, collectively known as mural cells, to the maturing vessel wall[6]. During maturation, endothelial cells recruit and promote the proliferation of mural cell precursors by secreting PDGFB (platelet-derived growth factor subunit b), which binds to and activates PDGFRB (PDGF receptor beta) expressed by mural cell precursors (Figure 4). Once mural cell precursors are recruited to the endothelium, they undergo reciprocal signaling by secreting ANGPT1 (angiopoietin1), which then binds to the TEK receptor expressed on the surface of endothelial cells[184–186]. Ultimately, this promotes the formation of cell-cell adhesions that allow mural cells to associate with the vessel wall[187]. In parallel, mural cell precursors undergo endothelial-contact dependent mural cell differentiation. This requires gap junction communication via GJA1 (gap junction protein alpha1)[188] and GJC1 (gap junction protein gamma1),[189] and TGFB1 (transforming growth factor-beta1) signal activation[190]. Currently, it is thought that the latent form of TGFB1 ligand is secreted by endothelial cells and differentiating mural cells. Upon endothelial-mural cell interaction, the latent form is cleaved and activated to induce TGFB1 receptor activation, in both cell types[191–193], through the TGFB1 receptors, ACVRL1 (activin A receptor, type2 like) and TGFBR1 (transforming growth factor, beta receptor 1). TGFB1 signaling has been shown to induce mural cell marker expression such as *Acta2* (smooth muscle alpha actin) via upregulation of *Srf* (serum response factor)[194], to inhibit proliferation of endothelial and mural cells[193], and to induce secretion of elastin from smooth muscle cells as well as fibroblasts[195–197]. It is worth noting that TGFB1 receptor activation appears to depend on gap junction formation (via connexins), but it is unclear how this occurs. Ultimately, the association of differentiated mural cells with endothelial cells allows the vessel wall to be

stabilized and creates quiescent vessel beds where remodeling has ceased[187]. This vessel stabilization is reversible to allow for further remodeling when needed. Secretion of ANGPT2, an antagonist of ANGPT1/TEK signaling that is expressed in regions of pronounced vascular remodeling, is thought to be one mechanism by which this disruption of vessel homeostasis is achieved[187, 198].

As development proceeds, mural cells become crucial for the maintenance of vessel integrity. Contractile pericytes and smooth muscle cells become critical for enabling the vessels to withstand the increasing pressures [199–201]. Fittingly, mural cell recruitment is differentially regulated according to endothelial subtype, as well as hemodynamics, where arterial endothelial cells exposed to higher shear stress recruit more support cells in order to strengthen the arterial wall and distribute the force of the flow[202, 203]. Furthermore, by coupling smooth muscle cells to endothelial cells with gap junctions, smooth muscle cell contractions induced by vasodilators and vasoconstrictors can be coordinated with the endothelium[204–207]. Later, this becomes an important method for regulating blood flow.

One aspect of mural cell biology that is still poorly understood, however, is the developmental origins of these cells. Intriguingly, fate mapping studies suggest that during development, vascular smooth muscle cells are derived from a wide variety of different cell types[208], including mesothelium[209, 210], mesoangioblasts[211], proepicardium[212–214], secondary heart field[215, 216], neural crest[217, 218], and somites[219, 220]. The mechanisms by which the members of this diverse set of cell types retain the competency to differentiate into mural cells are still largely a mystery, as are the implications that these lineage relationships have for our understanding of how mural cell types first arose during evolution. Additionally, the role that this heterogeneity plays in smooth muscle function in the adult is still under investigation. Studies examining TGFB1 signaling *in vitro* have shown that smooth muscle cells from different origins have different functional responses to TGFB1, and a few published studies offer clues that this is true for *in vivo* systems as well[221, 222], but a full understanding of this functional heterogeneity is still lacking.

