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Review

Regulation of blood vessel sprouting

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ABSTRACT

Blood vessels are essential conduits of nutrients and oxygen throughout the body. The formation of these vessels involves angiogenic sprouting, a complex process entailing highly integrated cell behaviors and signaling pathways. In this review, we discuss how endothelial cells initiate a vessel sprout through interactions with their environment and with one another, particularly through lateral inhibition. We review the composition of the local environment, which contains an initial set of guidance cues to facilitate the proper outward migration of the sprout as it emerges from a parent vessel. The long-range guidance and sprout stability cues provided by soluble molecules, extracellular matrix components, and interactions with other cell types are also discussed. We also examine emerging evidence for mechanisms that govern sprout fusion with its target and lumen formation.

Contents

1. Introduction

As the vasculature forms, blood vessels must expand and form interconnected networks to deliver oxygen and nutrients to developing tissues and organs. They do this primarily via sprouting angiogenesis [1]. Sprouting angiogenesis (shortened to "sprouting" in this review) is a reiterative process that seems simple at first glance, but in reality involves numerous levels of regulation that control critical signals and endothelial cell responses in both time and space. In fact, a coalescing theme of recent exciting research is that spatial organization of endothelial cell behaviors – and hence the signals that control those behaviors – is crucial to proper vessel sprouting and network expansion. Moreover, these behaviors must be integrated within the developing vessel network via cell–cell communication. Thus endothelial cells must "know" the status of neighboring cells in the developing vessel and adjust their behaviors accordingly. The emerging model is that the developing vasculature is analogous to a bee colony. Like individual bees in a bee colony, individual endothelial cells have different roles, or phenotypes, and different responses to incoming

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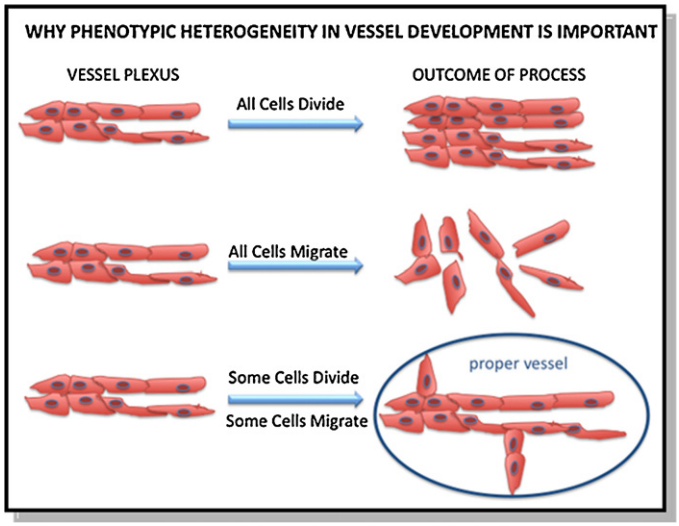

Fig. 1. Endothelial heterogeneity in vessel sprouting. In this model, endothelial cell heterogeneity is important for proper sprouting. In response to a stimulus, if all endothelial cells divide, no sprouting occurs, and if all cells migrate (sprout), a productive sprout does not form. When some endothelial cells migrate (i.e. become tip cells), while other endothelial cells divide and/or form lagging cells (i.e. stalk cells), proper blood vessel sprouts form.

information. For example, in response to angiogenic cues such as VEGF-A, some endothelial cells migrate and initiate sprouting, while others undergo cell division (Fig. 1). However, unlike most bees, many endothelial cells change their phenotypes over time, so that what was once the leading cell, or tip cell, of the sprout, becomes a lagging cell, or stalk cell. How endothelial cell phenotypes are specified, regulated, and dynamically modulated is the focus of this short review. Many excellent reviews cover vascular development more globally [2–5], and the other chapters in this volume cover other important aspects of blood vessel formation. We provide a description of the endothelial cell behaviors involved in sprouting angiogenesis, then cover in detail current information regarding initiation of vessel sprouting, sprout guidance, and sprout fusion to form new connections.

