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Veins and Arteries Build Hierarchical Branching Patterns Differently: Bottom-Up versus Top-Down

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Abstract

A tree-like hierarchical branching structure is present in many biological systems, such as the kidney, lung, mammary gland, and blood vessels. Most of these organs form through branching morphogenesis, where outward growth results in smaller and smaller branches. However, the blood vasculature is unique in that it exists as two trees (arterial and venous) connected at their tips. Obtaining this organization might therefore require unique developmental mechanisms. As reviewed here, recent data indicate that arterial trees often form in reverse order. Accordingly, initial arterial endothelial cell differentiation occurs outside of arterial vessels. These pre-artery cells then build trees by following a migratory path from smaller into larger arteries, a process guided by the forces imparted by blood flow. Thus, in comparison to other branched organs, arteries can obtain their structure through inward growth and coalescence. Here, new information on the underlying mechanisms is discussed, and how defects can lead to pathologies, such as hypoplastic arteries and arteriovenous malformations.

Keywords

angiogenesis; arteries; blood flow; branching morphogenesis; coronary vasculature; cxcr4; vein; notch

1. Introduction

How hierarchical patterned trees form in diverse tubular organs, such as the kidney, lung, and vasculature, has been of scientific interest for several centuries. As early as the 16th century, Leonardo da Vinci provided a comparative study that envisioned how arteries and veins might grow. In this view, the growth of tree-like structures starts with a main stem, from which a sprout emerges that would subsequently grow out into a branch that is smaller than the parental stem, followed by multiple iterations. This mode of branching morphogenesis applies to the tracheal system of *Drosophila*, the mammalian lung, and kidney.^[1–5] It also operates in many settings within newly forming blood vessels. However, the final topology of the vasculature is fundamentally different compared to other branched structures. It is not a blind-ending tree, but rather consists of arterial and venous trees that

are inter-connected via a capillary bed (Figure 1). Accordingly, establishment of the vasculature follows unique developmental processes, guided by distinct mechanisms important for obtaining proper hierarchical structure and optimal organ function.

Although all hierarchical in nature, arteries in different organs and different organisms exhibit slightly different structures. Frequently, in smaller networks, such as some vascular beds of the developing Zebrafish, a pre-existing vessel will undergo sprouting angiogenesis giving rise to a sprout that connects to another vessel and directly forms a new artery or vein.^[6,7] In larger vascular beds, it is common to see multiple sprouting vessels that dramatically proliferate and interconnect, forming a web-like network of small-diameter vessels called an immature vascular plexus.^[8,9] The plexus will subsequently remodel into recognizable arteries, capillaries, and veins. In both cases, initial sprouting resembles the development of other tubular organs, where the action of a growth factor stimulates an existing branch to bud out. However, recent findings have revealed that during the remodeling of an immature plexus, the growth of arterial trees is reversed, and new cells are instead recruited inward into newly forming arterial vessels.

Another difference between the vasculature and other branched organs is that the former receives hemodynamic cues from blood that flows through its lumen. Such cues play important roles during sprouting angiogenesis,^[10–13] remodeling,^[14,15] artery-vein differentiation,^[16,17] and blood vessel maintenance.^[18] They are also crucial to control blood vessel diameters^[15,19,20] and the pruning of unnecessary side branches within vascular networks.^[21–24] New results now suggest that cell migration in response to hemodynamic cues also contributes to proper artery formation through influencing the number of ECs in enlarging arterial segments.^[19,25–29] Here, we review these emerging themes on the relationship between angiogenesis, arterial specification, and blood flow during the formation of hierarchically patterned arterial trees.

2. VEGF and Notch Signaling Control Arteriovenous Differentiation During Vasculogenesis in Early Embryos

Vascular beds in most organs do not usually form *de novo*, but this is how the very first blood vessels in the early embryo must arise. *De novo* formation of blood vessels is referred to as vasculogenesis, which entails the differentiation of individual endothelial progenitor cells called angioblasts prior to their coalesce and lumen formation. This process has been well-studied and extensively reviewed.^[30–35] The morphogenesis mechanisms are dissimilar between vasculogenesis and angiogenesis; however, the two processes share several molecular pathways and key features. Specifically, Vascular Endothelial Growth Factor (VEGF) and Notch signaling play prominent roles. During vasculogenesis, angioblasts differentiate into either arterial or venous ECs prior to coalescence into their respective vessels, which can be visualized by Notch reporter activity as a marker of arterial identity^[36] and by following their fate.^[31,34]