PATTERNING BLOOD VESSELS DURING ORGANOGENESIS

Vasculogenesis, angiogenesis, remodeling, and mural cell recruitment are all mechanisms by which new vessels form and mature. However, the ultimate pattern that vessels adopt in various tissues can be quite different. Figure 5 shows examples of three different microvasculatures in the cerebral cortex, the heart, and the kidney, showing that even similarly-sized vessels can be arranged quite differently to adapt to tissue structure and function. Recently it has been shown that vessel pattern, like neuron outgrowth, can be guided by specific signals. For example, various transmembrane axonal guidance molecules (eph family receptor tyrosine kinases, roundabouts, slits, semaphorins, netrins, UNC5B, plexins, and neuropilins) can direct vessel outgrowth away from or towards non-vascular cells by juxtacrine or paracrine signaling[79, 80]. A paracrine approach is often utilized via the release of VEGFA from non-vascular cells that are already patterned in order to create a similar vascular pattern by guiding vessel sprouts[89]. Incidentally, *Vegfa* expression can be stimulated by a flow-induced genetic pathway, implicating a role for hemodynamic force in regulating vessel patterning as well[176]. Other molecules, such as the FGF2 that is released

from podocytes in the kidney, can induce intussusceptive microvascular growth[120]. In addition to promoting angiogenesis, signals from non-vascular cells can also specify endothelial heterogeneity. For instance, both VEGFA (which is expressed highly by the glomerular podocytes) and TGFB1 can regulate vessel permeability by inducing the formation of endothelial fenestrae in the glomerular capillaries[223–225]. Taken together, vessel patterning during angiogenesis seems to occur by distinct molecular interactions with neighboring cells and their environment.

SUMMARY

The last century of research has defined many of the events required for normal vessel development. These insights have led to a greater understanding of not only how vessels form and undergo repair, but also how the same molecular pathways influence adult vessel physiology and cardiovascular disease. However, despite the advances in our knowledge, there are still many open questions. As discussed above, the upstream mechanisms controlling initial endothelial cell specification are not well understood. Persistent mysteries surround the origins of pericytes, and how endothelial cells sense and respond to mechanical force still remains unclear. These and many other open questions continue to fuel research in this area with profound implications for clinical translation. New tools and methods to assess cell lineage relationships, to image angiogenesis or to measure how forces are interpreted by cells are leading the charge into the next decade of vessel research, and will undoubtedly add to our understanding of vessel development, likely adding additional unanticipated layers of complexity that drive future investigation.

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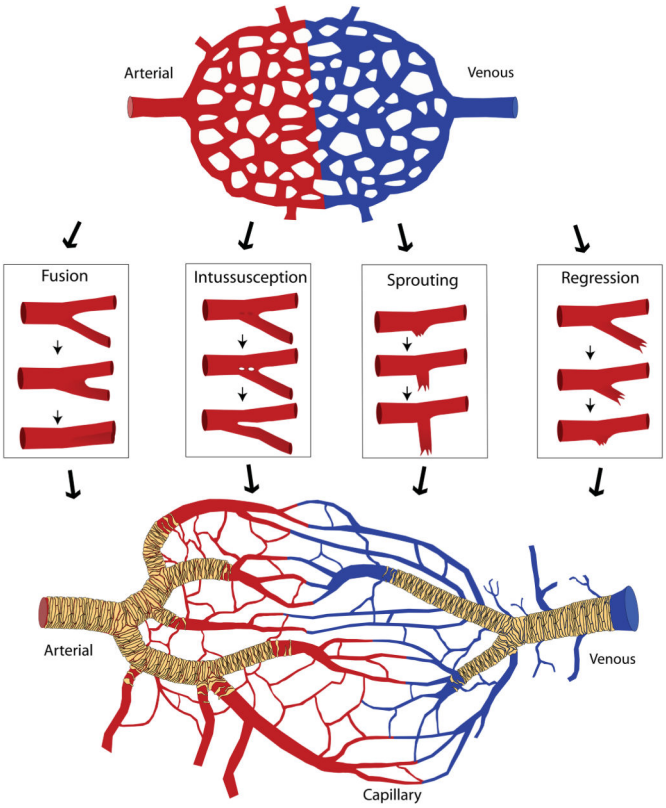

Figure 1.