1.1. Overview of blood vessel sprouting

Vessel sprouting is a process carried out by endothelial cells. A primary vessel, such as the dorsal aorta, forms via vasculogenesis, the coalescence and differentiation of endothelial progenitor cells. Sprout initiation involves one endothelial cell responding to angiogenic stimuli by extending filopodia, and then migrating outward from the parent vessel while still connected to its neighbors (Fig. 2). This endothelial cell may initiate sprouting because it experiences higher angiogenic factor signaling than its neighbors, or it may be a stochastic process. Nevertheless, the chosen initiating endothelial cell, now called a tip cell, initiates signaling that prevents neighboring endothelial cells from sprouting. It also likely signals to neighbors to provide local guidance cues that help direct the emerging sprout away from the parent vessel. As the tip cell moves further away from the parent vessel, neighboring cells remain attached to the tip cell and migrate behind it to form a stalk. The stalk cells are more proliferative than the tip cells, and they divide and reorganize along the stalk and within the parent vessel to increase the mass and surface area of the growing vessel. The parent vessel often has a central lumen, and as the sprout extends and explores the environment for new connections, a lumen begins to form in the sprout that eventually will connect with the lumen of the parent vessel and extend through the new connection. How the tip cell is guided at significant distances from the parent vessel is not clear. In some cases other embryonic tissues, such as somites, provide both a physical barrier and negative signaling cues, leaving the space between somites as the "path of least resistance" for emerging sprouts. In many other environments, however, these barriers and cues are not obvious. In these scenarios the forward motion may be more of a "trial and error" process whereby the tip cell samples the environment via its filopodia. How a point is chosen for connection and fusion is even less well-understood. Most sprouts eventually find another sprout or a vessel and set up cell junctions with one or more endothelial cells in that structure. As mentioned, the lumen eventually runs through the new connection to allow for new patterns of blood flow. This new stretch of blood vessel may then act as the parent vessel for another round of sprouting, setting up the reiterative nature of the process. Furthermore, the vessel network that forms as a result of sprouting is often remodeled in response to physiological cues such as hypoxia and blood flow, but the initial pattern – and sometimes the final pattern – of a particular vessel network is set up by these elegantly regulated endothelial cell behaviors.

2. Blood vessel sprout initiation

The VEGF-A (vascular endothelial growth factor-A) signaling pathway has been established as a potent and essential regulator of angiogenesis [1,6]. The VEGF-A ligand is expressed by many tissues and is induced by hypoxic conditions [7]. Endothelial cells express the primary VEGF-A signaling receptor, VEGF receptor -2 (VEGFR-2) (called Flk-1 in mouse), a tyrosine kinase receptor that positively drives the mitogenic and chemotactic responses of endothelial cells in response to the VEGF-A ligand. Interestingly, angiogenic spouts are composed of leading cells which are responsive to extrinsic stimuli (i.e. extend multiple filopodia) and neighboring cells that are largely unresponsive in terms of morphogenesis but respond to VEGF-A by dividing. This heterogeneous organization suggests that angiogenic vessels are composed of specialized cells. In the following sections, we discuss the mechanisms that set up and maintain this endothelial heterogeneity.

2.1. Tip cell selection and lateral inhibition

Tip cells are specialized cells that respond to environmental cues to direct the migration and patterning of adjacent stalk cells. Endothelial tip cells can be distinguished from their neighboring stalk cells by the expression of unique markers and extensive filopodia (Fig. 2). They are analogous to the growth cones of axons [8] and to the tracheal tip cells that contribute to Drosophila trachea formation [9] in that these specialized cells use filopodia to sense and respond to extrinsic cues. During Drosophila tracheal development, FGF (Branchless) is a chemoattractant that induces filopodial extensions in tracheal tip cells [9], and Notch signaling appears to regulate tip/stalk cell dynamics by affecting FGFR (Breathless) levels [10]. In vascular development, VEGF-A replaces FGF as the incoming signal.