VEGF and NOTCH are both required for angioblast differentiation into arterial cells. The signals that trigger venous differentiation at this stage have not been reported. However, the transcription factor COUP-TFII is necessary to block Notch expression in venous ECs.^[37]

As no blood flow is present at this stage, hemodynamic forces cannot contribute to de novo artery formation. In addition, because arteries and veins coalesce in situ from individually migrating angioblasts, the morphogenesis of these early forming blood vessels does not resemble those of other tubular organs. The result is the first vascular loop, consisting of the heart that pumps blood through a simple connection between arterial (dorsal aorta) and venous blood vessels (cardinal vein). Subsequent vessels can then develop through budding from this pre-formed vascular network, which is referred to as sprouting angiogenesis.

3. Angiogenesis in Early Embryos: Arteries Generate Arteries and Veins Generate Veins

Because angiogenesis is defined as sprouting from pre-formed vessels, one relevant question is: how do arteries and veins arise from vessels with already specified fates? This is particularly important given the fact that arteries and veins express mutually exclusive transcriptional pathways.^[38,39] One possibility is that arteries and veins sprout while retaining their identity, and eventually produce the arteries and veins of the new vascular bed. Another possibility is that ECs de-differentiate in terms of arteriovenous identity during angiogenesis and are re-specified at a later time point. Answering this question has been inherently difficult due to the limited number of markers used to assess arterial-venous identity and their ability to be localized in vivo. However, live imaging,^[40–43] lineage tracing,^[26,43–49] and single cell transcriptional analyses^[50–52] indicate that the processes of sprouting, cell fate reacquisition, and cell migration are heavily inter-twined, and are revealing general and organ-specific mechanisms.

One of the first sprouting angiogenesis events in the embryo is at the intersegmental blood vessels (ISVs), and imaging from Zebrafish has established that sprouts emerge from both the artery and vein (Figure 2A). Sprouts first develop from the dorsal aorta in response to VEGF and anastomose in the dorsal region of the embryo.^[13,53–56] This leads to a configuration in which arterial blood vessels are interconnected without a venous drainage (Figure 2A, 1–1.5dpf time points). Subsequently, BMP signaling stimulates sprouting from the cardinal vein^[57] (secondary sprouts; Figure 2A, 1.5dpf time point), and some of these connect to the artery-derived ISVs, providing a venous drainage (Figure 2A, 2dpf time point). Therefore, arterio-venous sprouting in this setting occurs in response to different cues.^[6] Although Notch synergizes with VEGF to stimulate arterial differentiation,^[38,39,58] it inhibits angiogenic sprouting.^[59–69] These findings led to the view that during artery formation, VEGF, and Notch signaling act in a common pathway, while in angiogenesis they play opposing roles.

4. EC Plasticity Allows for Arterial to Venous Cell Fate Conversion in Early Embryos

With respect to cell identity dynamics, analysis of two arterial markers (Flt1^{enh} and Notch activation) suggests that dorsal aorta ECs that ultimately form the arterial ISVs maintain their arterial identity during sprouting.^[36,70] However, during the remodeling that leads to the formation of venous ISVs, arterial-derived ECs can adopt a venous fate (Figure 2A,

remodeling), which involves loss of Notch signaling.^[36,62,71] Vein-derived ECs can also replace artery cells to help establish the alternating pattern of interconnected arteries and veins, a process that relies on the migration of ECs against the direction of blood flow in newly established venous connections.^[49,72] These findings are consistent with previous work showing that blood flow plays an important role in determining the patterning of arterial and venous ISVs^[55] and with observations in chick embryos, where arterial segments can be incorporated into the venous part of the circulation.^[17] Thus, at early angiogenesis stages, new blood vessel sprouts emerge from both arterial and venous precursor vessels and create new intersegmental arteries and veins. However, subsequently, there is some interconversion between fates that allows the establishment of proper connections between the two sides of the circulation. These results concerning the expansion of the early vasculature via sprouting from precursor vessels for both arteries and veins are in line with the general concepts of branching morphogenesis.

