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Ups and Downs of Guided Vessel Sprouting: The Role of Polarity

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Blood vessel networks expand to meet oxygen demands via sprouting angiogenesis. This process is heterogeneous but not random; as sprouts form and extend, neighboring endothelial cells do not sprout but divide. Sprouting is regulated by local sprout guidance cues produced by the vessels themselves, as well as extrinsic cues. Endothelial cells in developing vessels orient in several axes to establish migratory polarity, apical-basolateral polarity, and planar cell polarity. Although little is known about how polarity axes are set up or maintained, they are important for vessel formation and function. This review focuses on the current knowledge of how blood vessel sprouting is regulated and guided, the role of endothelial cell polarity in forming vessels, and how these processes affect vessel function and are potentially perturbed in pathologies with vascular components.

Blood vessel networks form via de novo formation of vessels, and subsequent sprouting from these vessels to form new conduits (see reviews in Refs. 11, 16, 60). These processes require the coordination of numerous cellular processes, and the endothelial cells that comprise primitive vessels proliferate, migrate, polarize, and fuse in response to a set of molecular cues that are integrated in both space and time. These guidance cues are produced by nearby tissues and the vessels themselves, and they are found in the local micro-environment. However, the individual endothelial cells of a target vessel often respond differently to these cues; in fact, this heterogeneous response is a requirement for proper vascular development (see reviews in Refs. 2, 13, 40). Sprouting migration of endothelial cells only produces new networks when some endothelial cells respond to cues by migrating and eventually fusing with other sprouts, whereas nearby neighbors proliferate to provide the building blocks for expansion.

Thus endothelial cells must know their position in relation to neighbors in developing vessels to provide the proper responses to guidance cues. This is accomplished in part by cell-cell communication that includes orienting along several axes. Although first responses to cues must be heterogeneous to set up sprouting, subsequent responses and orientation as the sprout forms a lumen, and eventually a conduit, are more homogeneous. First, the endothelial cell that responds to cues by migrating sets up a migratory polarity that orients the cell to move forward. Polarization of the tip cell, or leader cell, in turn is predicted to signal adjacent cells to adopt a follower, or stalk cell phenotype. As a nascent sprout migrates outward, cells behind the leading edge polarize along an apical-basolateral axis (also called the luminal-abluminal axis in a tube) to initiate the formation of a lumen. Finally, endothelial cells in developing vessels organize in the plane orthogonal to the apical-basolateral axis, and polarity in this plane is called planar cell polarity (PCP). This polarization takes the form of oriented cell divisions that set up the cleavage plane perpendicular to the vessel long axis and lengthen the vessel. Subsequently, blood flow provides a shear stress vector that polarizes the endothelial cell cytoskeleton relative to flow. All of these distinct orientations require the coordination of endothelial cell responses to inputs.

This review discusses how blood vessel sprouting is regulated, with a focus on the means by which spatial orientation of endothelial cells in developing vessels is accomplished to provide both heterogeneous and homogeneous responses to environmental cues. We first assess how the events of sprouting migration are set up, and how nascent vessels interpret signals. We discuss some of the guidance cues that are known to regulate sprouting, including VEGF and Notch. We next describe how endothelial cells set up their spatial orientation via polarization at several different levels. Finally, we discuss how these important developmental responses of endothelial cells impact blood vessel function and are potentially misregulated in disease.

Guided Vessel Sprouting

Initially, a single endothelial cell, termed a "tip cell," initiates a new sprout by migrating outward in response to angiogenic growth factors (27). It is not known how the tip cell is initially specified, but, once specified, the tip cell upregulates several markers, including the Notch ligand Dll4, endothelial-specific molecule 1, angiopoietin 2, the chemokine receptor CXCR4, and others (18, 32, 71). At the same time, adjacent cells adopt a "stalk cell" phenotype, likely in response to Notch signaling induced by Dll4.

