What Determines Blood Vessel Structure? Genetic Prespecification vs. Hemodynamics

Vascular network remodeling, angiogenesis, and arteriogenesis play an important role in the pathophysiology of ischemic cardiovascular diseases and cancer. Based on recent studies of vascular network development in the embryo, several novel aspects to angiogenesis have been identified as crucial to generate a functional vascular network. These aspects include specification of arterial and venous identity in vessels and network patterning. In early embryogenesis, vessel identity and positioning are genetically hardwired and involve neural guidance genes expressed in the vascular system. We demonstrated that, during later stages of embryogenesis, blood flow plays a crucial role in regulating vessel identity and network remodeling. The flow-evoked remodeling process is dynamic and involves a high degree of vessel plasticity. The open question in the field is how genetically predetermined processes in vessel identity and patterning balance with the contribution of blood flow in shaping a functional vascular architecture. Although blood flow is essential, it remains unclear to what extent flow is able to act on the developing cardiovascular system. There is significant evidence that mechanical forces created by flowing blood are biologically active within the embryo and that the level of mechanical forces and the type of flow patterns present in the embryo are able to affect gene expression. Here, we highlight the pivotal role for blood flow and physical forces in shaping the cardiovascular system.

Formation of the vascular tree is the result of a complex interplay between genetic factors and epigenetic factors including hemodynamics and tissue oxygenation. The process of cardiovascular development occurs in two steps, vasculogenesis and then vascular remodeling. During vasculogenesis, cells are specified as angioblasts, or endothelial cell precursors, that coalesce to form lumenized tubes. These lumenized tubes form an initial network for blood flow. During remodeling, this network becomes a hierarchical vasculature tree composed of arteries, veins, and capillaries. The initial specification of angioblasts is believed to be controlled by genetics, since blood flow is not present at this stage, but the role of environmental inputs such as mechanical forces and hypoxia in the remodeling phase remains unclear (48). More explicitly, the specification of arteries and veins was initially believed to be a result of flow; however, this theory has recently been put into question since studies in zebrafish and chick embryo indicate that angioblasts can express arterial and venous markers before the formation of tubes and the onset of flow (50). In fact, proper attribution of arterial-venous identity in early embryogenesis is essential for vascular remodeling. Without specification, expansion into a branched vascular network is impeded. There is therefore now reason to believe that genetically predetermined components must play a role in cardiovascular remodeling and not just the initial specification of angioblasts (for review, see Ref. 24). The question from a biophysical point of view, however, is whether the optimization of the vascular tree to be both hemodynamically efficient (to reduce the load on the heart) and functionally efficient (to deliver nutrients and oxygen) can be preprogrammed and, hence, controlled by genetically driven processes.

Environmental Cues in Remodeling

Two independent locations of vasculogenesis occur in the embryo: the blood islands in the extra-embryonic membranes and the major vessels in the embryo proper. The blood islands consist of hematopoietic cells surrounded by endothelial cells and form in the distal part of the yolk sac (FIGURE 1A). These endothelial cells of the blood islands expand from a thin band of endothelial and hematopoietic cells to cover the entire yolk sac, forming a vascular network.
known as the capillary plexus (FIGURE 1B). Within the embryo proper, the major vessels are formed through the differentiation and migration of angioblasts. These two locations eventually interconnect, allowing for a functional loop to be present in which blood can flow. As the heart begins to beat, blood flow is introduced into this network, and the vessels remodel into a more hierarchical vascular tree (FIGURE 1C). Remodeling involves changes in vessel diameter, branching morphology, and recruitment of peripheral cell types (pericytes, vascular smooth muscle) that stabilize the nascent vessels.

The evidence that remodeling of the vasculature, and therefore the final vascular architecture, is genetically predetermined derives from the observation that certain genes are selectively expressed in either arterial or venous vascular compartments. Before the onset of blood flow, expression of proteins from the ephrin or neuropilin family (51) demarcate arterial and venous angioblasts. Mutant mice for ephrinB2 and ephB4 die because of failure to form a proper arterial-venous network (17, 50). Many of these arterial and venous genes, such as Unc5B, are also expressed in endothelial tip cells that are involved in branching morphogenesis decisions in the early vasculature, and exposure of endothelial cell filopodia to ligands of these genes cause retraction of the filopodia (30). It is therefore postulated that arterial and venous genes expressed in the vascular system may control two crucial events: vessel identity and branching morphogenesis. Angioblast specification by ephrins and other arterial and venous genes is needed to allow arterial-venous differentiation, without which remodeling cannot occur. However, during later stages of development, it is readily observed that arterial endothelial cells are reused in growth of veins (chick embryo or zebrafish). This leaves open the question: To what extent is genetic prespecification before start of flow needed, and how does it affect the final architecture?