5. Angiogenesis During Later Embryonic Stages: Vein Sprouts Can Generate Arteries

In contrast to early embryonic stages, work in multiple systems has suggested that sprouting frequently arises from veins and not from arteries during later organogenesis stages and in tissue regeneration.^[40,41,43,45,46,48,73,74] These studies also suggested that there is a general migration of ECs from veins toward arteries. This has been observed in the zebrafish eye,^[73,75] the zebrafish brain (Figure 2B)^[40,41] and spinal cord,^[49] the regenerating fin^[43,45] (Figure 2C), the mouse retina,^[43,47] and during mouse coronary artery formation^[48,51] (Figure 2D). Thus, the ability of venous ECs to be reprogrammed into arteries appears to be a common theme. Angiogenic sprouting might occur mostly from veins to avoid leakage of blood, which could be more likely if cells emerge from an arterial blood vessel with high pressure and hemodynamic load. Regardless, these observations suggest that later appearing hierarchically patterned arteries and veins might rely on distinct mechanisms to those at play during early embryogenesis.

6. The Role of the Chemokine Receptor *Cxcr4* During Artery Formation

Examples of vein-derived arteries have been studied in detail in zebrafish. In the brain, veins running along the length of the head send off sprouts that connect to arteries and subsequently differentiate into arterial vessels, which, in this case, occurs without the formation of a vascular plexus intermediate (Figure 2B). In contrast to venous ISV sprouting, vein-derived angiogenesis in the brain relies on VEGF. Notch signaling is also required and both molecules activate arterial differentiation.^[40,41] Another difference is the requirement for the chemokine receptor *cxcr4a*, which is dispensable for ISV growth.^[76] In newly emerging brain sprouts, however, *cxcr4a* is specifically needed for connecting these sprouts to the pre-existing arterial pole of the vasculature. In *cxcr4a* mutants, vein sprouts only form connections to each other and consequently lack blood flow.^[40,41] This is consistent with the chemotactic CXCR4 ligand, CXCL12, being expressed adjacent to the artery (Figure 2B). Furthermore, *cxcr4a* expression is negatively regulated by blood flow,

suggesting a mechanism that ensures continuous *cxcr4a* expression in newly forming blood vessel sprouts until a functional connection to an artery has been made.

Studies of the regenerating fin vasculature showed similar responses. Upon fin resection, veins, but not arteries, are activated to sprout out. Vein-derived sprouts display a characteristic migratory behavior where tip cells at the leading edge of the sprouting front turn around and connect to the proximally located artery.^[43,45] Again, *cxcr4a* signaling is important because the cells respond to Cxcl12a expressed within the territory around the artery (Figure 2C). Thus, in the developing brain and during tissue regeneration, blood vessel formation in zebrafish occurs from veins to arteries, although the precise dynamics of arterio-venous fate transformations in these contexts has yet to be addressed.

Live imaging is extremely challenging in embryonic and neonatal mice, but by using genetic lineage tracing to track cell fates venous to arterial developmental progressions have been detected. This method allows one to label individual cell populations at a given time point and examine the fate of the cells' progeny.^[77] Red-Horse et al.^[48] used this technology to track ECs of the sinus venosus, the venous inlet to the embryonic heart. Venous sprouts migrate onto the hearts and proliferate to form a plexus that is subsequently remodeled into the coronary arteries, capillaries, and veins of the heart (Figure 2D). Although early coronary angiogenesis occurs in the absence of blood flow, the newly formed blood vessels must eventually connect to the aorta to become perfused. The mechanisms for this connection share similarities with those in Zebrafish. Specifically, mice deficient for *CXCR4* and its ligand, *CXCL12*, have a coronary plexus that fails to properly connect to the main aorta, resulting in an absence of perfusion.^[78] Cxcl12 is expressed in the cells surrounding the aorta, consistent with the chemotactic function of this protein. Other systems have a similar phenotype. Within the intestine, venous networks in Cxcl12 mutants appear unaffected, while connections to the larger arteries are disturbed.^[79,80] Consistent with its specificity for providing arterial connections from vein-derived vessels, *CXCR4* signaling is dispensable in settings in which angiogenesis generates only veins, such as the caudal vein plexus^[73] Thus, the *CXCR4* signaling axis appears to be a specific genetic module that is in place where arterial ECs need to connect to a pre-existing arterial circulation (Figure 2B–D).