The Notch signaling pathway is highly conserved [11–13]. There are five DSL (Delta, Serrate, LAG-2) ligands: Delta-like 1 (Dll1), Dll3, Dll4, Jagged-1 (Jag-1), and Jag-2 that bind to four Notch receptors (Notch1–4). The Notch receptors and ligands are all trans-membrane proteins. Consequently, Notch signal transduction requires cell–cell contact. Binding of a DSL ligand to a Notch receptor initiates proteolytic cleavage of the Notch intracellular domain (NICD). The NICD translocates to the nucleus where it co-activates downstream transcriptional targets such as Hairy/Enhancer of Split (Hes), Hes-related proteins (Hey/HRT/HERP), and Notch-regulated ankyrin repeat protein (Nrarp). The Notch signaling pathway utilizes the process of

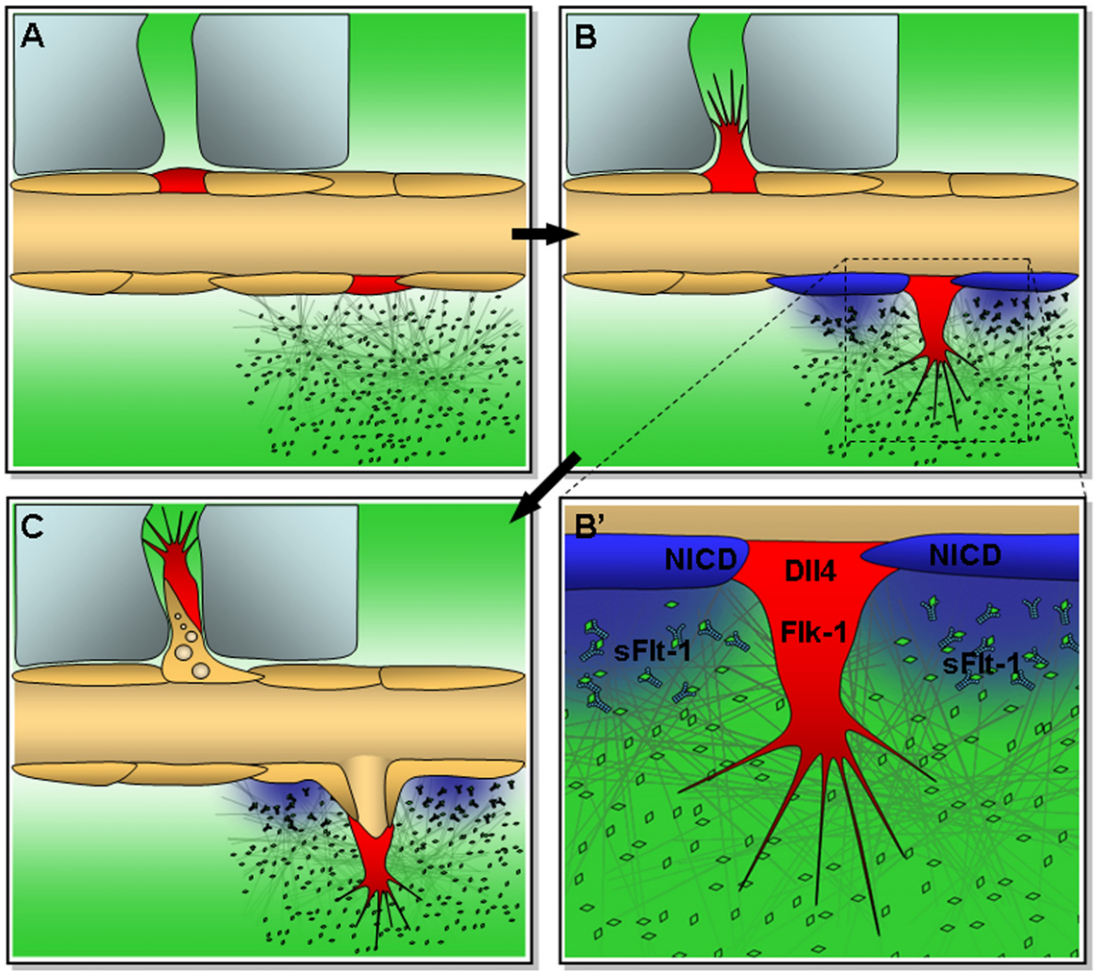

Fig. 2. Overview of stages of blood vessel sprouting. (A) Initiation of blood vessel sprouting occurs when soluble cues such as VEGF (green) "select" one endothelial cell to be the tip cell (red) and lead the outward extension of the sprout. (B) The local sprouting environment contains soluble factors, ECM components, and cell-based guidance cues to facilitate proper guidance of the emerging tip cell. (B′, inset from B) VEGF signaling through VEGFR-2 (Flk-1) increases Dll4 expression in tip cells, which engages with Notch receptors on adjacent lateral base cells (blue) and promotes signaling downstream of Notch intracellular domain (NICD) cleavage, including expression of soluble VEGFR-1 (Flt-1, blue Y's). (C) Strong negative guidance cues often facilitate stereotypical patterning of vessels (upper left sprout), while "free-form" patterning relies on vessel intrinsic guidance cues such as soluble VEGFR-1 (lower right sprout). Like patterning, lumen formation may be context-dependent, occurring via fusion of intracellular vesicles or through maintaining an existing lumen up to the tip cell.

lateral inhibition to regulate biological processes. Lateral inhibition is achieved when a cell expressing the highest levels of ligand activates Notch in the surrounding cells, which often induces in these neighboring cells a particular fate distinct from that induced in the ligand-expressing neighbor [14]. Increasing evidence suggests that during sprouting Notch-mediated lateral inhibition is important not in endothelial cell fate decisions, but in regulating tip and stalk cell phenotypes during angiogenesis. These phenotypes are dynamic and thus not literally cell fates.