VEGF is a strong and predominant vessel sprout guidance system (see reviews in Refs. 24, 57) (see FIGURE 1). A major cue is provided by the ligand VEGF-A, which is produced by tissues and organs as they form and grow, often in response to oxygen demands. VEGF-A binds two high-affinity endothelial cell receptors expressed in nascent vessels, VEGFR-2 (Flk-1 in mouse) and VEGFR-1 (Flt-1 in mouse). These are tyrosine kinase family receptors, with an extracellular domain that binds ligand, a transmembrane domain, and a cytoplasmic domain that, upon ligand binding and dimerization, stimulates signaling via numerous downstream effector pathways. Binding of VEGF-A to VEGFR-2/Flk-1 stimulates endothelial cell proliferation, migration, and survival, and also modulates the stability of cell-cell adhesions. Genetic loss of either VEGF-A or VEGFR-2/Flk-1 is embryonic lethal due to insufficient blood vessel formation (12, 23, 66). Genetic loss of VEGFR-1/Flt-1 also leads to embryonic lethality; however, rather than insufficient vessel formation, the embryonic vessels overgrow and become disrupted (25). This phenotype suggests that Flt-1 negatively modulates VEGF signaling. The finding that loss of Flt-1 signaling capacity via deletion of the cytoplasmic domain does not perturb vascular development (33), along with identification of a naturally occurring soluble version of Flt-1 (sFlt-1) that is generated via alternative splicing and secreted (44), suggested that Flt-1 may act as a competitive inhibitor to modulate signaling through Flk-1. This was confirmed at the molecular level (43, 61). Moreover, sFlt-1 selectively and positively affects branching morphogenesis in developing vessels, whereas both isoforms negatively modulate endothelial division rate (14, 43, 48). A model explaining this data posits that localized expression of sFlt-1 by developing vessels spatially modulates VEGF-A ligand accessibility such that emerging sprouts utilize a ligand corridor to move efficiently away from the parent vessel and thus enhance productive sprouting and branching (14).

An additional component of VEGF signaling that is important for guided vessel sprouting is VEGFR-3 (Flt-4 in mouse), although how it affects vessel sprouting is not entirely clear. The VEGFR-3 ligand VEGF-C influences angiogenesis, presumably through interactions with VEGFR-2 (51), and VEGF-D is not required for proper vessel development (reviewed in Ref. 50). However, VEGFR-3 appears to be important in blood vessel sprouting, since genetic loss of function is embryonic lethal with vascular defects, and receptor blockade suppresses angiogenic sprouting in the postnatal retina (19, 75).

