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Tubulogenesis during blood vessel formation

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Abstract

The ability to form and maintain a functional system of contiguous hollow tubes is a critical feature of vascular endothelial cells (ECs). Lumen formation, or tubulogenesis, occurs in blood vessels during both vasculogenesis and angiogenesis in the embryo. Formation of vascular lumens takes place prior to the establishment of blood flow and to vascular remodeling which results in a characteristic hierarchical vessel organization. While epithelial lumen formation has received intense attention in past decades, more recent work has only just begun to elucidate the mechanisms controlling the initiation and morphogenesis of endothelial lumens. Studies using *in vitro* and *in vivo* models, including zebrafish and mammals, are beginning to paint an emerging picture of how blood vessels establish their characteristic morphology and become patent. In this chapter, we review and discuss the molecular and cellular mechanisms driving the formation of vascular tubes, primarily *in vivo*, and we compare and contrast proposed models for blood vessel lumen formation.

Keywords

blood vessel; lumen; endothelial cell; adhesion; cell polarity; vacuole; vasculogenesis; angiogenesis; cord hollowing; sialomucin; Rasip1; NMHCIIA; Arhgap29

1. Introduction

The cardiovascular system is the first functional organ system to form in developing embryos across all vertebrate species, providing tissues with nutrient and gas exchange required for life. Formation of a cohesive, seamless and contiguous network of blood vessels to carry blood is therefore essential for proper cardiovascular function. While a growing understanding is emerging regarding the specification, patterning and sprouting of blood vessels, data still lags regarding the cellular morphogenesis and molecular pathways that direct formation of vascular ‘tubes’.

1.1 Blood vessel lumen formation and tubulogenesis

The initial, fundamental building block of the vascular system is termed the *angioblast*, or endothelial progenitor cell, and the first blood vessels to form in the embryo arise via

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vasculogenesis. Angioblasts are mesodermally-derived cells that emerge following gastrulation as scattered cells in both extraembryonic yolk sac blood islands [1] and within embryonic tissues [2]. During vasculogenesis, the initial vascular plexus is formed as angioblasts rapidly organize and coalesce to form solid cords, at distinct embryonic locations. These lumenless, linear aggregates must then transform into tubes so that they may carry blood.

During 'tubulogenesis', angioblasts transition from a cuboidal to a flattened shape, and they rearrange their junctional contact points to the cord periphery, as they mature into endothelial cells (ECs) which line central lumens [3]. Later, as embryonic tissues develop and become increasingly complex, new blood vessels sprout from pre-existing vessels of the primary plexus via *sprouting angiogenesis* [4]. Sprouting extends existing lumens within these new angiogenic vessels and expands the tubular network. In addition, when vessel networks acquire their recognizable hierarchical arrangement of large and small vessels via *remodeling angiogenesis*, lumens either expand or shrink in concert with the changing vasculature. The final cardiovascular system in the adult thus emerges from a rather astounding array of varied and divergent cellular mechanisms.

1.2 Vasculogenic tubulogenesis: 'cord-to-tube' transition into de novo lumens

Not surprisingly, vasculogenesis in mammalian embryos precedes both the initiation of heart beating and the formation of all other organs. It represents a first and critical step in a coordinated series of events that together drive vascular morphogenesis, ensuring that all organs acquire their proper vasculature as they form and grow. Initially, angioblasts emerge in the mesoderm throughout embryonic tissues. These specialized progenitor cells migrate and coalesce to form a primitive plexus of solid vascular "cords", sandwiched at the endodermal-mesodermal interface. This initial vascular network resembles a fisherman's net, with rope-like vessels that are for the most part relatively homogeneous in shape and size. Shortly thereafter, endothelial lumens appear at the heart of each solid cord, transforming them into functional tubes (Figure 1A). This morphogenetic process is rapid and remains poorly understood.

Strictly speaking, vasculogenic lumen formation and 'tubulogenesis' can actually be considered separable events. While lumen formation is a local event, involving a small number of cells opening up a space between them, tubulogenesis is the formation of a continuous lumen along the entire length of a vessel. Formation of continuous lumens is inherently required for blood circulation and cardiovascular function, both critical for tissue growth and viability.

The transformation of solid EC cords into 'canalized' vascular tubes has been the focus of growing interest. As different aspects of blood vessel formation become unraveled, new questions arise and preoccupy vascular developmental biologists. How are cells changing to form vascular lumens *de novo* at the heart of newly formed cords? Where do the forces that generate a central lumen come from, during this process? From within cells, or from surrounding tissues? Is anything pumped into the growing cavities prior to blood flow to keep them from collapsing? How and why do angioblasts 'thin' out as they become ECs, and contribute to the cylindrical vascular structure? These questions regarding fundamental cellular behaviors during vasculogenic and angiogenic lumen formation, as well as their molecular underpinnings, remain largely unexplored but are beginning to attract attention [5–7].

1.3 Angiogenic tubulogenesis: lumen extension

Similar to *de novo* lumen and tube formation in initial embryonic vessels, lumens must also form in new angiogenic sprouts. However, the mechanisms are likely to differ to some extent, as initial vessels start as solid cords with no connections to existing lumens, while angiogenic vessels grow from pre-existing tubes, with pre-existing lumens (Figure 1B). Much work has recently focused on sprout formation and the role of VEGF and Notch signaling during this process. The distal growing end of new angiogenic vessels consists of endothelial 'tip' cells, which extend long filopodia and migrate in a manner similar to axonal growth cones, sensing their microenvironment. The proximal end of these vessels, by contrast, consists of 'stalk' cells that initially form a trailing cord but often quickly becomes lumenized [8, 9]. The process that controls tip cell growth is dependent on a remarkable interplay of selective Notch ligand activation, as well as the establishment of well-controlled VEGF gradients [10]. To date, however, relatively little attention has been focused on the timing, mechanics or molecular regulation of lumen formation within stalk cells. Comparing and contrasting lumen formation in different types of vessels will reveal whether similar mechanisms apply to all vessels or whether locally distinct modes of lumen formation reflect inherent heterogeneity of vessels.

2. Cellular mechanisms of vascular tubulogenesis

As a tubular organ, the endothelial vasculature shares a lot of common features with epithelial tubes found in a number of other organs, such as lung, kidney, salivary glands and pancreas. A considerable amount of work has been carried out to elucidate epithelial tubulogenesis during the past several decades. Epithelial tubes are generally composed of a sheath of cuboidal or columnar epithelial cells, with defined apical membranes facing a central lumen, lateral edges interfacing with each other closely, and basal membranes making up the tube periphery (Figure 2A). Similarly, ECs of functional vessels consist of a luminal (apical) membrane facing the flowing blood and an abluminal (basal) membrane in contact with the basement membrane. The principal difference between epithelial and endothelial tubes is the smaller contact area comprising the junctional contacts (lateral membranes) between ECs (Figure 2B).

Nonetheless, a number of analogies between the two tissues can be made. Most epithelial tubulogenesis processes fall into one, or a combination of several, of the following categories: wrapping (i.e. vertebrate primary neurulation), budding (i.e. lung), mesenchymal-to-epithelial transition or MET (i.e. kidney tubules), cell hollowing (i.e. Drosophila trachea), cord hollowing (i.e. zebrafish gut), cavitation (i.e. salivary gland), and cell division and intercalation (i.e. zebrafish neural tube) [11–14]. While MET reflects the overall process that angioblasts must accomplish to aggregate and form tubes, at least three additional mechanisms have been implicated in the later stages of endothelial lumen formation: budding, cell and cord hollowing [6].

2.1 Budding

By definition, *budding* consists of formation and extension of a tube via growth from a pre-existing epithelium. During lung development, for instance, the pulmonary epithelium repeatedly buds and extends finger-like projections, until it becomes a contiguous, highly branched, ramifying, tubular tree. During blood vessel formation, sprouting angiogenesis is essentially synonymous with budding (Figure 1B). ECs along the wall of a blood vessel become locally activated, degrade the surrounding basement membrane, extend filopodia, change shape and migrate out, with tip cells at the leading front of the growing vessel, invading surrounding tissue. As angiogenic sprouting gives rise to patent vessels, it can thereby be considered one mechanism for lumen formation. However, what processes

specifically drive stalk cells to organize, extend and maintain the parental lumen remain unclear.