The vascular plexus, mechanisms of angiogenesis and the remodeled vasculature. The vascular/capillary plexus, composed of an interwoven capillary network with intervening avascular spaces, and in which the presumptive arterial (red) and venous (blue) sides have been specified, is primarily generated by vasculogenesis. Various mechanisms of angiogenesis – fusion, intussusception, sprouting, and regression – contribute to changes in vessel diameter and vessel density. Remodeling of the plexus results in the formation of a hierarchical vasculature tree with large diameter arteries and veins that are connected to progressively smaller diameter/distal capillaries. Simultaneously, vessels become stabilized by the recruitment of mural cells (pericytes and smooth muscle cells) (tan).

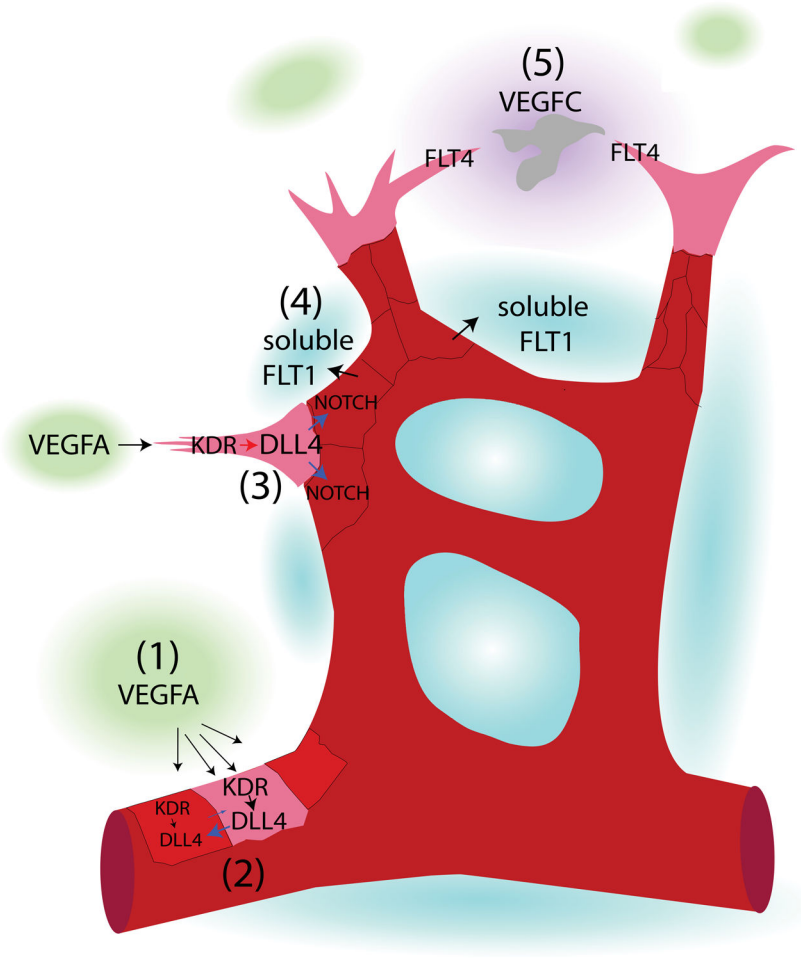

Figure 2.

The stages of sprouting angiogenesis. Sprouting angiogenesis occurs in stages. (1) Signaling to induce new vessel formation: VEGFA is secreted from tissues, resulting in KDR activation in nearby vessels. (2) Induction of a tip cell: an endothelial cell exposed to the highest concentration of VEGFA becomes a tip cell and exhibits the highest expression of DLL4. (3) Formation of stalk cells: in turn, the tip cell induces NOTCH activation in the juxtaposed neighboring cells to induce stalk cell fate. (4) Vessel outgrowth: stalk cells release soluble FLT1, which functions to bind available VEGFA in the vicinity. This increases the steepness of the VEGFA gradient, and stalk cell proliferation and tip cell pulling drives vessel outgrowth perpendicular to the length of the vessel. (5) Vessel fusion to other vessels: as the vessel grows toward VEGFA, release of VEGFC from macrophages (gray cell) activates FLT4 on endothelial tip cells to guide opposing tip cells together, to promote the conversion of tip cells to stalk cells, and to complete vessel anastamosis.