These and other guidance cues are likely interpreted by nascent vessels in the context of the status of Notch signaling (see reviews in Refs. 29, 34, 35, 40, 59, 62, 67, 68). Although the precise ways in which Notch signaling intersects VEGF signaling are a current focus of research, a model for which much of the data is consistent suggests that the initial VEGF signal leads to upregulation of the Notch ligand Dll4 in the nascent tip cell (see FIGURE 2A), perhaps via integrin engagement (20). How this particular endothelial cell is chosen to become the tip cell is not clear, but it may have higher levels of VEGFR-2 and thus respond more
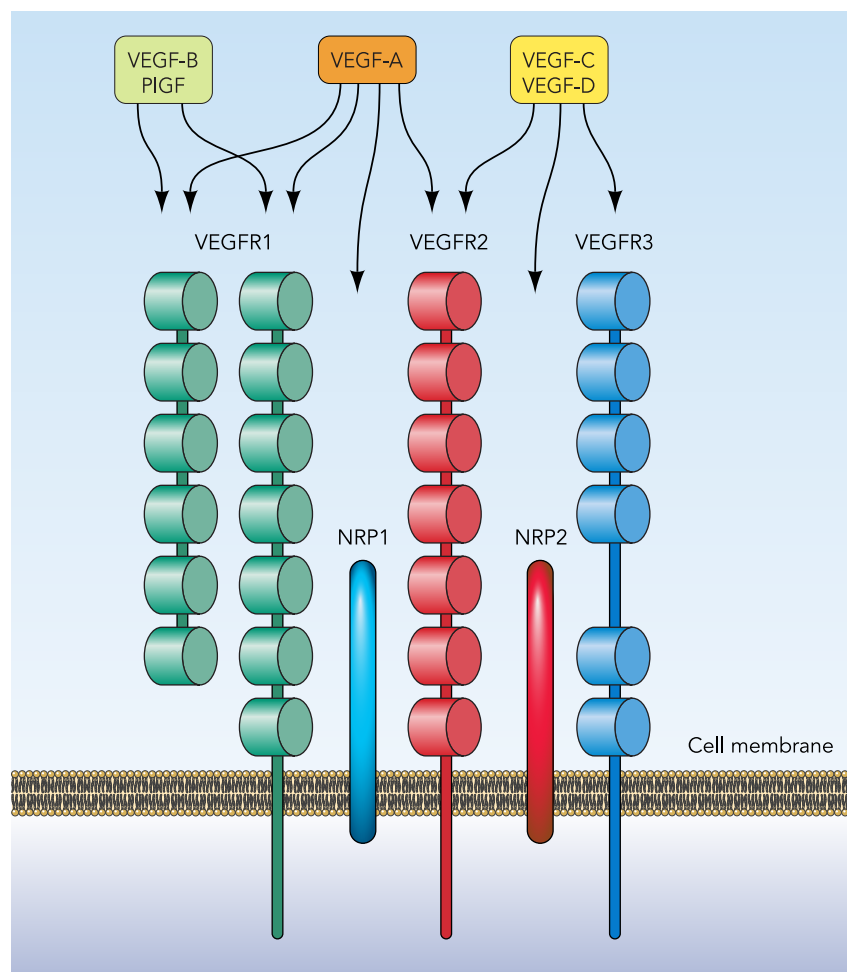

FIGURE 1. Components of the VEGF signaling pathway
Diagram shows the ligands (*top*), receptors, and co-receptors that are primarily involved in VEGF signaling in endothelial cells.

robustly to the VEGF signal. Nevertheless, the upregulation of Dll4 leads to elevated Notch signaling in neighboring cells, which has several consequences. Notch upregulates VEGFR-1/Flt-1 and perhaps downregulates VEGFR-2 and R3 receptors. These molecular changes, and perhaps others, lead the endothelial cells with elevated Notch signaling to adopt a stalk cell phenotype that is characterized by a proliferation response to VEGF signaling. These changes also allow the cells adjacent to the emerging sprout to inactivate near-field VEGF-A ligand and provide guidance cues via secretion of sFlt-1 (FIGURE 2A).

Once a sprout leaves the near-field environment, where environmental signals are interpreted by the parent vessel and the emerging sprout, it continues to respond to environmental cues, but the cues and the responses are less well understood. It appears that the levels of VEGF signaling perceived by endothelial cells and interpreted via Notch are still important, and cells with higher VEGF signaling assume the tip cell position at higher frequency than their neighbors with lower VEGF signaling (41). Interestingly, recent evidence from the mouse embryonic hindbrain and developing retinal vasculature indicates that tissue macrophages may physically bridge neighboring sprouts while they are anastomosing (22). Finally, in a process that is poorly understood, a sprout contacts and fuses with another sprout or vessel, and

formation of a lumen provides a new conduit for delivery of oxygenated blood. Using computational simulations, Bentley et al. predicted that the formation of new cell-cell junctions by sprout fusion disrupts Dll4/Notch-mediated tip/stalk cell patterns, causing the fusing tip cells to become inhibited for further sprouting, whereas neighboring stalk cells are predicted to reverse phenotypes and become tip cells (8).

Polarity and Initiation of Vessel Sprouting

Endothelial cell polarity is a key feature of angiogenic sprouting; however, the profound cellular remodeling events that must occur as vessels sprout and form new conduits are only beginning to be understood. Once the tip cell is specified, it reorganizes its cytoskeleton to become polarized for migration by extending numerous lamellipodia and filopodia at the leading front, and it lacks a vessel lumen (27, 32, 74) (FIGURE 2B). In contrast, the stalk cells following behind as the sprout extends are proliferative, adopt apical-basal polarity, and establish a lumen (17). The stalk cells are also relatively devoid of filopodia, downregulate expression of matrix metalloproteinases, express the apical marker podocalyxin, and secrete collagen IV basally (49, 65, 79) (FIGURE 2B).