2.2 Cell hollowing: vacuole fusion

In contrast to budding where new lumens extend directly from pre-existing lumens, *cell hollowing* represents a different cellular mechanism whereby new lumens emerge intracellularly. In this case, lumens initiate as multiple small vesicles or larger vacuoles, that fuse to produce a central lumen, which in turn becomes connected with similar lumens in adjacent cells (Figure 3A **and** Figure 4A). This type of lumen formation has been observed in Drosophila terminal tracheal cells [15, 16], as well as in ECs. Indeed, until recently vascular lumen formation had primarily been studied *in vitro*, and a large body of work using live imaging of ECs in 3D matrices has demonstrated that intraendothelial lumen formation occurs by cell hollowing via vacuole fusion [6, 17, 18]. Vacuole fusion has also been observed *in vivo*, during formation of intersegmental vessels in zebrafish [19]. In these studies, live imaging of quantum dots injected intravascularly into circulating blood demonstrates intercellular fusion of vacuoles during ISV morphogenesis. Together, these observations suggested that cell hollowing is an important mechanism in both endothelial and non-endothelial tubulogenesis.

2.3 Cord hollowing: vacuole fusion and cell rearrangement

A third type of mechanism by which some lumens form is termed *cord hollowing*. In this process, lumenal space is generated extracellularly between ECs, even as they remain joined peripherally (Figure 3B). Expansion of apical membranes to create an intervening extracellular space could be achieved either by addition of new lumenal membrane (Figure 4A), or by removal or clearance of junctions from the cord center. The result of either process is to build up the net surface area of the lumenal membrane.

During lumen formation, addition of new lumenal membrane has been to shown in some cases to occur via directed exocytosis of vesicles, which fuse with and expand the lumen at the cord center. This type of vectorial vesicle/vacuole fusion has been observed in cultured MDCK epithelial cells [12, 20, 21], and has also been shown in ECs and suggested to be dependent on Rab7 [3] (Figure 4B). Indeed, an interesting link was made between vectorial transport, cell adhesion and cell polarity in the ECs of arterioles in late gestation mouse embryos. In these vessels, loss of β1 integrin disrupted Par3 localization resulting in accumulation of excess $Rab7^+$ cytosolic vesicles and failure of vascular lumen formation. This finding reflects the need for EC polarity during lumen formation, as ECs must be able to define and/or determine the ‘inside’ from the ‘outside’ of the cord to correctly target vectorial transport of vesicles/vacuoles.

Another possible mechanism for creating extracellular space between ECs during lumen formation is clearance of adhesion at the cell center. This can be accomplished either by de-adhesion at the apical/luminal membrane, and/or by redistribution of junctional molecules away from the cord center. In the former case, cells within a cord de-adhere from each other locally at the luminal membrane, but remain tethered at the lumen periphery. This differential control of adhesion thereby alters EC shape and rearranges ECs relative to each other. Control of de-adhesion, with precisely controlled regional removal of junctions at the cord center, is likely to be a highly regulated process. In the latter case, ECs actively redistribute existing junctions to the periphery, away from the lumen. Such junctional redistribution has been observed in the gut epithelium of zebrafish [22] and more recently during vasculogenesis in mouse [23] (Figure 4C).

To date no definitive experimental evidence has clearly distinguished endothelial lumenal membrane expansion (via centripetal vesicle/vacuole fusion) versus either de-adhesion or clearance of junctions during vascular tubulogenesis. It is likely that a number of cellular phenomena occur coordinately during this process. Indeed, in late mouse arterioles, both directed Rab7-directed vesicle transport to the lumenal membrane and junctional redistribution (away from the center) occur concurrently [3]. It will be of great interest to assess whether tandem mechanisms apply more globally to forming vessels.

2.4. Vascular tubulogenesis: mechanistic heterogeneity

As the number of studies on vascular tubulogenesis has increased, our understanding of the variety of underlying mechanisms has evolved [7, 11, 24]. Until recently, cell hollowing was considered a common, if not predominant, mode of *de novo* endothelial lumen formation. However, additional mechanisms have since been identified. Here, we cover a few divergent examples.

Clear live imaging of ECs cultured in 3D matrices provided strong support for vacuole-based lumen formation [17]. Cells were shown to generate intracellular vacuoles that would align at the cell center and fuse with each other to create lumens. Similarly, vacuole fusion has been observed in the growing vessels of vertebrate embryos [19, 25–27] (Figure 4A). Live imaging of growing zebrafish intersegmental vessels (ISVs) identified fusing vacuoles during lumen formation [19]. This high resolution study examined the development of ISVs, which were thought to assemble stereotypically with three ECs in a head-to-tail cord along myotomal boundaries [28]. Two photon live cell imaging identified vacuoles during angiogenic sprouting and suggested intra-cellular fusion of endothelial vacuoles at the center of ISV ECs suggesting cell hollowing.

More recent ISV studies have confirmed vacuole formation and fusion as a basis for ISV lumen formation [26]. However, rather than intra-cellular vacuole fusion, they observed inter-cellular fusion in ISVs. Careful examination of membrane fusion events, using apical markers and injected dyes to outline functional lumens, revealed that vacuoles were fusing with and establishing the luminal membrane, ‘between’ rather than ‘inside’ cells, suggesting cord hollowing rather than cell hollowing. In addition, this study identified a requirement for the apical ERM domain protein moesin-1 and adherens junctions during this process. Cord hollowing was further supported by a recent study which examined tight junctions and adherens junctions in zebrafish ECs [27]. Adjacent ISV ECs were observed to share large cell-cell junctional contacts, and lumens were shown to form extracellularly, between closely apposed cells. Tools and fish lines developed for these studies will undoubtedly continue to refine our understanding of angiogenic lumen formation in the future.

One strikingly unique example of a divergent mechanism of vascular lumen formation involves aortal and caudal vein lumen morphogenesis in zebrafish during arteriovenous segregation [29] (Figure 4D). High-resolution live imaging of zebrafish vascular morphogenesis showed that the primary axial dorsal aorta (artery) arises first by vasculogenic aggregation of angioblasts. Secondarily, the caudal vein emerges via selective ventral sprouting and migration of angioblasts away from the dorsal aorta, a process they show to be regulated by EphrinB2-EphB4 signaling. Interestingly, the caudal lumen forms via angioblasts aggregating into a partially formed and dorsally ‘open’ vessel, which then rapidly fills with blood accumulated at the interface of the two vessels. Upon this ‘filling’ of the caudal vein, functional blood circulation is then rapidly established. The cellular mechanisms underlying this ‘open’ vessel formation remain to be examined, but support the idea that a variety of mechanisms underlie vascular tubulogenesis in different vessels.

Yet another unique mechanism for vessel lumen formation was reported during Drosophila heart morphogenesis [30, 31] (Figure 4E). In flies, the heart represents an open-ended and contractile endolymph 'vessel' of sorts, with tubular morphology and a distinct internal lumen. In these studies, formation of the cardiovascular lumen by two parallel rows of myoendothelial cells is shown to be modulated by membrane repulsion. Slit-Robo signaling downregulates E-cadherin at the lumenal cell-surface and prevents fusion between apposed cells. Instead, the two cells form junctions only at their dorsal- and then ventral-most regions, resulting in the formation of an internal central lumen, enclosed by two rows of "C-shaped" cells. Even more interestingly, and distinct from typical epithelial or endothelial tubulogenesis, the surface that lines the inner lumen in the fly heart lumen is the basal rather than apical cell surface. This difference suggests that fundamental differences can occur between different types of tube forming cells with respect to cell polarity features.

Similar apical/luminal surface repulsion has also been suggested during mouse dorsal aortae formation. Following cord hollowing, observations by Lammert and colleagues showed that accumulation of negatively charged sialomucins along apposed lumenal membranes results in the initial opening of slit-like spaces between ECs [32, 33] (Figure 4F). Specifically, they show that sialic acids of lumenal glycoproteins create repulsive electrostatic fields that result in membranes moving away from each other at the cord center. Neutralizing these charges with injection of cationic protamine sulfates inhibits normal lumen formation.

Taken together, these divergent examples of lumen formation mechanisms suggest that different blood vessels form via a range of different cellular mechanisms. It is not completely unexpected, as endothelium is known to display a high level of heterogeneity across different tissues [34]. We propose that distinct or even yet to be discovered 'novel' mechanisms of lumen formation will likely be identified as vascular beds of different organs are more extensively examined and understood.

2.5. Cord hollowing example: Embryonic dorsal aortae

Cord hollowing has recently been demonstrated and thoroughly characterized during murine dorsal aortae lumen formation [23, 33] (Figures 4C and 4F). These cellular events are striking in that they constitute the first embryonic vasculogenic event, occurring prior to heart beating or hemodynamic flow [23, 33]. Aortic cords form as early as the 1 somite stage (1S), as 2 or 3 adjacent ECs contact each other and adhere along their entire interface. ECs at this stage are cuboidal and plump. Around 1-2S, junctions are cleared from the central region of the cord and EC luminal membranes de-adhere from each other. ECs then bend, forming a slit-like space between them. At 2-3S, the slit enlarges, eventually forming a lumen encircled by 3–4 more flattened ECs. From this time onward, the aortic lumens continue to enlarge and ECs continue to flatten and proliferate. By 8S, the aortic lumens are quite large and between 6–8 cells make up the aortic vessel circumference.