The problem with viewing these genetics aspects of remodeling in isolation, without the role of epigenetic influences, is that the presence of flow is known to be essential for remodeling to occur. Nearly a hundred years ago, Chapman showed by surgically removing the heart of chick embryos before the commencement of circulation that the peripheral vasculature formed but failed to remodel without blood flow and pressure (6). Manner et al. surgically removed the heart of young chicken embryos and incubated the embryos in high levels of oxygen to remove the effects of hypoxia and found that, although there were fewer deformities in the embryo proper, the remodeling of the vasculature still did not occur (31). In the Ncx1 knockout mouse, the heart is formed but does not beat. In these embryos, the plexus forms in the yolk sac but is never remodeled into vessels, even though other aspects of development, such as limb and organ development, are normal (49). At present, it is widely accepted that there must be blood flow in an embryo for remodeling.
to occur. The essential signal that flow imparts, however, is not known. Blood flow carries nutrients, oxygen, and signaling molecules to the vessels as well as creating physical forces on the endothelial cells of the vessel wall. Therefore, the initiation of blood flow brings many different signals to the embryo.

Reductions in tissue oxygen availability and hypoxia have been linked to changes in network configuration. Hypoxia, an environmental and therefore nongenetic signal, is known to be able to trigger vessel sprouting into avascular regions through induction of VEGF production (33, 39). VEGF is one of the most important proteins during vascular development and can induce migration, proliferation, and the survival of endothelial cells (12). Hypoxia-induced genes are regulated by a transcription factor known as hypoxia inducible factor-1, or HIF-1. Embryos that lack HIF-1α fail to undergo the remodeling process (45). Paradoxically, the oxygen-carrying ability of the blood does not seem to be required for remodeling during the first few days in which the heart is beating. In the yolk sac, the mesenchymal tissue and the blood vessels of the yolk sac have equal access to oxygen since the yolk sac is the link between the maternal and embryonic environment before the formation of the placenta. When zebrafish (38), Xenopus (48a), chick (8a), or mouse embryos (E8.5–E9.5; unpublished results) are cultured in carbon monoxide, effectively ablating oxygen transport by the blood, early stages of remodeling occur normally. Hypoxia increases the number of blood vessels by inducing sprouting. The heart can increase perfusion volume and pressure by increasing stoke volume or heart frequency; however, both parameters are limited in their degree of adaptation, and there needs to be a method to prune vessels, not only create new vessels. The final vascular structure must be an optimization between regulation of peripheral oxygen diffusion and cardiac activity. Such optimization principle has been put forward several decades ago as Murray’s law and seems to hold in many flow transportation systems (37). For a functional vascular system to be established, a mechanism to reduce vascular volume and prune inefficient vessels must therefore also be present for remodeling to occur.

The initiation of blood flow creates mechanical forces on the developing endothelial cells. As a fluid flows through a tube, it exerts a force tangential to the tube, called shear stress, and another that is perpendicular to the tube wall and is caused by the pressure in the vessel, called circumferential stretch (FIGURE 2). Mature vessels can react to both these forces. Shear stress from blood flow has been shown to cause changes in morphology, cytoskeleton organization, ion channel activation, and gene expression within endothelial cells in vitro (7). Observation of endothelial cells in flowless embryos, such as the Titin−/−, show that endothelial cells are more globular and do not flatten, reminiscent of cell shapes associated with migrating angioblasts rather than differentiated endothelial cells (32). Many genes upregulated by abnormal flow patterns in adult circulatory systems are important to cardiovascular development, such as PDGF-β (41), connexin43 (14), and Flk1 (14). Our work in the mouse embryo has shown that the levels of shear stress present during the remodeling process are within the range known to activate these pathways (21). Pressure also activates certain genetic pathways (reviewed in Ref. 28) and leads to changes in smooth muscle cell coverage in adult blood vessels (5). In these mature systems, chronic elevations in shear stress lead to increased vessel diameter, whereas chronic increases in pressure lead to decreased vessel diameter (13, 36). This work has led to a pressure-shear hypothesis, whereby vessels adapt to shear stress as a function of local pressure (40). Thus the two blood flow-induced forces, pressure and shear stress, are associated with long-term rearrangement of the cardiovascular system in adults. The question remains, however, whether embryonic vasculature has the same mechanisms as adult vasculature to alter in response to these forces. It is known that postnatal arterial development is affected by shear stress (9, 52), although the stage of development at which shear sensitivity is initiated is not known. The response to pressure in adults is largely driven by smooth muscle cells.