A Guidance cues of vessel sprouting
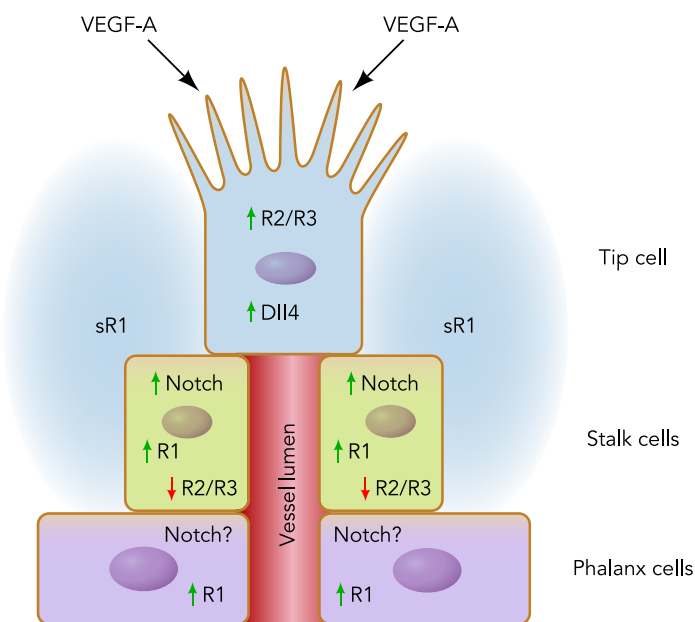
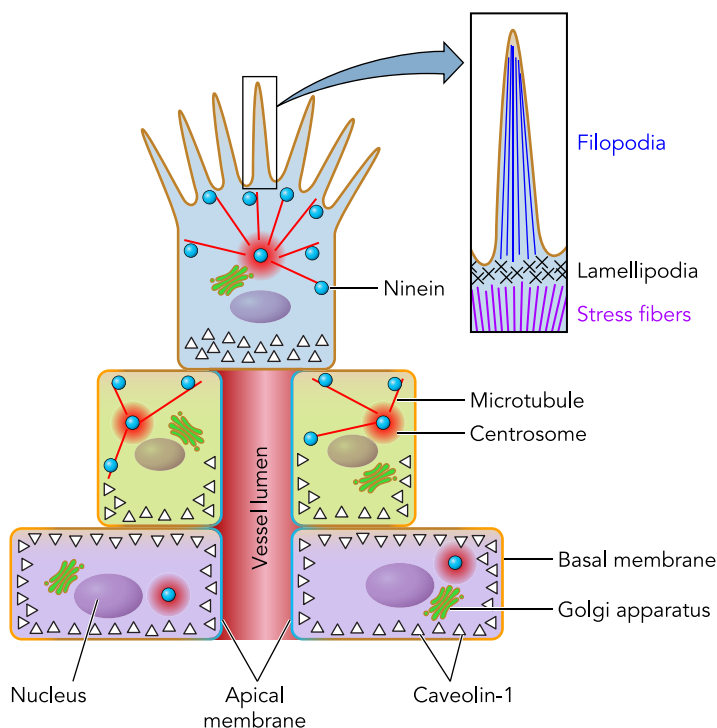**B** Endothelial polarization and cytoskeletal arrangements

FIGURE 2. **Guided blood vessel sprouting: signaling and polarity**
A: diagram of a vessel sprout illustrating major signaling axes (see text for details). B: diagram of a vessel sprout showing migratory and apical-basolateral polarization (see text for details).