Cord hollowing appears to take place in a similar manner in the single midline aorta of zebrafish [35]. However chick aortae display large vacuoles suggesting possible cell hollowing [36]. Studies of aortae formation in additional species, and comparison with other vessel types, will determine the extent of the use of cord versus cell hollowing. It will also be interesting to establish whether this mechanism is distinct to early vasculogenic vessel formation, or whether it will prove more universal and be observed in additional vessel types.

2.6. Relationship of vascular tubes with surrounding microenvironment

One open question is the influence of surrounding cells on blood vessel tubulogenesis. An interesting relationship between ECs and surrounding mesenchyme has been recently noted

in murine aortae, where ECs are tightly associated with the underlying endodermal epithelium and encased in the overlying paraxial mesoderm [23]. In one model of vascular tubulogenesis failure, Rasip1 null embryos, aortic vessels never transition from cords to tubes, and interestingly, the mesoderm surrounding the failed aortic cords appears to autonomously 'open' a cavity at the location where aortic vessels would normally have formed. This suggests an active contribution by the mesoderm in providing support to ECs and perhaps 'pulling open' aortic lumens.

An alternative explanation for the open mesodermal 'space' is that ECs may digest surrounding mesenchyme-derived ECM to clear space for tube growth and expansion. This idea is supported by observations in an *in vitro* 3D system, where ECs cultured in a collagen matrix digest canals via membrane bound matrix metalloproteinase (MMP) dependent proteolysis [37, 38]. ECs subsequently flatten against the walls of these canals and organize into vessel-like tubes, assuming a characteristic cobblestone appearance along the wall of these spaces through EC-matrix contacts [39]. The EC-generated physical spaces, or 'guidance tunnels' within the 3D ECM thus facilitate lumen and tube network formation [5, 6]. Additional studies however will be required to elucidate the reciprocal influence of ECs and surrounding cells or matrix *in vivo*, during vasculogenesis and vascular tubulogenesis.

3. Molecular mechanisms of vascular tubulogenesis

3.1. Cell-cell junctional molecules

Like epithelial cells, ECs interconnect to form cell-lined networks of tubes. In both tissues, cells adhere via adherens junctions (AJ) and tight junctions (TJ). These junctional molecules are specialized transmembrane and intracellular protein complexes that promote homophilic interactions and tether cells to each other in a zipper-like manner [40]. Proper regulation of cell junctions, including adhesion and de-adhesion, is critical for proper vascular lumen formation. One principle difference between epithelial and endothelial junctions however is that the interface of adjacent ECs is much narrower than epithelial cells, with small 'lateral' or junctional surfaces (Figure 6). While smooth muscle and adventitial coverage supports vessels as they mature and withstand sometimes high pressure loads, it is nonetheless remarkable that these relatively narrow interfaces maintain tight control over permeability and extravasation of immune cells.

3.1.1. Adherens junctions (AJ)—The primary components of adherens junctions (AJs) are members of the cadherin and catenin families. Cadherins mediate homophilic transmembrane adhesion via multimeric complexes at cell borders, while catenins link cadherins to the actin cytoskeleton. A few cadherins show strong enrichment in ECs.

Vascular endothelial cadherin (VE-Cadherin) is the principal endothelial-specific AJ molecule present across vessels in all vascular beds (Figure 7A). Expression in developing mouse vessels is observed in angioblasts, as early as E7.5, and throughout embryogenesis to adulthood. Loss of VE-Cadherin in mice results in embryonic lethality at E9.5 due to cardiovascular failure. Specifically, although the initial plexus was relatively normal, vessel remodeling and maturation were blocked. In addition, vascular lumen defects were observed as early as E8.5, including both lumenless (thoracic dorsal aortae, anterior cardinal vein) and dilated vessels (cephalic vessels) in different vascular beds [41, 42]. Recently work has also demonstrated that VE-Cadherin is critical for maintenance of proper EC polarization [33], suggesting another link between polarity and lumen formation.

Interestingly, another adhesion component, N-Cadherin, has been observed both in endothelial cell-cell AJs, but also along the abluminal surface of vessels [43, 44]. This peripheral N-Cadherin expression is likely responsible for tethering ECs to ECM. Along

those lines, N-Cadherin has been shown to enhance vessel stability by promoting EC adhesion to surrounding mesenchyme and mural cells, and has been shown to be required for proper lumen formation [39, 43]. Endothelial deletion of N-Cadherin leads to embryonic lethality and mutant embryos display severe vascular remodeling defects, including smaller lumens in the dorsal aortae [43]. An interesting observation is that N-Cadherin regulates VE-Cadherin levels, bringing up the possibility that it indirectly regulates cell-cell adhesion. Therefore, given this regulatory relationship and the fact that N-Cadherin also regulates EC adhesion to mural cells, it remains to be determined which failure is causative of lumen defects.

3.1.2. Tight junctions—Tight junctions (TJs) constitute the most closely associated points between adjacent cells, forming a continuous seal between cells and creating a relatively impermeable barrier. TJs generally consist of claudins, occludins and junctional adhesion molecules (JAMs), with ‘endothelial cell selective adhesion molecule (ESAM) being particularly enriched in EC-EC TJs. Claudin-5 is also specific to endothelial TJs (Figure 7B). In addition, there are many intracellular components associated with TJs, including zona occludens 1 and 2 (ZO-1 and ZO-2) (Figure 7B), afadin (or AF6) and the cell polarity determinant Par3 (Figure 7J). However, while TJs are critical components of EC junctions, none to date have proved explicitly required for the building of vascular lumen formation [7].

Interestingly, many of the same TJ molecules are used by both endothelial and epithelial cells. However, while TJs are located at the apical aspect of columnar and cuboidal epithelial cell junctions (Figure 2A), clearly separating apicolateral from basolateral regions of the cells, in ECs they are confined to a more compressed interface (Figure 2B, 7B and 7C). Indeed, the flattened and elongated morphology of ECs means that TJs and AJs appear to singlehandedly separate luminal (apical) from abluminal (basal) membranes, and thus cannot serve as reliable markers of either. However, the contact area is in fact more extensive than it appears (Figure 7L), with large flat areas often overlapping and covering each other and adhering via a mixture of TJs and AJs intermingled with other adhesion molecules [40].

3.2. Lumenal de-adhesion molecules: apical sialomucins

A key process for extracellular lumen formation during cord hollowing is de-adhesion of ECs at the cord center. Recent work shows that this process is mediated in part by apical/luminal delivery of CD34-sialomucin containing vesicles to the luminal membrane [32], a process likely facilitated by VE-cadherin [33]. CD34-sialomucins are a group of single-pass transmembrane glycosylated cell surface molecules whose functions remain largely unknown [45]. Mucins and other proteoglycans coat many lumenal membranes, such as in the gut tube and other tubular organs like lung and pancreas. In leukocytes and hematopoietic stem cells, CD34 exerts an anti-adhesive function through charge repulsion mediated by its negatively charged extracellular domain [46]. In endothelial tubes, CD34 localizes to the luminal EC membrane (Figure 7D).

One CD34-sialomucin family member podocalyxin is located along the luminal (apical) side of both epithelial [47] and endothelial tubes [33, 48] (Figure 7E), suggesting it plays an important role in regulating lumen formation. In epithelial lumens, podocalyxin is required for apical compartmentalization and lumen formation in MDCK cells, as either cytoplasmic mislocalization or siRNA knockdown of podocalyxin leads to failure of proper cell polarization and lumen formation [49]. Similarly, recent studies show that the sialic acids of apical glycoproteins such as podocalyxin localize to apposed lumenal EC surfaces and generate repelling electrostatic fields at the center of EC cords [32]. This repulsion creates

an initially small slit that later enlarges to a functional vascular lumen as a result of numerous intracellular events, including subcellular moesin association (Figure 7F) and activation of actomyosin complexes (Figure 4F). The presence of negatively charged glycoproteins on most luminal surfaces of tubular organs suggests that the electrostatic repulsion may be a more general regulating mechanism in tubulogenesis.

3.3. Cell-ECM adhesion molecules

While proper control of cell-cell adhesion and de-adhesion is critical to vascular lumen formation, so is interaction between ECs and surrounding tissues. During mouse aortic vasculogenesis, ECs are in contact with the endodermal basement membrane and with matrix components secreted by trunk mesenchymal cells [23] (Figure 7G–I, K). This close association of ECs with ECM raises the question of how ECs interact with this surrounding ECM and whether it impacts basic vascular development, including lumen formation.