7. Single Cell Sequencing and Genetic Lineage Tracing Identify Venous to Arterial Cell Fate Conversions

Using single cell RNA sequencing, Su et al. were able to interrogate the venous to arterial fate conversion during coronary plexus remodeling. These remodeling events were thought to occur in response to the ensuing blood flow after the plexus has connected to the arterial stem (Figure 3A).^[9] Surprisingly, Su et al.^[51] now showed that the switch from venous to arterial fates is initially gradual such that the immature plexus is composed of cells existing along a continuum of arterio-venous identity. Arterial differentiation occurred within the immature plexus, which produced pre-artery cells that displayed a transcriptional profile similar to fully mature coronary arteries. Even though morphologically indistinguishable from their neighboring cells, lineage tracing showed that pre-artery cells subsequently build coronary arteries. The early immature coronary plexus is not connected to the circulation,

indicating that genetic specification of coronary arterial ECs occurs prior to the onset of blood flow. This shows that similar to early vasculogenesis stages, and, in contrast to the current model, important arterial differentiation events occur outside of the arterial vessel independent of hemodynamic cues from blood flow (Figure 3B).

Another well-studied model of blood vessel plexus remodeling is the mouse retina.^[81,82] Following birth, an immature plexus develops from the optic nerve toward the periphery of the mouse retina. In current models of retinal artery formation, new arterial cells are thought to be specified close to the pre-existing artery, thereby extending it distally^[60] (Figure 3C). Lineage tracing and mutant analyses have revealed arterial specification away from the established artery and a general migration of cells in a vein-to-artery direction (Figure 3D). Labeling endothelial tip cells at the growing front revealed that they are later preferentially found in arteries, while they infrequently populate veins.^[43,47] This indicates that tip cell positioning pre-specifies cells to become arterial, and suggests that part of the specification is to potentiate their directional migration back toward developing arteries. These pre-specified cells are experiencing high Notch signaling and express *Cxcr4*, hallmarks of mature arterial cells, and require Notch to polarize toward and migrate into arteries.^[83]

Recent results also suggest that these tip cells arise by migration from the venous side of the plexus. This is exemplified by mutations inhibiting general EC migration. Deletion of the small GTPase, *cdc42*, which is important for cell migration, causes ECs to accumulate in and around veins, while arteries mostly contain wild-type cells.^[46] A similar distribution can be found in endoglin mutant ECs that display defective cell migratory behaviors.^[26] Thus, accumulating evidence in the mouse retina indicates that ECs that produce arteries originate in venous territories before they transit through the tip cell position where they become pre-specified to join arteries. Thus, in this setting, arterial and venous blood vessel trees appear to grow in opposing directions, where arteries grow in reverse, and genetically pre-specified cells are added from side-branches to build the larger arterial stems. This mode of blood vessel expansion is furthermore distinct from ISV sprouting in early embryos, where sprouts emerge from both, the arterial and venous poles.

8. How Important is the Regulation of EC Proliferation for Artery Formation?

What are the mechanisms that drive or inhibit pre-specification within the plexus and tip cell position? New studies show that an attenuation of EC proliferation is a prerequisite of arterial specification.^[16,51] One pathway controlling EC proliferation is Notch.^[84–88] Inheriting higher Notch signaling activates arterial pre-specification in the mouse retina^[47] and in the zebrafish trunk.^[36] In addition, tip cells exhibit low proliferation rates.^[89] During coronary artery development, the gradual transition from vein to pre-artery includes a progressive increase in Notch pathway genes, and expression of activated Notch (NICD) in coronary plexus cells pushes them into an arterial fate.^[51] Inhibitors of pre-artery specification include the transcription factor COUP-TF2, which induces the expression of cell cycle genes and thereby activates cell cycling.^[51]

In the retina, it has been shown that shear stress-induced Notch signaling activates Cx37 expression.^[16] Cx37 inhibits EC cycling through expression of the cell cycle inhibitor p27, which is required for arterial differentiation. It will be important to investigate what characteristics of cell cycling limit arterial differentiation. One possibility is that cell cycle specific chromatin structures facilitate or repress the expression of arterial cell fate determinants. By analogy to embryonic stem cell differentiation, another possibility is that cell cycle specific proteins directly regulate gene expression.^[90,91]