The tip cells of angiogenic sprouts can be distinguished from stalk cells by the absence of a lumen, the extension of numerous prominent filopodia [15–17], and heightened expression of Dll4, platelet-derived growth factor (PDGF)-b, UNC5b, VEGFR-2, and Flt-4 [17–20]. Imaging of angiogenic sprouts demonstrates that once a sprout emerges, endothelial cells compete for the tip cell position, highlighting the dynamic nature of the molecular mechanisms regulating tip-stalk cell selection among neighbors [21]. The tip cell presumably experiences higher VEGF signaling than its neighbors, and Notch signaling conveys the status of VEGF signaling among neighboring cells.

Treatment of developing vessel networks with γ-secretase inhibitors such as DAPT, which inhibit Notch signaling by blocking the cleavage of NICD, causes excessive vessel sprouting and branching in zebrafish and leads to the hyperfusion of the capillary networks in mice [22]. Point mutations of Dll4 in zebrafish and haplo-insufficiency of Dll4 in mice abrogate Notch signaling and phenocopy treatment with γ-secretase inhibitors. In addition, antisense morpholinos against Notch signaling factor *Dll4* [20,23], *notch1b* [23], and *rbpja* (recombining binding protein suppressor of hairless) [20] induce excessive branching in zebrafish. Collectively these findings demonstrate that active Notch signaling modulates angiogenesis by inhibiting sprouting and branching.

Increasing evidence suggests that Notch signaling coordinates angiogenesis through transcriptional regulation of multiple angiogenic factors [24]. Neuropilin-1 (Nrp1), a co-receptor of VEGF-A, is negatively regulated by Notch activation [25]. Flt-4, a VEGF-C receptor that is a critical regulator of lymphangiogenesis, is strongly expressed at the vascular front. Blocking Notch signaling leads to widespread Flt-4 expression and excessive tip cell activity, and blocking antibodies against Flt-4 partially restored normal sprouting [20,26]. Notch activation also negatively regulates VEGFR-2 expression [27], and the downstream Notch transcription factor HESR1 (CHF2) can directly repress the VEGFR-2 promoter [28]. In contrast, VEGFR-1 (Flt-1) is positively regulated by Notch signaling [29,30]. VEGFR-1 acts as a competitive inhibitor for the VEGFR-2 receptor [31,32], and its increased expression in stalk cells may

restrict the responsiveness of these endothelial cells to VEGF-A [33,34]. Thus Notch signaling appears to regulate tip cell dynamics through its effects on multiple angiogenic factors.