It is indeed rather interesting that the tubular aortae develop more or less uniformly around its circumference, given the different dorsal versus ventral microenvironments that it contacts. For instance, the underlying basement membrane is composed of a number of proteins, including laminins (Figure 7K), collagens (Figure 7I), perlecan, nidogens, and vWF. In contrast, a very different cohort of proteins is present on the mesenchymal side, including fibronectin (FN) and collagen IV (Figure 7H, I). It is conceivable that these different ECM components influence ECs within the aortae differently, dorsally versus ventrally. It is known that ventral ECs of the aorta later become ‘hemogenic’ and give rise to blood, while dorsal ones do not. Future studies in ECM impact on endothelial fate and morphogenesis during lumen formation, as well as generation of hemogenic endothelium, may reveal such localized matrix influences during embryogenesis.

3.3.1 Extracellular matrix (ECM)—All blood vessels, but especially the larger ones, are intimately sheathed with thick layers of ECM, both during their development and in their mature form. Interestingly however, during vasculogenesis, both the avian and mammalian dorsal aortae are initially devoid of ECM [23, 50]. The first matrix laid down by ECs of the aortae is FN (Figure 7H). It is only later that additional ECM components, such as laminin accumulate and form a basement membrane around the circumference of blood vessels (Figure 7K). This sequence of events by definition dictates that early, but not later, vascular matrix molecules may play important roles during vasculogenic tubulogenesis.

Indeed to date, few basement membrane proteins have been found to be required for blood vessel lumens. Most null alleles examined for basement membrane components, such as nidogens, vitronectin, perlecan and vWF, displayed no deleterious effects on vascular morphogenesis [51]. While it is possible few are critically required, it is also likely that defects are masked by functional redundancies among related components within molecular families. One study attempted to circumvent functional redundancy by deleting a common subunit for the majority of laminins in ES cells, however embryoid bodies still formed lumenized blood vessels [52], confirming that laminin does not play a significant role in vasculogenic tubulogenesis. One interesting exception is FN, a common ligand for integrin receptors. FN is amongst one of the earliest ECM components secreted by ECs during vascular development [53]. Targeted ablation of FN in ECs impaired endocardial lumen formation and aortic ECs attachment to surrounding mesenchyme [54], demonstrating the critical requirement for EC-ECM adhesion during vascular tubulogenesis. FN and its integrin receptors will be discussed in greater detail below.

3.3.2 Integrins—Growing evidence points to cell adhesion as a critical requirement for vascular lumen formation. Whether it is integrity of EC-EC contacts, or proper adhesion to

surrounding cells or ECM, it is clear that failure of adhesion impairs the integrity of forming cellular tubes. The predominant receptors that mediate cellular adhesion to surrounding ECM are integrins. The integrin family is composed of 24 heterodimeric transmembrane complexes formed by alpha and beta subunits that mediate both cell-cell and cell-ECM adhesions [55]. Up to 9 integrins have been shown to have roles in vascular development, including integrin receptors to collagens (α1β1, α2β1), laminins (α3β1, α6β1, α6β4), FN (α4β1, α5β1), and the multi-ligand binding αv receptors (αvβ3, αvβ5) [51]. Many of these have been of keen interest, as they are expressed on growing vessels and may prove to be good targets for anti-angiogenic therapies. Relevant to this review, αvβ3 and β1 have been proposed to play roles during vascular lumen formation (Figure 7G).

3.3.3 FN and its integrin receptors—As mentioned above, mammalian vasculogenesis takes place in a FN-rich environment [50]. During development and maturation of embryonic blood vessels peri-vascular FN expression decreases, as laminin (Figure 7K) and collagen IV (Figure 7I) levels increase [53, 56]. FN and its receptor α5β1 integrin have both been shown to be essential for vasculogenesis, as genetic ablation in mice resulted in lethality due to vascular defects [53, 57], including regional lumen failure and loss of vessel integrity, respectively. Specifically, the endocardial/endothelial tube within the heart fails to form a lumen.

Classical experiments carried out decades ago showed that β1 integrin was critical to vascular lumen formation. Injection into chick embryos of a monoclonal antibody (CSAT), that prevents β1 integrin from binding its ligands, resulted in a complete block to aortic lumen formation [58]. Aortic ECs remained cuboidal and junctions did not clear from the cord periphery. This initial finding was not recapitulated by recent genetic approaches to β1 deletion. A number of recent studies report that endothelial-specific deletion of β1 integrin with Tie2-cre leads to early lethality due to blood vessel failure [59, 60], however lumen defects were not reported. Interestingly, when deletion is carried out later, using VE-cadherin-Cre, lumen formation in arterioles of older vessels is blocked via defective expression and localization of the polarity determinant Par3 and loss of Rab7 dependent apical vesicle transport [3]. It is possible that, in contrast to chick, mouse vascular lumen formation depends on a wider array of redundant integrins, or that the CSAT blocking antibody affects additional integrins. However, together the findings point to integrin importance during vascular tubulogenesis.

A second FN receptor, αvβ3, has also been shown to regulate vascular lumen formation. In similar chick experiments, Drake and colleagues injected blocking antibodies to αvβ3 and demonstrated that, like with the β1 blocking CSAT antibodies, aortic lumen formation was completely inhibited [61]. Of note, lumen formation was inhibited more quickly and effectively with CSAT than with the αvβ3 antibody LM609, arguing again that perhaps CSAT blocks a wider array of integrin functions than does LM609 and that lumen formation requires parallel integrins. In support of this idea, recent work in mouse showed that multiple integrins are likely to be required simultaneously during lumen formation, including both αvβ3 and β1, but also probably additional integrins as well [23]. Nonetheless, both findings support the notion that cell-ECM adhesion is critical to building vascular lumens and suggest that many factors work redundantly in endothelium to support morphogenesis.

3.4 Cell polarity complex components

Decades of work in epithelial lumen systems have demonstrated that interactions between cells and surrounding ECM environment (adhesion) anchor the epithelial cell cytoskeleton and thereby establish polarity of the cell [62–64]. Signals from the ECM essentially provide

an axis, or reference point, from which to orient the positioning of the lumen, often located perpendicular to the ECM-contacting cell membrane. This establishment of apicobasal polarity allows spatiotemporal coordination of basic cellular processes, such as directed exo/endocytosis and localized adhesion or de-adhesion, processes shown to be critical to epithelial lumen formation [14]. Recent work in endothelial lumen formation identifies important analogies with epithelial lumen formation and demonstrates that these fundamental cellular processes are also involved in blood vessel morphogenesis [3, 23].

Acquisition of epithelial polarity is modulated by polarity complexes composed of Par3, Par6 and aPKC, and these complexes are in turn regulated by Rho family small GTPases [47]. A region on the shared membrane between two cells that forms a focal target site for this complex molecular machinery and initiates lumen formation has been referred to as the 'apical membrane initiation site' or AMIS [14, 47]. The AMIS represents the pre-lumenal membrane where tight junctions have not yet segregated away from lumen membrane components, such as podocalyxin. Recent live cell imaging studies using MDCK cells similarly examined pre-lumen formation between cells and identified the formation of 'preapical patch' (PAP), which represents slightly more 'matured' luminal membrane where tight junctions have resolved away from podocalyxin and E-cadherin has cleared [65]. The formation of the AMIS and PAP involves coordinated apical targeting of Par3-aPKC polarity complex components, and other proteins such as annexin II, PIP3 and PTEN, via Rab8 and Rab11a expressing vesicles. Interestingly, formation of the PAP was dependent on Cdc42, but suppressed by ROCK/myosin II activity [47, 65]. Similarly, aortic endothelial lumen formation has been shown to be suppressed by excess RhoA/ROCK signaling [23]. Together, these studies underline the importance of proper regulation of cell polarity and internal contractility for lumen formation.

During epithelial tubulogenesis, cells form a polarized monolayer with apical sides oriented towards the central lumen and basal sides forming the tube periphery (Figure 2A). Polarization of epithelial cells is relatively easy to recognize using many standard polarity 'markers', which are localized to either the apical or basal surfaces. For instance, ZO-1 and Par3 localize to apico-lateral junctions, while aPKC and podocalyxin mark the entire apical membrane (lumenal) and laminin marks the basement membrane at the basal surface (ablumenal).