Polarity and Migratory Tip Cells

Endothelial cell filopodia play a crucial role in the polarized migration of tip cells by sensing and responding to guidance cues (27, 64). Retinal vessels treated with exogenous VEGF-A and vessels of VEGF120/120 mice that presumably do not form in a VEGF gradient exhibit a loss of migratory polarity, and ectopic filopodia extend in random directions from both tip and stalk cells (14, 27). Substantial evidence in other cell types suggests that this phenotype results from perturbed downstream signaling through the small GTPases Rac1, Cdc42, and RhoA, which regulate cytoskeleton dynamics, cell polarity, and focal adhesions (reviewed in Ref. 10). In addition, tip cell number and filopodia density on endothelial cells are regulated by Dll4/Notch1 signaling (32, 74). This may be in part due to cross talk with the VEGF pathway, but it is unclear whether Dll4/Notch1 signaling also interacts with the cytoskeleton independent of VEGF to induce the tip cell phenotype.

Polarized migration of endothelial and other cell types requires proper orientation of the Golgi apparatus and the microtubule organizing center (MTOC) (28, 56). More recently, the membrane-associated proteins, angiomotin and angiomotin-like protein-1, were localized to endothelial lamellipodia and tight junctions, respectively. These proteins influenced formation of filopodial extensions, positioning of the Golgi apparatus, and the stability of cell-cell junctions (1, 82). Furthermore, Matsumoto and colleagues showed that localization of ninein, a centrosomal microtubule-anchoring protein, changes in developing vessels. Ninein is largely localized at the centrosome in the base and stalk cells, whereas it is abundantly expressed in the cytoplasm of migrating tip cells (53). It is proposed that its release from the centrosome allows for microtubule reorganization that is required for forward movement of the tip cell.

Meanwhile, the rear of the tip cell must maintain contact with and exert pulling forces on stalk cells to prevent branch disintegration. Another protein that may be involved is caveolin-1, a major component of endothelial cell surface caveolae. Caveolin-1 protein concentrates at the trailing end of aortic endothelial cells induced to migrate by chemotaxis or chemokinesis (7, 38), where it may participate in focal adhesion disassembly that is required for forward movement (see reviews in Refs. 9, 26). Beardsley et al. noted an absence of lamellipodia at sites of caveolin-1 polarization and also found that loss of caveolin-1 prevented cell migration. However, there appear to be complex mechanisms driving caveolin-1 polarization that depend on its phosphorylation state and the migration mode (7, 58). It

is still unclear how downstream signaling through VEGF coordinates these rapid and dynamic polarization events.

Polarity in Sprout Fusion and Lumen Formation

Following tip-cell selection and migration, sprouts from different vessel segments meet and subsequently fuse, or anastomose, to form a functional vessel connection. Although we currently have a poor understanding of the overall process, particularly in regard to how a sprout determines its target for fusion, it is likely to also be regulated by guidance cues and polarity changes, similar to vessel sprouting.

Finally, the developing vessels must generate a lumen and connect to the circulation. Studying this process has been relatively difficult due to the lack of appropriate models; however, our understanding is quickly expanding by recent studies using three-dimensional extracellular matrices in combination with in vivo data. Studies thus far suggest that lumen formation is governed by different processes depending on the specific vessel bed, with the two main mechanisms being cell hollowing and cord hollowing (reviewed in Ref. 37). Briefly, cell hollowing involves the generation and coalescence of intracellular vacuoles of individual cells, which then interconnect with neighbors to form a multicellular lumen. In vitro studies show that this process involves the small GTPases Rho, Rac, and cdc42 (5, 6). In contrast, cord hollowing involves a cord of packed cells undergoing dramatic cell shape changes to create a lumen (FIGURE 3). For either process to occur, the establishment of apical-basal polarity is essential.

The complex composed of the Par proteins, Par3 and Par6, and atypical protein kinase C (aPKC) is crucial for apical-basal polarity in numerous cell types and organisms (reviewed in Ref. 21). Iden et al. showed that, in cultured endothelial cells, the Par complex physically associates with adherens junctions (36). It was later discovered that this interaction is needed for activation of the small GTPase Cdc42 and aPKC activity, which are crucial for proper vessel formation (47, 49). Indeed, disrupting any member of the Par3-Par6-aPKC complex in vitro leads to defective endothelial polarity and vessel lumenization (47). In vitro studies also show that polarized Cdc42/Par6/aPKC activity is required for flow-dependent reorientation of the MTOC, suggesting another method by which blood flow contributes to vessel polarization (76).