9. How Do the Observations That Tip Cells Can Generate Arteries Fit with the Current Tip-Stalk Cell Concept?

The observation that tip cells are pre-specified arterial cells adds an additional layer of complexity to the current tip-stalk model of sprouting angiogenesis. The tip-stalk cell concept was originally inspired by lateral inhibition observed in *Drosophila* tracheal branching morphogenesis.^[2,3,92] In this setting, the Fibroblast Growth Factor homologue *branchless* (*bnl*) functions as a positive growth cue that binds its receptor *breathless* (*btl*) on tip cells of a growing tracheal sprout. *Delta* expression on the tip cell then activates Notch signaling in trailing stalk cells inducing lateral inhibition that inhibits the stalk from becoming a tip cell.^[93] A similar relationship has been proposed to exist between the EC growth cue, VEGF, and Notch signaling in the vasculature.^[8,65,67,94–98] The currently accepted model is that VEGF induces migration of leading endothelial tip cells and expression of the Notch ligand *dll4*.^[63,99,100] *Dll4* then activates Notch signaling in trailing stalk cells.^[61,62,68] This inhibits the stalk from becoming tip cells, partly via downregulation of VEGF receptor expression^[69,101] (Figure 3E). This system functions to balance the angiogenic growth induced by VEGF.

How does this model fit with the latest observations that Notch signaling is high in select tips cells that are pre-specified for arterial differentiation?^[47,73] The lateral inhibition model predicts that activation of the Notch pathway should lead to the downregulation of its ligand *dll4*.^[102] However, during artery specification, the Notch transcriptional effector Rbpj activates *dll4* expression in ECs.^[103,104] Also, during the artery pre-specification phase in the retina, overall levels of Notch signaling appear more critical than activation within individual cells.^[47] These studies also showed that, in mosaic analysis experiments, individual cells lacking *dll4* could still obtain the tip cell position. The lateral inhibition model would predict that *dll4* deficient cells could not be tips because they would be outcompeted by wild-type cells due to their inability to induce Notch signaling in adjacent stalk cells. *Dll4* deficient cells were also observed in the tip position in Zebrafish ISVs, showing that this phenomenon is not retina specific.^[73] Taken together, these data suggest that the role of Notch during angiogenesis may be more complex than previously appreciated.

In light of these data, it may be that, in a subset of tip cells, there is activation of Notch signaling that links the termination of angiogenesis with artery formation.^[47,73] In this modified concept, VEGF would activate Notch signaling in new blood vessel sprouts. Depending on the degree of Notch signaling pathway activation, ECs would then either

continue to sprout (low Notch pathway activity), or start an artery differentiation program (high Notch pathway activity). Thus, once some tip cells activate Notch signaling, they stop being angiogenic and instead form arteries (Figure 3F). This interpretation would also reconcile the role of Notch signaling during tip-stalk cell selection with its function in early ISVs and vasculogenesis stages, where Notch signaling induces artery formation, while at the same time inhibiting angiogenesis. It would also be consistent with previous findings reporting shuffling between tip and stalk cells.^[99] Future experiments will continue to refine our understanding of Notch during angiogenesis and arterial differentiation.

10. ECs Migrate Against the Blood Flow Direction to Support Artery Formation

The initial steps of artery cell differentiation can occur in the absence of blood flow, as observed in the early embryo, in the heart, and in retinal tips cells. However, once the heart starts beating or after a plexus connects to an artery, the new vascular bed begins receiving blood. Multiple studies have shown that flow is required for full remodeling of the blood vessel plexus into properly sized arteries, capillaries, and veins.^[14,15,17,105–107] This is because blood flow imparts mechanical forces (largely shear stress, but also circumferential strain) on vascular cells, and the cells directly sense and respond to these stimuli.^[108,109] It has also been proposed that blood flow may distribute growth factors in patterns that regulate remodeling.^[11]

Blood flow impacts two essential morphogenic events within the remodeling plexus to increase vessel diameter during artery formation. The first is vessel fusion, where two small size branches merge to form a larger vessel. This has been mainly observed in the developing yolk sac vasculature.^[15] The second is the vein-to-artery EC migration discussed above, which is against the direction of blood flow. This migration occurs because ECs sense the precise direction of blood flow.^[110] Vessel fusion and EC migration against blood flow has been directly observed in vivo during time-lapse imaging of yolk sac remodeling in mice^[15] and quail embryos,^[111] and in the regenerating Zebrafish fin.^[43,45] In systems not amenable to time-lapse imaging, such as the developing retina and heart, it has been observed that ECs are polarized against the direction of flow throughout the vascular bed.^[22,25] Cells polarize in the direction in which they migrate, hence suggesting that a general movement from veins through capillaries and into arteries also occurs in these systems.