In contrast to epithelial tubes, establishment and recognition of cell polarity in endothelial tubes has been much more challenging. A quick glance at the morphology of vessel ECs reveals that their apical and basal surfaces display few distinguishable characteristics beyond their location, interfacing either with the lumen or the basement membrane (respectively). In addition, their compressed interfaces have made it difficult to assess apico- from baso-lateral junctions. Standard apicobasal markers (such as aPKC for instance) are expressed at low or insignificant levels, and most standard apical markers have simply not been reported in aortal ECs during vasculogenesis (Figure 7J). Even basal markers, such as laminin, known to encircle later blood vessels during angiogenesis, are absent in early aortae (Figure 7K). Nonetheless, useful markers have been identified (Figure 7), with CD34, podocalyxin and moesin [23, 33, 48] marking the early aortic lumen surface, collagenIV, FN and β1 integrin lining the peripheral abluminal surface and claudin5, ZO-1, VE-cadherin and Par3 highlighting EC-EC junctions, at least in vasculogenic aortic ECs [23].

Epithelial and endothelium tubes thus present a number of useful analogies, in terms of junctional relationships and cell polarity, however they also present many significant differences. Both represent cells in sheets (rolled up into tubes), which closely associate to channel either fluids (i.e. blood vessels, kidney tubules), food (i.e. gut tube) or gases (i.e. lung branches), allowing controlled exchange across either their junctions or cell bodies.

However, each also displays unique characteristics. Given the divergence of their functional roles (transport, secretion or absorption), it is perhaps not entirely surprising that cell polarity and junctions of epithelial and endothelial cells display significant differences in their establishment, molecular underpinnings and dynamics during lumen formation. One elegant example that distinguishes endothelial from epithelial systems is the unique sensitivity of endothelium to hemodynamic flow, which can cause it to regress or expand [6]. Nonetheless, comparing and contrasting the similarities and differences between different types of epitheliums and the endothelium, which has often been referred to as an 'atypical' epithelium [66], will undoubtedly further our understanding of their ontogeny and functional morphology.

3.5. Intracellular signaling molecules - GTPases

One family of regulatory molecules that has been shown to include key regulators of vascular tubulogenesis is the intracellular Rho monomeric G protein GTPase family. These molecules are part of a large family of hydrolase enzymes that act as molecular switches that control many basic cellular processes, including cytoskeletal dynamics, actomyosin contractility, proliferation, cell adhesion and migration, as well as cell polarity. GTPases include many critical modulators such as Ras, Rap, Rho, Rac and Cdc42, which are widely expressed across most tissues. Rho, Rac and Cdc42 have been repeatedly linked to both epithelial [47, 67–69] and endothelial lumen formation [23, 70, 71]. A large body of work on the mechanistic roles of GTPases is reviewed elsewhere [5], we thus cover here only select highlights of their roles during EC tubulogenesis.

Early studies using 3D extracellular matrices *in vitro* established that Cdc42 and Rac1 were critical regulators of EC lumen formation [70]. Downstream effectors of these GTPases, such as Pak2 (activated by Rac1 and Cdc42) and Pak4 (activated by Cdc42), as well as a number of signaling components including Yes, Src, B/C-Raf and Erk1/2, are all similarly required *in vitro* for various aspects of EC tubulogenesis [71]. Interestingly, Cdc42 signaling and MT1-MMP-dependent proteolysis were recently shown to be interdependent processes, required for creating 'vascular guidance channels' within the surrounding matrix that facilitated attachment of ECs and development of vacuole-derived lumens [18]. In addition, a key role of Cdc42, in both epithelial and endothelial systems, is to regulate cell polarity signaling by directly binding to a number of junctional and polarity effectors including VE-cadherin, Par3/6 and aPKC, thereby influencing lumen formation [71, 72].

Tightly regulated activity of RhoA has also recently been shown to be required for proper EC lumen morphogenesis in the mouse dorsal aortae [23, 33]. It appears that RhoA may play different roles during temporally distinct steps of vascular lumen tubulogenesis. During initiation of lumen formation in the mouse aorta, RhoA, via ROCK and under the control of VEGF signaling, promotes the recruitment of non-muscle Myosin IIA (NMHCIIA) to apical F-actin, 'bending' the membrane and providing the force to further 'open up' the initial slit between ECs formed via sialomucin-induced repulsion [33]. In a sense, this represents a 'positive' role for RhoA function at the earliest stages of lumen formation.

In contrast, it was also shown that RhoA activity must be suppressed during vascular lumen formation, suggesting a 'negative' role. Indeed, high RhoA activity is known to be associated with high internal cell contractility and for ECs to flatten and lumen morphogenesis to occur that contractility must be relaxed. In Rasip1 null embryos, where angioblasts remain rounded and vascular lumens fail to form, it was shown that Rasip1 acts as an endothelial-specific modulator of GTPase signaling [23]. Specifically, Rasip1 brings the GTPase activating protein (GAP) Arhgap29 in proximity to NMHCIIA/myosinII, suppressing RhoA activity and thereby suppressing EC actomyosin contractility. Loss-of-function of either Rasip1 or Arhgap29 lead to overactivation of RhoA, increased stress fiber

formation and actomyosin contractility, rounded EC morphology on both matrigel and *in vivo*, consequently resulting in decreased integrin-dependent cell adhesion and tubulogenesis failure [23, 73].

Together, these data suggest that coordinated control of multiple GTPases is essential to direct a number of cellular processes that dictate EC cell shape and adhesion dynamics during blood vessel morphogenesis and tubulogenesis.

4. Summary

Vascular tubulogenesis is critical for blood vessel development and subsequent cardiovascular function. Elegant *in vitro* and *in vivo* work during the past decade, as well as important concepts borrowed from epithelial studies, have together contributed to a growing understanding of this complicated process. Using different models of EC lumen formation has provided valuable insights as to how vascular lumens take shape and mature in different places and at different times. Given the intrinsic complexity of underlying cellular and molecular mechanisms, and the uniqueness of this intricate and dynamic tissue, it is likely that we have only scratched the surface in our attempts to draw a bigger picture.

Despite what we already know about vascular tubulogenesis, major questions remain to be answered. Why and how do cell-cell junctions de-adhere only at the cord center, while those at the periphery remain? Where does the initial circulating plasma come from, as vascular lumens first form, and is it required for initial lumen formation? Do growth factors, such as VEGF, play a role in stimulating lumen formation as suggested by gene targeting studies? Although it is clear that certain regulatory pathways are shared in lumen formation in different vascular beds, others are likely to be context-dependent in specific types of ECs or vessels. It is remarkable, albeit not altogether surprising, that vascular functional and regional heterogeneity is reflected by heterogeneity of lumen ontogeny mechanisms. Finding the common themes that unite lumen formation will be an exciting challenge.

To date, the study of lumen formation and/or maintenance in adult blood vessels has received relatively little attention. Given the fact that Rasip1, for instance, is also expressed in postnatal and adult established vessels [23, 73, 74], it remains to be seen whether it is also required to maintain or extend preexisting vascular lumens. Is Rasip1 similarly required during physiological and pathological conditions? Initial blood vessel tubulogenesis is clearly a normal and beneficial phenomenon that occurs in all growing vessels allowing proper blood circulation in the growing tissues of embryos/neonates or healing wounds. However, in the tumors of cancer patients, acquisition of lumens in growing vessels provides ‘escape’ routes for metastasizing cancer cells, as well as sustenance for the tumor. In this case, vascular tubulogenesis is deleterious to human health. The possibility that targeting angiogenic blood vessel lumen formation could prevent functional vessels from growing into tumors is an enticing one. Overall, understanding and potentially manipulating blood vessel lumen formation holds great promise for both pro- and anti-angiogenic therapies.