Strilic and colleagues showed that, in the developing mouse aorta, endothelial cells first adhere to each other (FIGURE 3). CD34-sialomucins are next localized to internal cell-cell contacts in a VE-cadherin-dependent manner. Moesin and F-actin are

subsequently recruited to these contacts, thus defining the apical surface. VEGF signaling then enables F-actin and non-muscle myosin II interactions to induce cell shape changes and initiate lumen formation (72). In addition, the negative charge of apical glycoproteins on opposing endothelial cell surfaces generates repelling electrostatic forces that also contribute to lumen expansion (73). Another report indicates that VE-cadherin, with CCM1 and Rap1, is also required for localized activation of the Par complex and organization of adherens junctions (49). Furthermore, β1-integrin-null endothelial cells lose their characteristic squamous shape, mislocalize the polarity determinant Par3 and cell-cell adhesion molecules, and display luminal occlusion (83). Ras interacting protein 1 (Rasip1) was recently identified as a vascular-specific regulator of Rho GTPase signaling, and, interestingly, mice lacking Rasip1 failed to form lumens in all blood vessels (78). The primary defects included excessive actomyosin contractility, failure of Par3 and junctional proteins to redistribute to the periphery, and loss of integrin adhesion to the surrounding extracellular matrix. Taken together, these findings suggest that a number of distinct determinants are essential for proper polarization and lumen formation, but we still do not fully understand how these factors interact to coordinate cell polarization for lumen formation during vascular development.

Planar Cell Polarity in Angiogenesis

In contrast to the well established importance of Wnt/β-catenin signaling in angiogenesis, the role of the noncanonical Wnt/planar cell polarity pathway that regulates Rho, Rac, and JNK is poorly understood. Planar cell polarity (PCP) describes the coordinated polarization of cells within the plane of an epithelial sheet that is perpendicular to the apical-basal axis and commonly involves asymmetric localization of protein complexes within individual cells (3). Well studied examples of PCP include the precise arrangement of bristles on the fly wing and the uniform orientation of stereocilia in the sensory hair cells of the mouse cochlea. Further vertebrate studies have provided evidence of PCP regulation in a variety of developmental contexts, including cellular processes such as directed migration, cell division orientation, and tissue morphogenesis (for detailed reviews, see Refs. 69, 77, 80).

It is thus tempting to speculate that PCP signaling may coordinate at least some of the necessary cell polarity information between neighboring cells that is required for the complex processes of angiogenesis.