In addition, Notch signaling is regulated downstream of growth factor signaling pathways. Wnt/β-catenin signaling up-regulates Dll4 transcription and activates Notch signaling in blood vessels. Consequently, β-catenin over-expression resembles Notch over-expression (excessive stalk cell) phenotype, and Wnt disruption resembles the Notch loss-of-function phenotype (excessive tip cells) [35]. VEGF-A signaling also induces Dll4 expression in endothelial cells, demonstrating that Notch mediated angiogenesis involves complex regulatory loops [36–38].

Mosaic analysis was used to determine the role of Notch-mediated lateral inhibition in tip cell-stalk cell dynamics in angiogenic sprouts. Notch1-deficient endothelial cells preferentially adopt tip cell characteristics in mice [22], and cells that over-express constitutive active NICD are excluded from the tip cell position in zebrafish [20]. These findings demonstrate that Notch activation induces the stalk cell phenotype. Meanwhile the absence of Notch signaling results in the tip cell phenotype, suggesting that the tip cell phenotype is the default state of angiogenic endothelial cells. Cells with low Notch activity have high VEGFR-2 and low VEGFR-1 levels and preferentially become tip cells [21].

2.2. Effects of blood flow on vessel sprouting

Hemodynamic forces play a critical role in the maturation and patterning of vascular beds, but the relationship between sprouting and blood flow is complex [39,40]. For example, the remodeling of the mouse yolk sac from a honeycomb-like plexus into a hierarchical vascular network temporally coincides with the initiation of circulation and the cessation of sprouting [41]. Embryos without circulation or lacking erythroblasts in circulation fail to remodel, and restoration of blood viscosity rescues vessel remodeling, demonstrating that the hemodynamic forces mediate yolk sac remodeling [42]. The remodeling of the aortic arch is also dependent on hemodynamic forces, but in this case these forces are required for proper sprouting [43]. Flow induces the mechanosensitive zinc finger transcription factor *klf2a* in zebrafish. *klf2a* induces the expression of *mir-126* which positively regulates VEGF signaling, and mediates the angiogenic sprouting of aortic arch vessels [44].

3. Vessel sprout guidance

Following sprout initiation, the leading tip cell likely utilizes multiple near-field guidance cues to establish a trajectory outward. As the sprout continues extending outward, long-range molecular factors likely instruct vessel trajectory for fusion and eventual branch formation, and they may also stabilize the sprout or induce regression. These short- and long-range guidance cues likely contribute to vessel patterning differentially depending on the tissue bed, as some regions of the vasculature pattern in a highly stereotypical manner while other regions exhibit more “freely-formed” patterning (Fig. 2). We discuss these aspects of sprout guidance and vascular patterning in the following sections.

3.1. Local sprout guidance cues

Among the most important of guidance cues for endothelial sprouts is VEGF-A [45]. Alternative splicing yields three primary VEGF-A isoforms, each with unique extracellular matrix (ECM) binding affinities based on the presence or absence of heparin-binding domains [46]. This variable affinity for the ECM results in the proper spatial distribution of VEGF-A and thus provides important vessel patterning cues that are lost when VEGF-A isoforms are genetically perturbed [16,47]. We have recently found evidence for further refinement of local VEGF-A gradients through increased expression of soluble VEGFR-1 (sVEGFR-1) by endothelial cells adjacent to a nascent sprout [33]. These localized counter-gradients of sVEGFR-1 reduce the availability of VEGF-A in the regions adjacent to the sprout and create a more directed vector of VEGF-A to properly guide the sprout away from the parent vessel, a behavior that is disrupted when the lateral base cells cannot express sVEGFR-1 (Fig. 2). Near-field gradients of available VEGF-A might also be reinforced through the release of matrix-bound VEGF-A by protease cleavage [48,49] or endothelial VEGF-A production [50,51]. In contrast, VEGF-A retained by the ECM likely enhances sprout guidance, resulting in more productive branch formation [52,53]. Thus, a number of mechanisms positively and negatively regulate the spatial presentation of VEGF-A for the proper guidance of endothelial sprouts and vessel morphogenesis.