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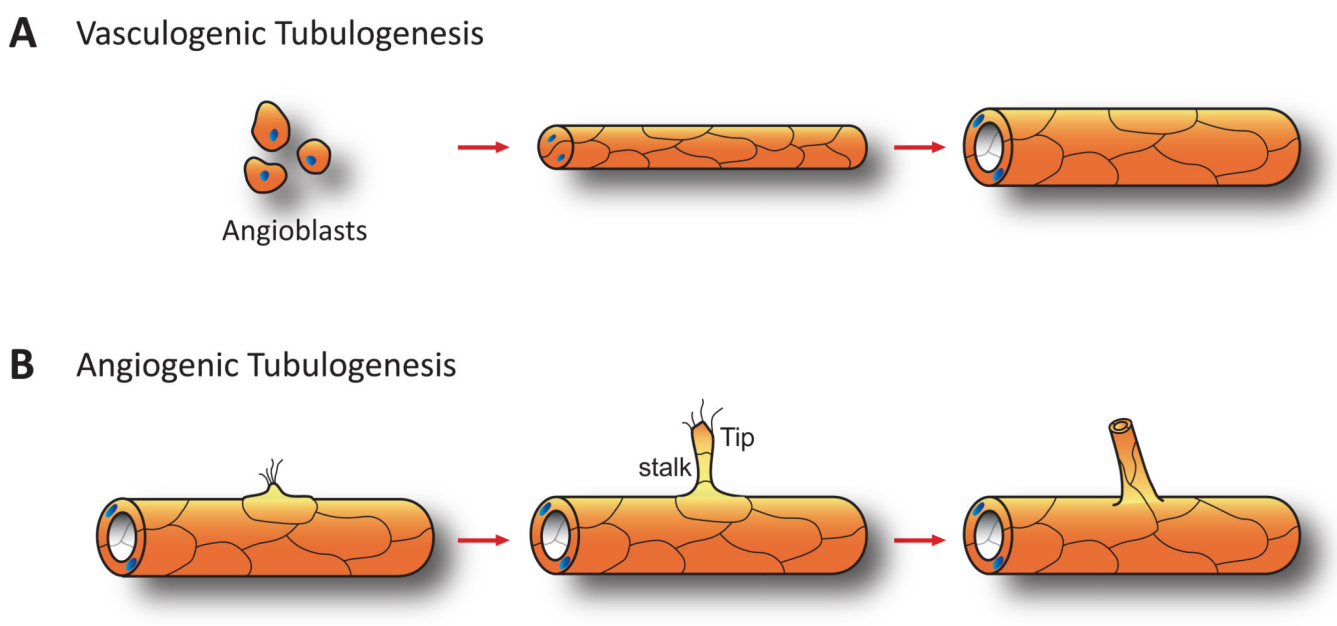

Figure 1. Vasculogenic versus angiogenic tubulogenesis

(**A**) During vasculogenesis, angioblasts within the mesoderm aggregate into lumenless vascular cords. These rope-like vascular structures then undergo a cord-to-tube transition, opening up *de novo* lumens. (**B**) During angiogenic sprouting, endothelial 'tip' cells grow from pre-existing vessels and extend lumens into newly formed branches.

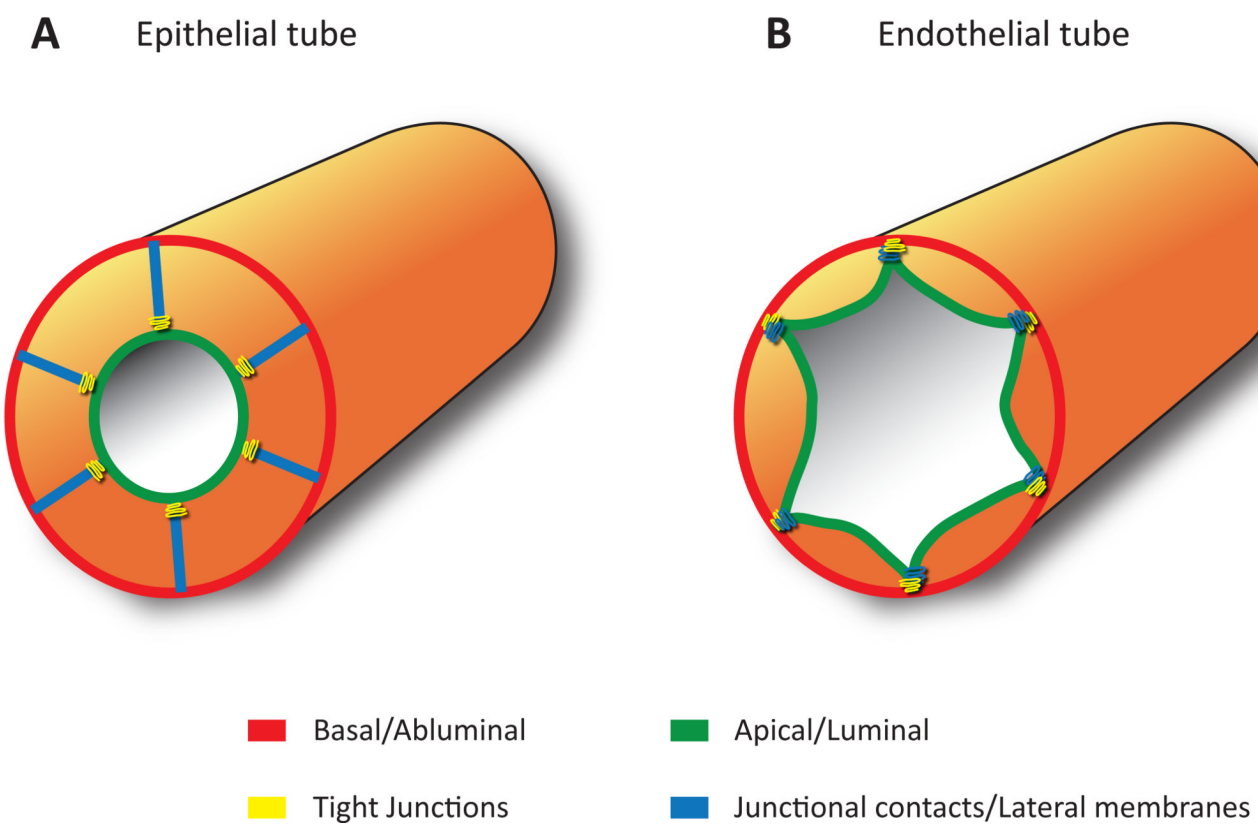

Figure 2. Junctional contacts in epithelial versus endothelial tubes

(**A**) Epithelial tubes are formed by cuboidal/columnar epithelial cells with well-defined apical, lateral and basal surfaces. (B) Endothelial cells constitute an atypical epithelium, with cells displaying a 'flattened' morphology and reduced junctional areas at the tube periphery.

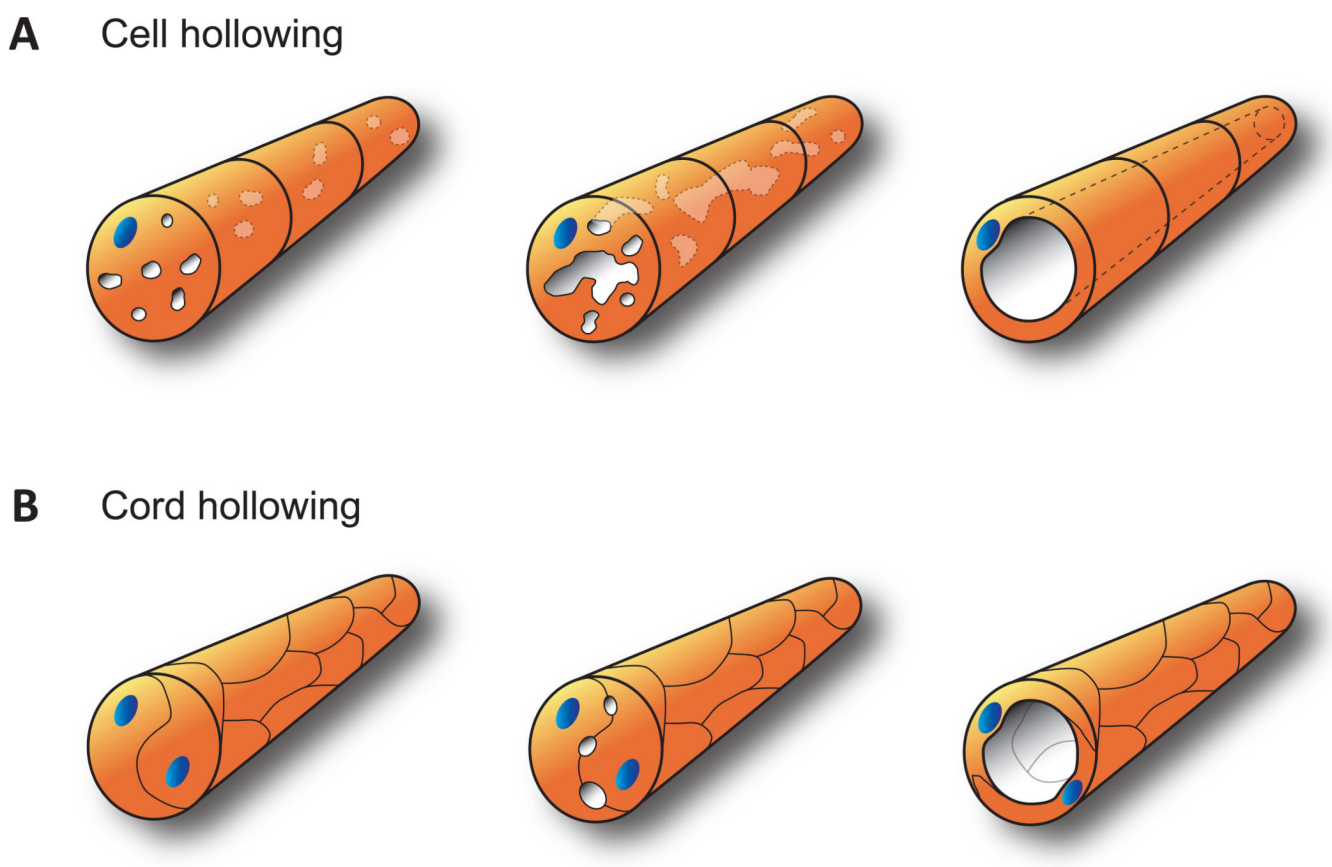

Figure 3. Vascular morphogenesis: Cell hollowing versus cord hollowing

(A) Cell hollowing: lumen formed between aligned ECs through intracellular vesicle fusing.
(B) Cord hollowing: lumen formed by slits initiation and fusing between polarized ECs.