Recent studies have begun to elucidate the mechanisms that allow ECs to polarize and migrate against the direction of flow during artery development. Deletion of Apj, a receptor for the peptide hormones Apelin and Elabela, inhibits EC polarization against flow in the zebrafish vasculature and in cultured human ECs.^[112] Whether this relies on ligand binding or direct mechanical stimulation of Apj has not yet been reported.^[113] Dach1, a transcription factor that regulates organ size in *Drosophila*^[114] potentiates EC migration against the direction of flow in cultured human ECs^[25] (Figure 4A). It does so, at least in part, by inducing the expression of the chemokine CXCL12 in ECs. Suggesting a similar role in vivo, Dach1 deletion results in decreased CXCL12 expression, decreased EC polarization

against flow, and small coronary arteries. These findings suggest that EC migration against flow supports artery growth during development.

11. TGF-Beta Signaling Affects Artery Morphogenesis Through Effects on Cell Migration: Implications for Hereditary Hemorrhagic Telangiectasia (HHT)

Similar to *Dach1*, SMAD signaling is also involved in flow-guided migration (Figure 4A). Knock down of SMAD4 abrogates cell alignment and migration against flow in culture.^[27] Deletion of Endoglin, a co-receptor for TGF-beta receptors, which signal through SMADs, also inhibits EC migration against the direction of flow, while Endoglin overexpressing cells are more migratory in this direction^[26] (Figure 4B). Indicating a similar function in vivo, Endoglin deficient cells in the developing retina accumulate in veins while overexpressing cells accumulate in arteries.^[26] Alk1 is one of the TGF-beta receptors that interacts with Endoglin, and its deletion also inhibits migration against flow in zebrafish arteries.^[19] This leads to a maladaptation of arterial diameters, which coincides with aberrant EC numbers within these arteries. In *alk1* mutant zebrafish, arteries close to the heart are too small, containing too few ECs, while distal arteries retain too many ECs. Consequently, distal arterial segments are too large. Thus, the proper redistribution of arterial ECs from distal to proximal locations is an important driver during the establishment of hierarchically patterned arterial trees. Of note, these defects in EC migration likely contribute to arteriovenous malformations seen in animal models and humans with mutations in the Endoglin, Alk1, or SMAD4,^[20,28,29,115–124] as enlarged vessel segments display aberrant flow patterns that can secondarily affect proliferation and cell fates within these segments.^[26] These findings also match the above-discussed observation that migration-deficient ECs in *cdc42* mutant mice accumulate in veins (Figure 4B).

Endoglin-, Alk1-, and SMAD-deficient animals also have larger arteries due to additional functions in arterial vessels. In response to blood flow, arteries in the zebrafish trunk undergo a contraction mediated by EC shape changes.^[20] Specifically, ECs elongate, which ultimately decreases lumen diameter. Endoglin mutant ECs do not elongate and actually increase their size in response to flow. A similar phenomenon is seen in the developing mouse heart and retina. In these organs, SMAD4 deletion results in increased EC sizes and proliferation, which, in the heart, is accompanied by increased artery diameters.^[27,115] These studies indicate that one role of SMAD signaling is to restrain flow-induced increases in cell size and proliferation because the changes in mutant cells were not seen in the absence of flow.^[20,27]

Blood provides circulating Bmp9/10, and its signaling through Alk1 is enhanced by shear stress.^[125] Therefore, BMP9/10 may be the activating ligand that drives SMAD activation during arterial remodeling.^[126] Future studies should investigate how signaling from circulating and/or secreted ligands translates in directional migratory cues, the mechanistic connection between SMAD signaling and cell shape changes, and if SMAD-mediated transcription is required. It would also be important to investigate to which extent *alk1* and *endoglin* might function in a ligand-independent manner.