A Early stage of vascular lumen formation
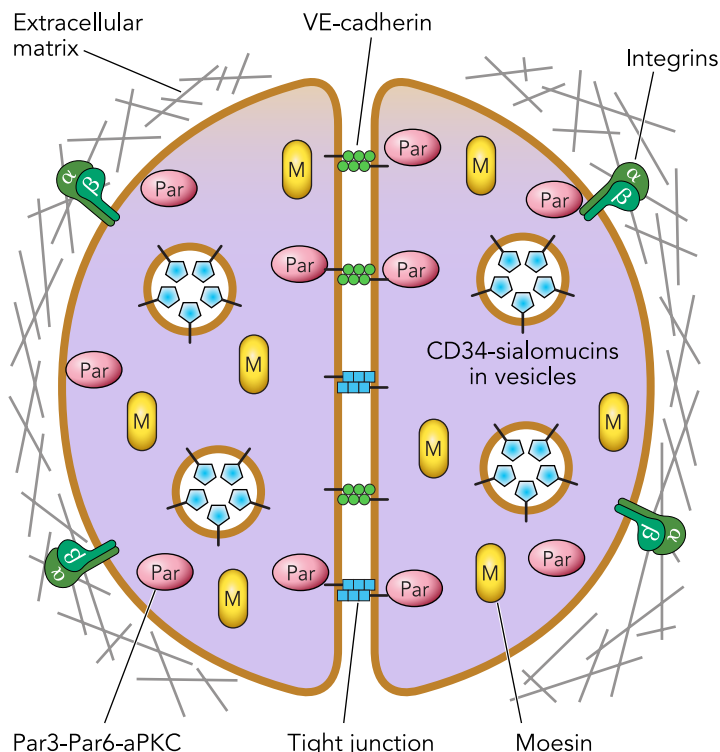
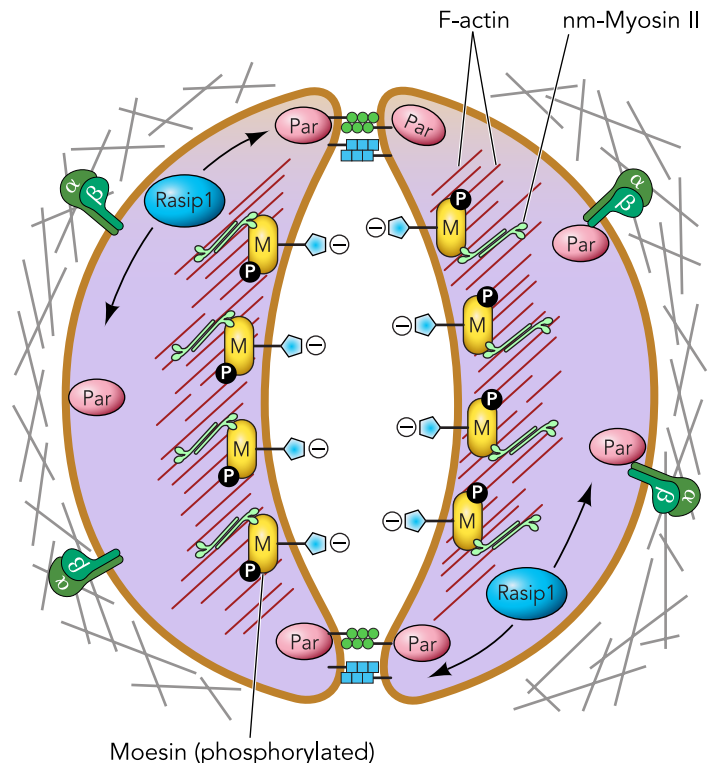**B** Later stage of vascular lumen formation

FIGURE 3. **Cord hollowing mechanism of vessel lumen formation requires vessel polarity**
A: at early stages of lumen formation, junctional and Par proteins are uniformly expressed on the endothelial cell surface. B: at later stages of lumen formation, junctional and Par3 proteins are excluded from the apical membrane, sialomucins lead the apical surfaces to repel each other, and actomyosin contractility induces shape changes that extend the lumen diameter.

For example, developing blood vessels display oriented endothelial cell division that is regulated in a flow-independent manner (81). Moreover, the term PCP has been used to describe the fluid shear stress-induced phenotypes of cultured vascular endothelium, which include the reorganization of actin stress fibers, the redistribution of junctional complexes, and the reorientation of the MTOC on the downstream side of the nucleus (55). In vivo evidence is less clear, however, because studies have suggested that endothelial microtubule polarity is either cardiocentric (upstream in arteries and downstream in veins) or differs with vessel age and identity (46, 55, 63).

Using cell culture assays, Cirone et al. (15) recently showed that selective inhibition of β-catenin-independent Wnt signaling by either the anti-angiogenic drug TNP-470 or a ΔDIX-Dvl2 mutant resulted in disrupted endothelial cell growth, polarity (defined by caveolin-1 localization), and cell migration. Interestingly, activation of the PCP downstream effectors Daam-1, Diversin, or Inversin rescued all these phenotypes (15). It was also found that overactivation of Daam-1 selectively inhibited endothelial cell proliferation by regulating microtubule assembly and stabilization and resulted in impaired migration and vessel network formation (42). Furthermore, Wnt5-deficient zebrafish with impaired noncanonical Wnt signaling display gross morphological vascular defects, including defective intersegmental and cranial vessels (15).