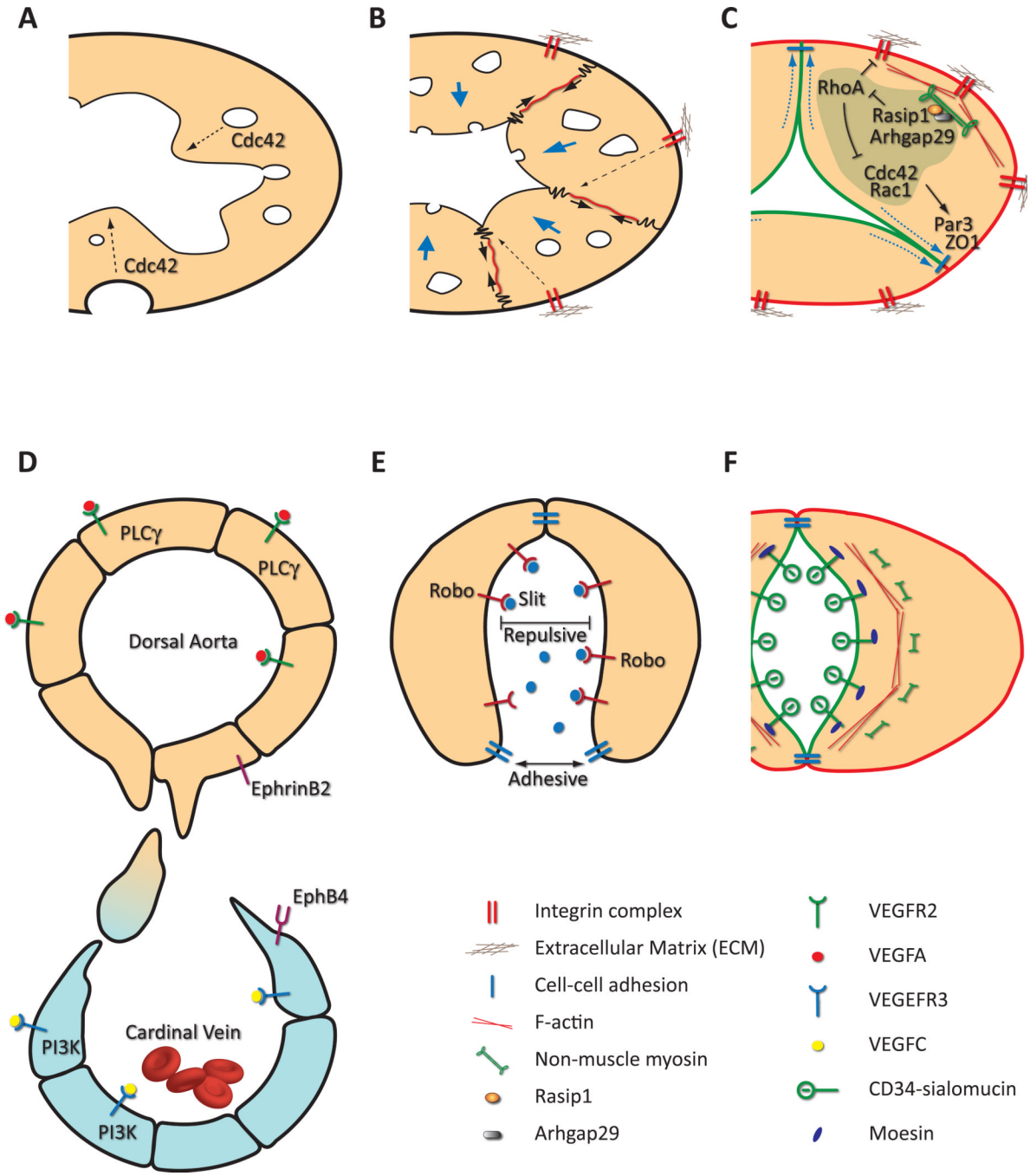

Figure 4. Vascular tubulogenesis: mechanistic heterogeneity

(A) Vacuole fusion mediated by Cdc42 signaling pathway. (B) Lumen formation requires integrin induced cell polarity formation. (C) Rasip1-Rho family small GTPases regulate EC-ECM adhesion and actomyosin activity in ECs. (D) Zebrafish cardinal vein is formed by ECs pinched off from the dorsal aorta. (E) Robo-Slit repulsive signal mediates Drosophila heart tube formation. (F) Vascular silt initiation regulated by cell surface negative charges and cell contractility downstream of VEGF signaling.

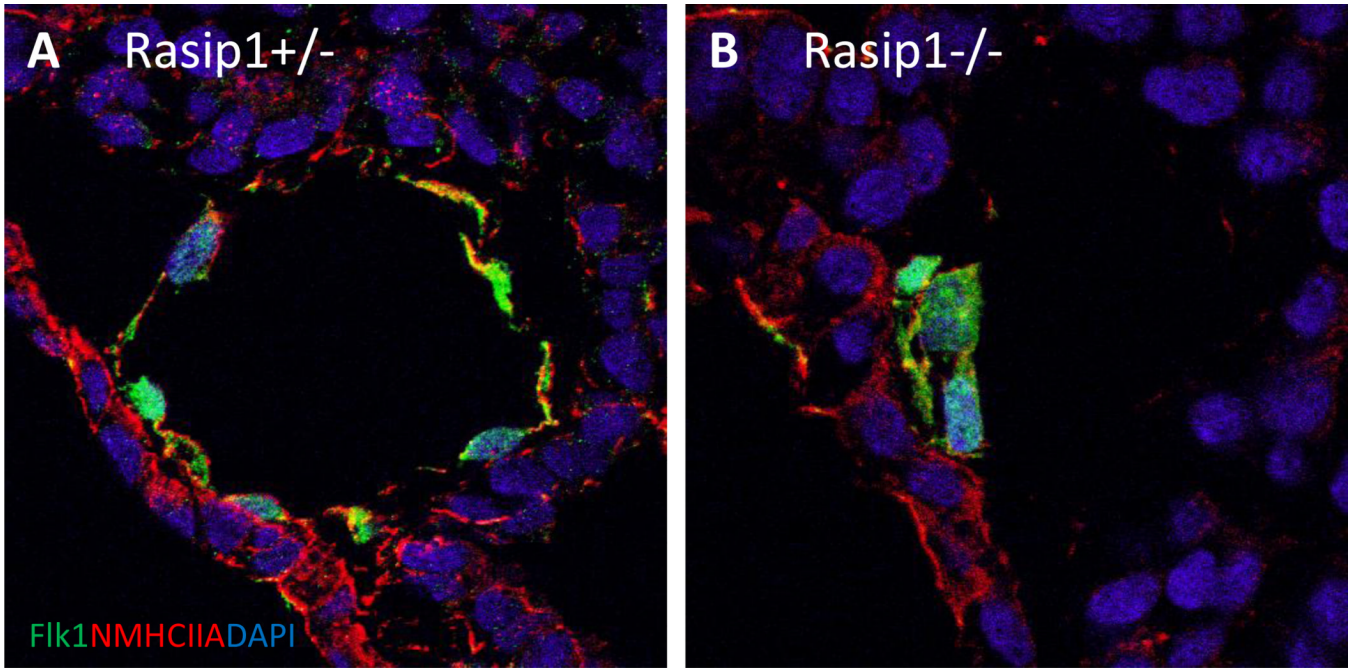

Figure 5. Rasip1 ablated ECs fail to adhere to surrounding ECM

(A) Wild type aortic ECs are thin and tightly associated with surrounding cells, while (B) Rasip1 null ECs are cuboidal, fail to open a central lumen and de-adhere from surrounding mesenchyme.