12. Do Piezo1 and Notch Play a Role in Mechanosensing During Artery Formation?

Exactly how ECs directly sense the mechanical forces of blood flow to activate cell behaviors, such as migration is still poorly understood.^[127] In contrast, a significant amount has been learned about the critical pathways downstream of sensing changes in shear stress, particularly using in vitro systems, and this has been reviewed elsewhere.^[128] One more recently identified candidate for how ECs directly sense shear stress during artery remodeling is the mechanosensitive cation channel, Piezo1. Piezo1 was initially identified in a screen for mechanically activated ion channels using a neuroblastoma cell line.^[129] Subsequent studies found it to be specifically expressed in ECs in developing mouse embryos,^[130] and deletions led to severe defects in plexus remodeling, including artery formation.^[130,131] These data suggest that signaling through Piezo1 is one of the critical means by which ECs sense shear stress. More detailed analysis of shear stress-directed migration of arterial cells will identify its precise role in artery development.

Notch1 has also recently been proposed as a mechanosensor.^[87,132] Interestingly, this function was independent of its transcriptional activity. Shear stress-induced Notch1 proteolysis allowed association of its transmembrane domain with a complex including VE-cadherin, LAC, and TRIO, which stimulated adherens junction assembly.^[132] This functioned to increase the EC barrier. A separate study also showed that Notch1 acts as a mechanosensor.^[86] Here, Notch was activated by shear stress to support junctional integrity and suppress proliferation. Mack et al.^[86] also detected a polarized localization of Notch1 in response to flow, suggesting it could be an orienting signal during flow-guided migration. It will be interesting to investigate whether Notch activation in pre-specified artery cells is part of the program that allows the cells to migrate into and build arterial vessels in addition to its role in suppressing EC proliferation. The above-mentioned cell migration results might also provide an elegant solution to the problem that arterial cells do not divide any more, while at the same time arterial diameters need to increase. In this view, new arterial cells would be added via migration against the direction of blood flow into arteries, circumventing the need of continuous proliferation in this growing vessel subtype.

13. Conclusions and Outlook

Advances in our understanding of how arteries are constructed has revealed that arterial trees can form in a unique manner with respect to other hierarchically branched structures—via inward growth rather than outward branching morphogenesis (Figure 4C). This process of reverse directional growth of an organ undergoing branching morphogenesis is characterized by several key features. One is the initial genetic pre-specification of arterial cells outside of the artery, often occurring distally within immature vessels originating from venous sprouts. This pre-specification of arterial cells happens through activation of the Notch pathway and a loss of cell cycling. One key gene also activated during this process is the chemokine receptor *Cxcr4*, which is necessary for newly emerging sprouts to connect to pre-existing arteries. Once connected, blood flow ensues and arterial cells can then sense the direction of blood flow-induced shear stress, potentially using Piezo1 and Notch1, followed by migration

against this direction using the Eng/Alk1/SMAD4 and DACH1/CXCL12/CXCR4 signaling pathways. This ultimately leads to a redistribution of arterial cells from segments that are distal to the heart to more proximal ones, which is necessary for obtaining proper vessel diameters within the hierarchical arrangement of arterial blood vessel trees. Together, these findings show that distinct mechanisms can be responsible for the establishment of hierarchically patterned organs.

They also suggest that it might be necessary to target EC proliferation and migration in ways that were previously not appreciated when enhancing blood flow as a therapeutic aim to diseased or regenerating tissue; and that manipulating these parameters within ECs must be done with caution, because they might affect the formation of venous and arterial trees in opposite ways. Furthermore, it is now clear that genetic and hemodynamic factors interact during artery formation. However, it still needs to be determined exactly how these behaviors result in the exquisitely defined hierarchical branching of the final structure of mature arteries. These new insights are sure to be the subject of exciting studies in the near future.

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Two trees connected via a capillary bed

Figure 1.

The vasculature consists of two interconnected trees. Schematized drawing of arterial and venous blood vessel trees. Note that the two trees are interconnected at their tips, allowing blood to flow from the arteries to the veins.

Figure 2.