**FIGURE 2.** Forces created by flowing blood

Fluid flow within a tube creates two types of forces: shear stress, which is a force tangential to the vessel wall, and pressure, which creates a circumferential stretch perpendicular to the vessel wall. The velocity profile (shown by the arrows) within the vessel and the viscosity of the fluid determine the amount of shear stress to which the endothelial cells are exposed. The pressure at any given point in the vasculature is related to pressure at the outlet of the heart and the pressure losses that occur given the flow path to that point.
These cells are not present initially on embryonic blood vessels, and how pressure would affect a bare vessel segment is not known. Therefore, adult vasculature and embryonic vasculature may not react similarly to these forces, but it seems unlikely that hemodynamic forces would have such profound effects on the geometry of adult vasculature and be completely inert within the embryo.

Understanding the role of shear stress and forces in biology is complicated by the fact that different types of flow seem to induce different patterns of gene expression (for review, see Ref. 42). It is therefore not sufficient to analyze the level of shear stress present; one must also look at whether the flow is laminar or turbulent and at the role of oscillatory shear stress. The presence of laminar flow, which is a type of fluid flow where the streamlines of the fluid motion are parallel, has been found to have atheroprotective effects on mature blood vessels (4). Laminar shear stress reduces levels of apoptosis (10) and induces many anti-apoptotic genes, such as Bcl-XL (3). Although laminar flow prevents apoptosis, it also keeps cells from proliferating by inhibiting DNA synthesis (2, 29). Through microarray analysis, it appears that physiological levels of laminar or turbulent shear stress downregulate more genes than it upregulates and that gene expression profiles are significantly different depending on the type of flow endothelial monolayers are exposed to (16). Disturbed flow, a term that includes both turbulent flow and eddies caused by laminar flow separation, is much more biologically active flow. It is rarely seen in the mammalian cardiovascular system and is generally indicative of disease. Vessel bifurcations are prone to flow separation and eddy formation, and it has been observed that atherosclerotic plaques formed preferentially at these locations (9). Microarray analysis has identified genes, such as KLF2, that are differentially expressed when endothelial cells are exposed to flow typical of arteroprotected regions of the vascular tree compared with flow profiles similar to arteroproximate regions (37b). KLF2 is essential for the upregulation of many genes associated with endothelial response to disturbed flow, such as eNOS. In zebrafish embryos, KLF2 is upregulated after the onset of flow but absent in zebrafish mutants that lack blood flow (37b), and lack of this protein results in embryonic lethality in mice (50a). Disturbed flow is not, however, indicative of disease within the embryo and has been observed in normal development (21). Disturbed flow is important during the formation of cardiac valves in zebrafish embryos (18a). Only by localized assessment of gene expression changes in regions where disturbed flow is observed can a possible role for flow patterns in embryonic development be assessed.

The role of hemodynamics does not negate a role for genetics in the remodeling process. The signals that are created by flow are sensed through integrins and the cytoskeleton (42). How a cell attaches to surrounding cells and the composition of the extracellular matrix, something that is genetically determined, therefore, mediates how cells react to flow. It has recently been shown that cotransfection of only three proteins (VE-cadherin, PECAM-1, and VEGFR2) can endow nonendothelial cells with the ability to align with the direction of flow in vitro (48a). The flow that endothelial cells are exposed to is also genetically determined by the development of the heart. Since oscillatory flow is more biologically active, the formation of heart valves, which stops retrograde or backward flow, induces an important change in the hemodynamics of the early embryo (21). Thus, even when an external nongenetic signal such as flow is considered, it is important to keep in mind that it is the genetics of cell fate determination that set up the initial conditions to which the cells are reacting and that determine the type and location of these forces.

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**Plasticity in the Embryonic Vasculature**

Recent evidence has shown that proper establishment of vessel identity, whether arterial, venous, or capillary, is intrinsically linked to the remodeling process. Vessel identity can be defined by a set of markers, such as ephrinB2 (50) and Dil4 (11, 15, 46) for arteries and EphB4 (50) and neuropilin-2 (18) for veins. These genes are believed to be important in guidance, boundary formation between arteries and veins, and other functions. Abolition of these genes interferes with vascular remodeling in the yolk sac vessels (1, 11, 23, 50). The importance of these genes is not clear, however, since changes in cardiac activity also interfere with yolk sac vascular remodeling, even if arterial and venous markers are properly expressed. This indicates that arterial/venous identity alone is not sufficient to induce proper branching morphogenesis. Instead, branching requires a physical cue generated by hemodynamics and cardiac output regulation. Recent evidence has revealed a high level of plasticity within the networks, such that flow dynamics are able to change identity (27, 35). The idea of flow-driven plasticity is essential, because plasticity could in fact be the vital link between the genetic theories and epigenetic theories of vascular formation.