Explorative filopodia extend from an emerging tip cell, and they may be enriched in VEGFR-2 to detect the chemotactic VEGF-A gradients described above [17]. In addition, the filopodial surface presents integrins such as $\alpha_1\beta_1$, $\alpha_2\beta_1$, and the α_v integrins to engage binding sites within the ECM and facilitate migration along the scaffold [54,55]. In some tissues, sprouting endothelial cells likely directly interact with other cell types in close proximity to the sprout initiation site. For example, developing zebrafish intersegmental vessel sprouts interact and migrate between the trunk somites [56,57], and mouse retinal vessel tip cells migrate along the underlying astrocyte network [17,58]. Overall, an emerging sprout integrates information from local guidance cues including soluble factors, ECM components, and cell–cell contacts, to initiate and maintain a proper outward trajectory away from a parent vessel.

3.2. Vessel sprout extension and stability

Moving beyond the local micro-environment, a vessel sprout is likely guided by longer-range patterning cues that also affect sprout stability. Attractive and repulsive signals can come from cell–cell interactions located at a distance from the sprout initiation site. For example, recognition of similarities between endothelial tip cells and axonal growth cones has grown in recent years, and guidance cues that pattern growing nerve fibers also attract and repel endothelial sprouts [59,60]. Four classes of axon guidance cues have emerged as important regulators of blood vessel patterning: Ephrin-Eph, Slit-Robo, Netrin-UNC, and Semaphorin–Plexin–Neuropilin [60]. For example, endothelial expression of the Netrin receptor UNC5b provides repulsive signaling that prevents aberrant extension of vessel sprouts into the developing somites of mice and zebrafish [19]. In contrast, an axonal guidance molecule that provides an attractive guidance cue in angiogenic sprouting is the VEGF-A co-receptor Neuropilin-1 (Nrp-1). Perturbed Nrp-1 activity impairs directional migration of endothelial tip cells, perhaps by disrupting the binding of ECM-sequestered VEGF-A and the formation of a signaling complex with VEGFR-2 [61,62]. Other cues may pattern via induction of apoptosis; for example, macrophage Wnt7b is involved in regression of the hyaloid vessels that initially surround the developing eye [63]. Thus, both attractive and repulsive cues likely coordinate with growth factor signaling pathways to regulate sprout stability and reinforce a growing sprout or induce its retraction.

Notch-Delta signaling may also help ensure proper vessel guidance by longer range cues, as the tip cell may be replaced by a trailing stalk cell, via a Notch-mediated process, if this leading cell becomes misdirected and encounters lower VEGF-A concentrations [21]. This mechanism may also contribute to sprout regression, resulting in empty sleeves of ECM as seen in tumors following VEGF inhibition [64]. A poorly guided tip cell experiencing decreased VEGF signaling may in turn receive increased lateral inhibition

signals from its neighbors, causing the sprout to retract and re-engage in the competition for the tip cell position. However, in some models, such as the developing mouse retina, Notch inhibition leads to hyper-sprouting but not necessarily to an obvious loss of vessel guidance, suggesting differential regulation of sprout initiation and guidance by the Notch pathway [22,38]. Thus, Notch signaling may integrate growth factor signaling with other guidance cues to help redirect a straying sprout or even induce its regression and ensure proper vessel morphogenesis.