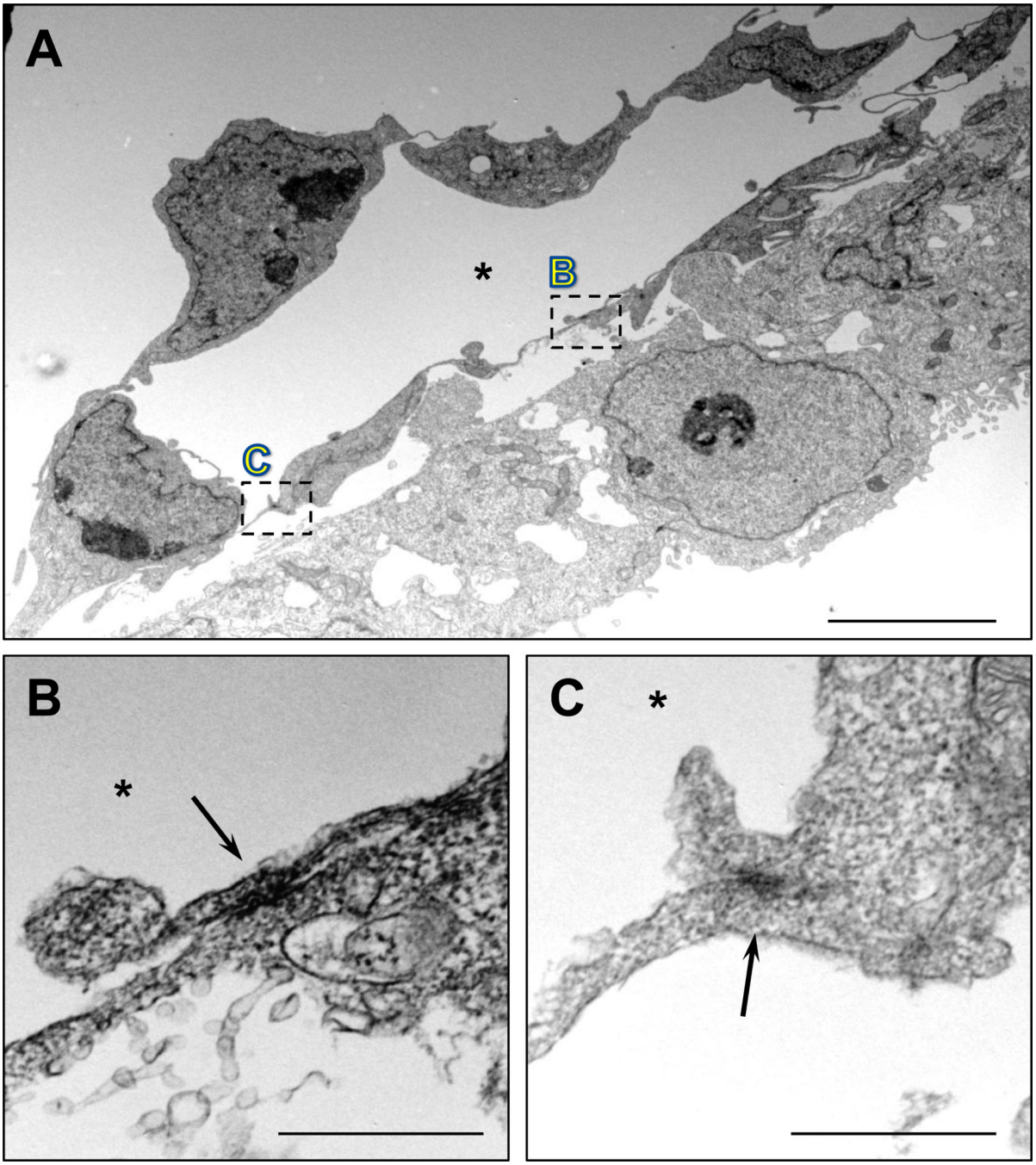

Figure 6. Cell junctions in mouse aortic ECs at E8.5 revealed by TEM

(A). lateral narrow tight junctional surfaces highlighted by high magnification insets in selected areas (B, C). Scale bars: 5μm in A and 0.5μm in B,C

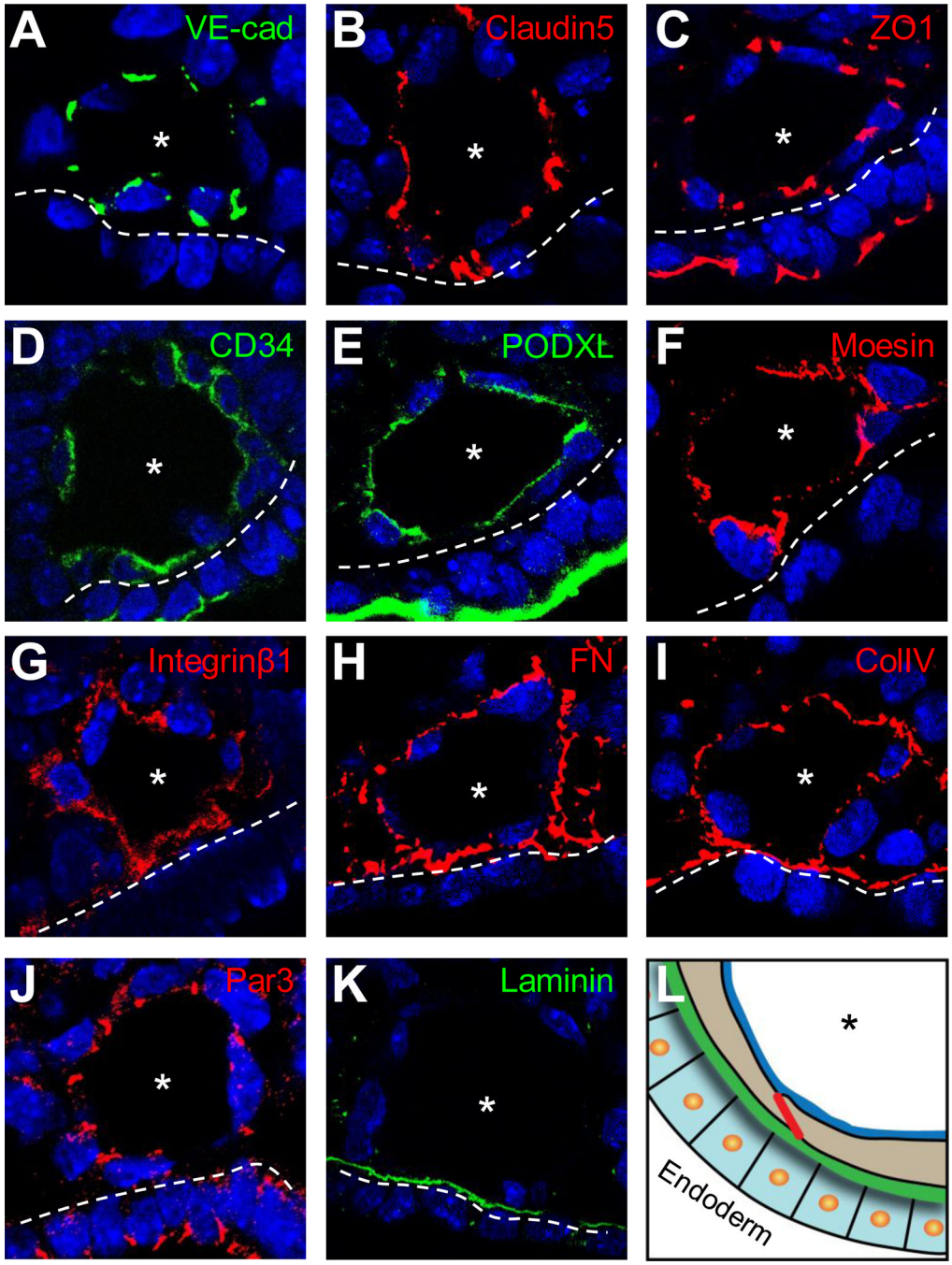

Figure 7. Selected apicobasal and adhesion molecules in mouse aortic ECs, at E8.5

Junctional: (A) VE-Cadherin (B) Claudin5 and (C) ZO1. Luminal: (D) CD34, (E) PODXL, and (F) Moesin. EC-ECM adhesion: (G) Integrin beta1, (H) Fibronectin, and (I) Collagen IV. Polarity: (J) Par3, and (K) Laminin. (L) schematic cartoon showing the luminal surface (Dark blue), contacting ECs (Grey), EC-EC junctions (Red), EC-ECM contacts/abluminal surface (Green) and Endoderm (Light blue). Asterisks represent lumens.