These findings provide support for PCP-dependent regulation of microtubule and other cytoskeletal elements in endothelial cells, but additional studies are needed to further elucidate the roles and mechanisms of PCP in endothelial cell polarity and blood vessel morphogenesis. For example, present studies have not yet investigated endothelial cell functions of unique PCP proteins, such as Vangl2 or Prickle. Other open questions include: What is the spatiotemporal significance of PCP regulation in vascular development? Are there specific defects resulting from perturbed vascular PCP signaling, and, if so, what mechanisms are affected? It will also be interesting to gain further understanding of the potential cross talk between these polarity complexes (both Par and PCP) and the better understood VEGF and Dll4-Notch signaling pathways in coordinating angiogenesis.

Guided Vessel Sprouting: Implications for Disease and Conclusions

Sprouts must be formed, guided, and the proper polarity programs initiated to make a functional vessel network that efficiently delivers oxygen and nutrients to tissues. Here, we highlight a few of the many instances where aberrant vessel formation and/or function are an integral part of the pathology of disease.

Diabetes is characterized by perturbed vessel function, and the retinal vasculature of the eye is particularly susceptible to the perturbed metabolism seen in diabetic individuals (reviewed in Refs. 52, 70). Diabetic retinopathy is associated with the overgrowth and dysmorphogenesis of retinal vessels, sometimes even leading to blindness. In diabetic animals, the retinal vessels are leaky with reduced barrier function, and they have a tortuous morphology. These initial defects lead to vessel overgrowth via mechanisms that are not well understood but are thought to involve VEGF signaling. Overgrown vessels in a rodent model have randomized endothelial cell divisions (31), suggesting that these defects are accompanied by perturbed vessel polarity. It will be interesting to see whether this is the case and whether polarity can be restored downstream of the initial input.

Solid tumors develop in the context of blood vessels recruited from adjacent tissues (reviews in Refs. 30, 45). Although the vessels that enter tumors are normal, once in the tumor environment the vessels become abnormal in several ways. They are leaky and tortuous, they do not recruit pericytes efficiently, and they are poor at relieving the hypoxia of the tumor environment. This inability to relieve hypoxia maintains high levels of several growth factors, including VEGF, and this is thought to further exacerbate the tumor vessel phenotype (39). In fact, when hypoxic signaling is short-circuited genetically, tumor vessels "normalize" and tumors are less metastatic (54). Scanning electron micrographs of the luminal (apical) surface of tumor vessels show that endothelial cells have protrusions and overlap with other cells that are not seen in vessels from normal tissues (4). Tumor vessels have a luminal surface, but other aspects of polarity have not been directly assessed, so it is tempting to speculate that the phenotype and reduced function of tumor vessels are associated with aberrant polarity of tumor endothelial cells. Although any polarity defects would likely be downstream of excess signaling in the tumor environment, it may be that restoration of endothelial cell polarity would act to normalize tumor vessels.

We now realize that blood vessel formation is organized at many levels, and we have some understanding of both the extrinsic and intrinsic molecular cues that regulate this process. Extrinsic signals such as VEGF are interpreted by endothelial cells in complex ways that depend on the behaviors of neighboring cells and lead to heterogeneous but integrated responses. A soluble form of VEGFR-1 is produced intrinsically but is secreted to provide guidance cues to nearby sprouts.

Finally, endothelial cells in developing vessels exhibit polarity in several axes, also likely as a result of both intrinsic and extrinsic cues. Some of these polarities, such as migratory polarity, are set up heterogeneously, whereas others, such as apical-basolateral polarity, are set up homogeneously by groups of cells in vessels. The next several years are sure to reveal important new mechanisms whereby these behaviors are set up and integrated, and perhaps new molecular players that are involved. Both extrinsic and intrinsic mechanisms are potential therapeutic targets. ■

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