3.3. Stereotypical vs. free-form vascular patterning

The spatial organization of sprout guidance factors within certain tissues yields highly stereotypical blood vessel networks, while other tissues lack obvious guidance cues and the vessel network appears to be more freely formed (Fig. 2). An example of stereotyped vessel patterning is found in the intersomitic vasculature. Intersomitic vessels, such as the intersegmental vessels of the zebrafish, are constrained in their response to positive guidance cues such as VEGF-A by the physical barrier provided by the somite tissue and by repulsive cues generated by the somites [57,65–67]. In contrast, "free-form" vessel patterning occurs in tissues lacking obvious chemo-attractant gradients. In the developing mouse yolk sac, for instance, VEGF-A is secreted by the endoderm and mesoderm, and formation and patterning of yolk sac vessels is impaired when endoderm expression of VEGF-A is lost [68]. Blood vessel expansion in the developing yolk sac lateral plane occurs initially through angiogenic sprouting, and the onset of blood flow subsequently induces extensive vessel remodeling [41]. The initial yolk sac vessels are evenly spaced and appear to have comparably sized lumens, yet little is known as to the mechanisms regulating this architecture. Notch signaling has been implicated in regulating yolk sac vessel formation [69,70], but it is precise role remains unclear. Negative cues from the endothelium, such as soluble VEGFR-1, may refine localized gradients of available VEGF-A to facilitate proper vessel sprout guidance and may also contribute to patterning this well-branched plexus.

4. Sprout maturation into a vascular branch

The fusion of a tip cell with a target vessel or sprout is an essential step in the formation of a new vessel segment. After the anastamosis of two vessels occurs, this nascent branch acquires a lumen to facilitate the flow of blood. These resolution phases of vessel sprouting are currently not well understood, but recent observations shed some light on the mechanisms underlying sprout fusion and lumen formation.

4.1. Blood vessel sprout fusion

A properly guided, stable sprout begins the transformation into a nascent vascular branch by fusing with an existing vessel or sprout. Fixed image analysis and computational modeling of endothelial tip cells in the developing mouse retina suggests that interactions between filopodia from two approaching cells initiates the formation of a junction [71]. Consistent with these data, dynamic imaging of sprouting endothelial cells in developing ES cell-derived vessels has revealed that, as a tip cell approaches a potential fusion site, the target cell extends filopodial protrusions that appear to engage filopodia from the sprouting tip cell [Chappell JC, Bautch VL, unpublished observations]. In this way, these cells presumably establish and reinforce their connection via increased cell–cell junctions [58,72]. Alternatively, failure to strengthen junctional contacts may lead to repulsion of the sprouting tip cell, diverting the sprout to another destination or inducing retraction. For example, tip cells in zebrafish intersegmental vessels that lack proper Notch signaling remain highly motile and thus do not form proper connections with the dorsal longitudinal anastomotic vessel (DLAV) [23]. Recent evidence from Ruhrberg and co-workers suggests that sprout fusion may be regulated further by embryonic macrophages that bridge the connection between a sprouting endothelial cell and its target [73]. Loss of macrophage activity perturbed branching complexity of blood vessels in the mouse embryonic hindbrain and postnatal retina, and macrophages were observed at presumptive tip cell fusion sites in the developing vasculature of mice and zebrafish. In contrast, Stefater et al. have recently identified a role for myeloid cells in repelling growing sprouts to pattern the retinal deep vascular layer, suggesting that macrophages have distinct roles at different phases of blood vessel formation [74]. Sprout fusion may therefore result from filopodia interactions and adhesions, and this increased cell–cell contact potentially enhances Notch signaling to reduce tip cell motility and stabilize the connection for further maturation. However, how the partner is recognized and chosen for fusion, especially in the "free-form" vessel patterning described above, is unknown.