In avian models, endothelial plasticity was demonstrated in transplantation studies with isolated arterial and venous vessels of quail embryos grafted into a chick embryo host (FIGURE 3A). The cell fate of the
quail cells could be assessed using the monoclonal antibody QH1, which selectively recognizes quail endothelial cells. In early stages of development, quail endothelial cells of either arterial or venous origin could colonize both chick arteries and veins (35). The expression of arterial- and venous-specific genes changed according to the vessel identity where the grafted endothelial cells integrated. This plasticity was, however, progressively lost at later stages of development. After embryonic day 11, grafts of arteries yielded endothelial cells that colonized only arteries, whereas venous grafts colonized mainly host veins. Signals in the vessel wall may at least partially regulate endothelial identity. When arterial endothelial cells from later stage embryos were dissociated from the vessel wall, these cells colonized both host arteries and veins. The responsible paracrine signaling pathways remain to be determined but could involve nerves since functional innervation of the arterial wall starts around that stage (43, 44).

Using in vivo time-lapse imaging of developing chick embryos, we recently demonstrated that vessel plasticity is controlled by hemodynamics (27). We observed that prior to the onset of flow, endothelial cells expressing arterial or venous markers are localized in a posterior-arterial and anterior-venous pole. After the onset of cardiac activity and the start of perfusion, the vitelline artery forms in the posterior arterial pole by flow-driven fusion of preexisting capillaries branching from the aorta at the level of somite 21. During the subsequent stages, the arterial network expands and some small capillary side branches are selectively disconnected from the arterial network. These segments do not regress or apoptose; instead, the disconnected vessels reconnect to the venous plexus. The disconnected segments lose their arterial identity and start to express venous markers. Detailed high-magnification intra-vital imaging shows that the disconnected segments, which are filled with blood and pressurized due to connections with more distal parts of the arterial network, grow and extend over the arteries before reconnecting to the veins of the primary network (26, 27). The disconnected segment lacks tip cells or filipodia and is instead driven by its luminal pressure and guided by the strain fields generated by the surrounding large caliber vessels (37a). The relatively high pressure in the arteries repels the expanding disconnected segments, which avoid the arteries and can only reconnect to lower pressure veins. Such avoidance of the arterial segments is also observed in the zebrafish parachordal vessel, which starts at the venous level, crosses the intersegmental artery without fusing to it, and reconnects to the cardinal vein in the trunk (20).

Arterial and venous identity can also be altered by changing the flow environment of the endothelial cells (FIGURE 3B). Rerouting flow by artificially obstructing the vitelline artery results in perfusion of the arterial tree with blood of venous origin, which transforms the arteries into veins, both morphologically and with respect to gene expression. Perfusing veins with arterial blood can likewise transform them into arteries (27). It is undetermined whether flow-regulated vessel identity is controlled by the physical properties of the flow signal itself (related to the pressure profile) or by chemical differences in arterial and venous flow such as oxygen tension. Remarkable differences in pressure profiles between arteries and veins exist, but any molecular signaling cascade able to recognize such differences is unknown, and the levels present in the embryo are significantly lower, around 1–2 mmHg, compared with the adult (18b). Therefore, any genetic cascade directly activated by arterial or venous profiles in the adult may not activate similarly in the embryonic situation. Even if pressure profiles cannot be directly “sensed,” plastic deformations of endothelial cells and the cytoskeleton, the concept of tensegrity (19), may provide a rational framework to explain changes evoked by arterial or venous flow, even in the relatively low-pressure environment of the embryo.

In vivo studies of the developing trunk vasculature in zebrafish have shown that endothelial cell plasticity