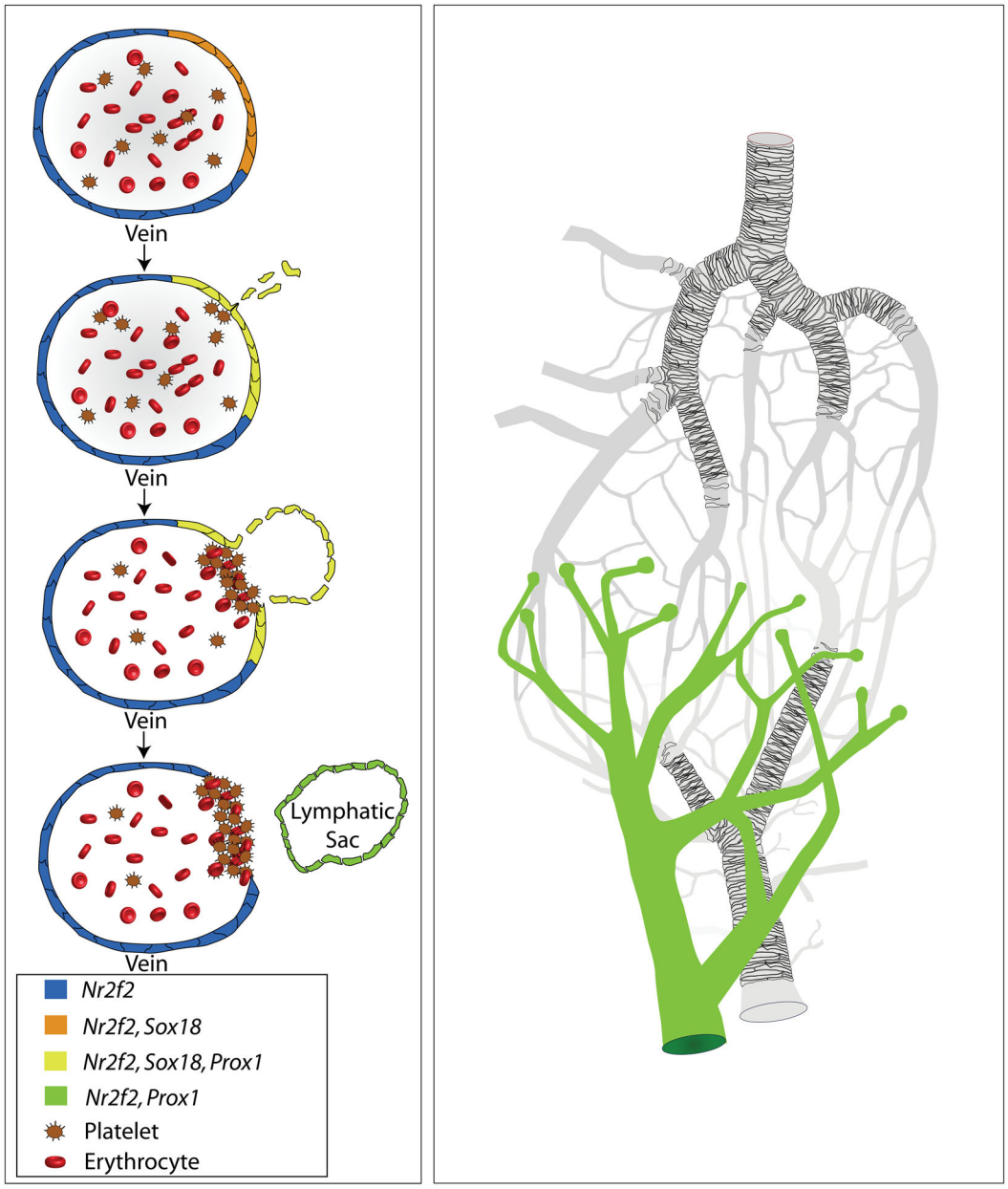

Figure 3.