4.2. Lumen formation

The vascular lumen must expand through a stably connected sprout so that blood can flow through the new branch [75]. Several recently published studies on lumen formation in developing vessels suggest that mechanisms governing this process are likely tissue-specific. In the mouse retina, the lumen extends to just behind the tip cell as the sprout is migrating outward [17]. Thus pressure from the blood or other unidentified mechanisms maintain lumen patency up to the tip cell so that primarily the fused tip cells undergo changes to form new luminal connections. Lammert and co-workers demonstrated that in the developing mouse aorta endothelial cells polarize and set up cell shape changes that result in lumen formation [76]. Cleaver and co-workers showed that perturbation of a Rho activator prevented lumenization of all vessels, suggesting that polarized shape changes may underlie vessel lumenization more globally [77]. Additionally, an investigation of the sialic acids found on vessel apical surface glycoproteins showed that loss of the negative charge impairs luminal expansion, suggesting that electrostatic repulsion normally acts to force apart adjacent cells and expand the lumen [78]. Alternatively, vessels formed in 3D collagen gels *in vitro* acquired lumens through the formation and fusion of intracellular and intercellular vesicles or vacuoles, while zebrafish intersegmental vessels have complex cell–cell interactions and lumenization patterns [79,80]. While these studies have begun to shed light on potential mechanisms underlying vascular lumen formation, our knowledge of these mechanisms remains incomplete, especially with regard to endothelial polarity cues and their role in establishing a patent lumen.

5. Conclusions and perspectives

While many gaps still remain in our understanding of blood vessel formation, we have made great strides in our knowledge of blood vessel sprouting, and the molecular regulation in both space and time that co-ordinates the endothelial cell behaviors involved in this process. The concept of phenotypic heterogeneity among endothelial cells of developing vessels, such that some endothelial cells becoming tip cells and others stalk cells, based on models proposed for tracheal development in the fly [10] and described in blood vessels by Gerhardt and Betsholtz [17], has allowed for a detailed molecular dissection of the cross-talk involved in establishing these endothelial phenotypes. We now know that, in addition to VEGF signaling, endothelial cross-talk via Notch–Delta–Jagged signaling is critical to proper vessel sprouting.

We are beginning to appreciate that other signals, such as BMP and Wnt, are also involved in regulation of vessel sprouting.

There are still many open questions and areas where improving technologies will lead to new insights. One important question regards the spatial organization of signals and responses – our ability to place signals and pathway readouts in a spatial grid is primitive. Once signals leave the source cell they are very difficult to track in biological systems, and reporter readouts of pathway activity are often insensitive. Moreover, our ability to document such events in time is even more primitive, so that most spatial information is gleaned from fixed images, and dynamic changes are extrapolated. As the next generation of imaging tools comes on line, we should be able to obtain a much more accurate picture of important dynamic events in vessel sprouting.

We know very little regarding how different cellular processes are coordinated as vessels sprout. For example, while endothelial cell polarity is clearly important for the proper formation and function of vessels, we are just beginning to understand how and when polarity is initially set up. There are numerous open questions. Is polarity achieved via distinct mechanisms in different vessel types and places? How is polarity modified and re-established as new sprouts form? How is apical-basolateral polarity integrated with planar cell polarity in developing vessels? The answers to these questions will provide exciting new insights into blood vessel sprouting.

The regulation of the resolution phases of vessel sprouting is still a black box. We do not understand how sprouts find a partner for fusion, and how the fusion with other sprouts or vessels and formation of contiguous lumenized vessels occurs. As with the tip cell concept, in this arena paradigms first described in the fly trachea are providing templates for investigations of these events in developing vessel networks.

Finally, we do not know how general or tissue-specific are the paradigms of vessel sprouting. Blood vessels sprout in many different environments and situations during the course of development. This suggests that the process is robust, which implies that there may be multiple ways to initiate and regulate sprouting. This is especially important to keep in mind because most of the recent work has utilized a limited number of models, including the post-natal mouse retina and intersegmental vessel formation in zebrafish. Our recent finding that sprouting from the caudal vein of the zebrafish requires BMP and not VEGF-A [81] suggests that there may be multiple ways to make a sprout. This is a good thing, since blood vessel sprouting is necessary for development and function of all vertebrate organisms.

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