Sprouting angiogenesis at different developmental stages. A) Intersegmental blood vessel (ISV) sprouts emerge from the dorsal aorta in response to VEGF signaling, migrate dorsally and anastomose to form a network of arterial vessels that lacks a venous connection at this time point. Sprouting from the posterior cardinal vein in response to BMP signaling generates secondary sprouts (1.5 dpf). About half of the secondary sprouts connect to arterial ISVs at 2 dpf, allowing blood to flow from arteries into the venous circulation. Endothelial cells within arterial ISVs can change their identity to a venous fate or be

replaced by venous cells through migration against the direction of blood flow. dpf: days post fertilization. B) Angiogenesis in the zebrafish brain. New sprouts emerge from two laterally located veins (PHBC) and connect to a pre-existing artery (BA) located medially. Note expression of the chemokine Cxcl12b around the artery (orange). Sprouting tip cells sense Cxcl12b via the chemokine receptor cxcr4a and migrate toward the artery. PHBC: primordial hindbrain channel; BA: basilar artery. C: Live imaging of artery formation during fin regeneration in zebrafish. Vein-derived cells at the distal tip of the advancing vascular front change their direction of migration and become incorporated into newly forming arteries. This is dependent on the chemokine receptor Cxcr4a. Cxcl12 is also expressed in the vicinity of the artery. D) Genetic lineage labeling of sinus venosus (sv) endothelial cells at embryonic day (E) 10 (green) traces into the coronary vessel plexus and coronary arteries at later stages. Ao, aorta; pt, pulmonary trunk.

Comparison between current and newly proposed models for artery formation during blood vessel plexus remodeling. A) Current model of coronary plexus remodeling involves artery differentiation triggered by blood flow. B) Proposed model shows artery differentiation is initiated prior to the onset of blood flow via genetic pathways. This pre-specification of arterial fates would subsequently be refined via the ensuing blood flow. C) Current model of artery differentiation within the mouse retinal blood vessel plexus. Artery differentiation would occur in capillaries close to the existing artery, thereby expanding the artery. D)

Proposed model involving pre-specification of arterial cells at the tip of the advancing vascular front. These would then grow toward the pre-existing artery, against the blood flow direction. E) Current model of the function of Notch signaling in the angiogenic front. VEGF leads to expression of the Notch ligand *dll4* in tip cells. In turn, *dll4* induces Notch signaling in neighboring stalk cells, preventing them from becoming a tip cell. Ensuing induction of Notch signaling leads to artery formation in locations deeper within the plexus. F) Proposed model linking the role of Notch signaling in angiogenesis and during artery formation. In this model, the tip cell initially displays low Notch signaling and expresses *dll4*. However, this does not lead to an induction of Notch signaling in the stalk cell for an unknown reason. Subsequently, some tip cells activate Notch signaling, presumably due to *dll4* signaling from stalk cells. This leads to the onset of an arterial differentiation program that destines these tip cells to form new arteries. Tip cells change their direction of migration, possibly due to expression of the chemokine receptor *cxcr4*, and stop proliferating (possibly regulated via the transcription factor *COUP-TFIII*). Finally, cells before present at the tip position connect to a pre-existing artery. In this model, initiation of artery formation occurs in tip cells at the angiogenic front. Both models are not necessarily mutually exclusive.

Figure 4.

Function of genes involved in cell migration during artery formation. A) In vitro data showing that overexpression of Dach1 leads to an increased response to shear stress, causing enhanced endothelial cell migration against the direction of the flowing media. Cells lacking Endoglin or SMAD4 function show an impaired response to the flowing media and fail to migrate properly. B) Cdh5-CreERT2-mediated activation of GFP lineage marker within endothelial cells of the mouse retina reveals an equal contribution to arteries, veins, and capillaries. Deletion of the BMP co-receptor Endoglin results in fewer cells in tip position and in arteries, while overexpression of Endoglin causes cells to contribute more to arteries, an effect that is amplified by a deletion of Endoglin in the surrounding retinal endothelial cells. Deletion of the small GTPase *cdc42* leads to an accumulation of mutant cells within and around veins, while mutant cells are less abundant at the angiogenic front and in arteries. C) Summary of cellular behaviors within arterial and venous blood vessel trees that might contribute to their hierarchical patterning. In both trees, endothelial cells migrate against the direction of the flowing blood (indicated by arrows). This distributes cells from smaller, more distally located segments toward larger proximal vessels. Endothelial cells within arteries show low proliferation; thus redistribution of cells necessary for assigning proper blood vessel calibers can only occur via migration. Endothelial cells within venous blood vessel trees proliferate and migrate distally toward smaller branches. Here, some cells become genetically specified as pre-artery cells, which subsequently build newly forming arteries.