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<th>FIGURE 3. Experimental manipulation of arterial-venous fate</th>
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<td>Quail arterial cells are grafted into a chick host between day 2 and day 14 of gestation. Before day 7 of gestation, these cells, which can be identified by antibody staining for quail-specific proteins, can be found in the aorta or the veins of the chick host. Similarly, if venous cells are grafted, they migrate to either the artery or vein of the host before day 7. These cells change expression of arterial and venous markers to match the new environment in which they are found (A). Arterial and venous markers can also be changed by rerouting flow (B). In chick embryos, if the major artery (red) is ligated, flow reroutes such that venous flow passes through these vessels. In response, these endothelial cells begin to express venous marker (black) and downregulate arterial markers (red).</td>
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is not only present in the yolk sac but also within the vascular network of the embryo proper. In early stage embryos, primary vessel sprouts originating from the aorta grow dorsally between the somites to form the intersomitic vessel, which subsequently branches laterally into the caudal and cranial regions to form the dorsal lateral anastomosing vessel (DLAV) (25). At this stage, the intersomitic vessel is connected to the aorta and is therefore of arterial origin. During subsequent stages, secondary sprouts form from the cardinal vein and grow parallel and in close proximity to the intersomitic artery. Occasionally, these secondary sprouts fuse with the preexisting intersomitic artery, which progressively loses its connection with the aorta as the intersegmental vessel turns into a vein. As a consequence, the flow direction in the intersomitic arteries is reversed (20). The exact nature of this process is not understood, but it is possible that the fusion of the secondary sprouts with the intersomitic artery branching from the aorta is stochastic, needed to balance local perfusion and probably related to local pressure profiles (20). Although these vessels are initially arterial, plasticity during the remodeling process is essential for the formation of a functional vascular loop.

Taken together, arterial-venous differentiation and branching morphogenesis in the yolk sac plexus is flow driven and requires a high degree of endothelial cell plasticity. In perfused networks, the expression of arterial- and venous-specific genes is the consequence of local hemodynamic cues that may regulate vessel wall remodeling.

Remodeling as a Force Equilibrium

One remaining factor that must be considered with regard to vascular remodeling is the principle of force equilibrium and changing tissue material properties. Flowing blood creates a force on surrounding mesenchyme. The mesenchyme itself exerts a force through tissue growth onto the vessels. As the physics of the situation change, the conformation must be energetically feasible or else the vessel location would require an active and energy-consuming effort by the tissues for the entire lifetime of the organism.

Mesenchymal tissue growth within the yolk sac is rarely considered when remodeling is studied. The
yolk sac is, however, expanding throughout the remodeling process, and the growth of these tissues pushes against the immature vessels. In chick embryos, if outgrowth of the yolk sac is prevented by physical insertion of a metallic ring around the yolk sac, vascular remodeling does not occur normally (unpublished results). In vivo time-lapse imaging of mice embryos that express GFP in their endothelial cells reveals that the expansion of mesenchymal tissues seems to pinch off or disconnect certain vessels within the yolk sac (movie 1, online, FIGURE 4). Thus growth of the mesenchymal tissue may be able to affect branching geometry within the yolk sac (FIGURE 4B). Similar longitudinal forces affect remodeling in adults. If an artery is exposed to longitudinal strain, it remodels to adapt to this strain. If strain on an artery is released, vessels take on a more tortuous trajectory (20a). A classic theory is that the vessels that carry the largest flow enlarge and vessels with the smallest flow regress (48). If true, the mechanism for vessel-diameter adaptation during remodeling could arise from a balance between the outward push created by blood flow and the inward force created by the growth of mesenchymal tissue.

Even if vascular remodeling is seen to have a component of force-driven deformation, this does not negate a role for genetics in the remodeling process. This is because the deformation of a tissue by a force is defined by its material properties, and those are all genetically encoded. The more stiff a tissue is, the less it can be deformed when exposed to flowing blood, and the stiffness of a tissue is given by its attachment to the extracellular matrix, the expression of adhesion proteins, and the nature of the cytoskeleton.

Conclusions

The remodeling and maturation of the embryonic cardiovascular system is a complicated process requiring both genetic and environmental inputs. The initial specification of vasculature is genetic predetermination to enable guidance and formation of proper connection between vessels. Remodeling, however, is both genetic and environmentally controlled. Ablation of certain genes results in a failure of remodeling, but lack of physical cues, whether oxygen tension or hemodynamics, also causes failures in vascular remodeling. It is very difficult at present to separate genetics factors from the epigenetic factors, since most genes expressed in the vasculature are also expressed in the heart. If the abnormal cardiac expression causes even small changes in the flow conditions, this could result in significant vascular abnormalities. The flow in embryos where arterial or venous genes have been ablated has not been analyzed, making the interpretation of gene ablation studies difficult. It will therefore be essential to develop techniques to expose endothelial cells in which important genetic signals are ablated to normal flow or to expose wild-type cells to abnormal flow. This may be possible through studies using heart-specific or vascular-specific conditional ablation of genes or through the use of embryonic stem cells and other in vitro models. In this way, the relative importance of genetics vs. epigenetics can be investigated.

References