Lymphangiogenesis. During development, lymphatic vessels form from veins. Veins entirely expressing Nr2f2 (blue and orange cells) exhibit a subpopulation of cells that also express Sox18 (orange cells). The coexpression of Nr2f2 and Sox18 confers expression of Prox1 (yellow cells). Prox1 expressing cells migrate away from the vein and downregulate Sox18 (green cells). These cells then connect to one another to form the lymphatic sac, which ultimately forms the lymphatic vasculature.

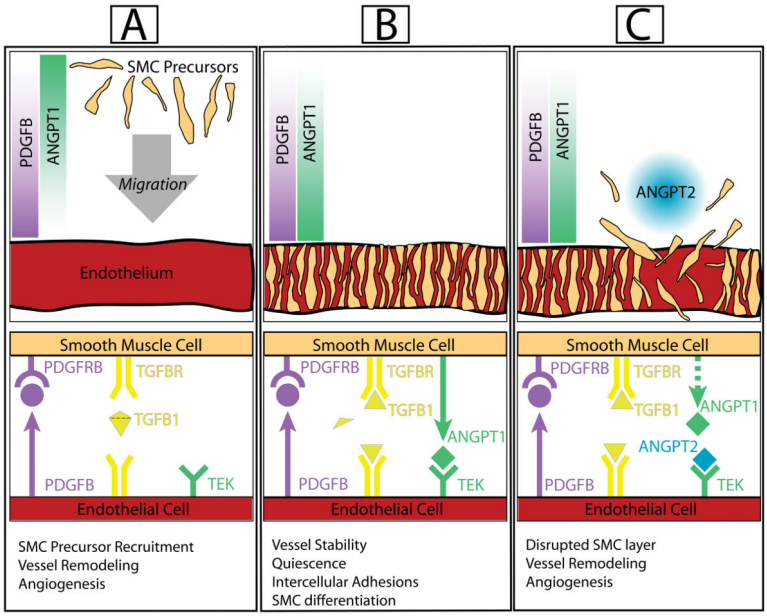

Figure 4.

Vessel maturation. New vessels (created during remodeling and angiogenesis) begin maturation by the recruitment of differentiating mural cells (pericytes and smooth muscle cells). For example, differentiating smooth muscle cells (SMC precursors) migrate toward a gradient of PDGFB (released from endothelial cells) resulting in activation of PDGFB receptors on SMC precursors (a). Upon the close proximity of SMC precursors to endothelial cells, SMC precursor-released ANGPT1 induces TEK activation on endothelial cells, which enhances SMC-endothelial adhesion. Simultaneously, the close proximity of these cells allows for latent TGFB1 (released by both cell types) to become activated. This induces proliferation arrest (quiescence) in SMC precursors and endothelial cells, and induces differentiation of SMCs, yielding vessel stability (b). When a new remodeling or angiogenic mechanism is enacted, competition of ANGPT2 with ANGPT1 for the TEK receptor can disrupt the endothelial-SMC interaction (c).

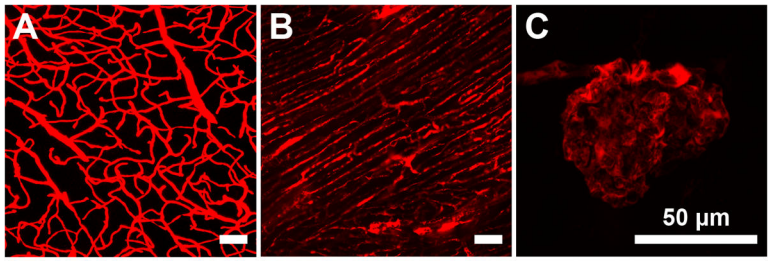

Figure 5.

Variability in vessel morphology and function. Differences in vessel bed morphology, induced by organ-specific patterning, are easily discernible. Vessels of the adult mouse cerebral cortex show a tree-like pattern that maximizes blood flow to all of the cells in the brain (a). Vessels of the adult mouse heart are organized in a manner that aligns with the cardiomyocytes (b). Finally, vessels in the adult mouse kidney show a convoluted vascular structure (c).