# Chapter 1
# Molecular Control of Capillary Tube Morphogenesis and Maturation Through Endothelial Cell-Pericyte Interactions: Regulation by Small GTPase-Mediated Signaling, Kinase Cascades, Extracellular Matrix Remodeling, and Defined Growth Factors

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## 1.1 Introduction

Considerable progress has been made in our understanding of molecular events underlying the development of the vasculature and how it is regulated in postnatal life, particularly in the context of tissue injury and tumorigenesis [1, 18, 19, 30, 31, 36, 55, 89, 92]. A key point is that the major advances in the field appear to be directly related to our understanding of basic events that control these processes such as cell survival, proliferation, migration, invasion, and morphogenesis. Also, it should be pointed out that both in vitro and in vivo studies have played major roles in advancing this understanding, and it remains essential that both types of approaches are utilized to approach the complexities inherent in the developing and postnatal vasculature.

The molecular control of the vasculature is affected by many factors and signals, and in this chapter, we will focus on the influence of extracellular matrix (ECM), matrix metalloproteinases (MMPs), small GTPase-mediated signal transduction, defined growth factors, and endothelial cell (EC)-pericyte interactions in controlling vascular development and postnatal vascularization events. In general terms, the ECM is a fundamental regulator of vascularization, in that it presents a physical scaffold containing adhesive and growth factor modulatory signals that are necessary for blood vessels to form and mature [34, 35, 57, 58, 81, 89]. Vascular cell

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recognition of ECM occurs through a variety of receptors including both integrin and non-integrin adhesion receptors [34, 58, 89, 97], and these mediate the complex signals that are delivered. There is considerable evidence that ECM can provide both stimulatory and inhibitory signals [34, 89], and thus, the ECM composition, the vascular cell types, and the biological context of the signaling dictate the cellular response that occurs. An important regulator of ECM structure and function are MMPs which can degrade matrix components [31, 33, 35, 47, 52] but can also release liberate factors such as growth factors and cytokines from these matrices to affect vascular cell behavior. Within the vascular wall, homotypic interactions between ECs [37] and heterotypic interactions of ECs and mural cells affect ECM production and deposition [92, 94], as well as its ability to be degraded by MMPs [87]. Many new studies are now focused on such interactions to understand how mural cells affect EC behavior during development and under various disease conditions [1, 5, 56, 92, 94]. In this chapter, we will review past and present work that addresses mechanisms by which ECM, MMPs, small GTPase signaling, defined growth factors, and EC-pericyte interactions influence vascular tube assembly and remodeling, tube stabilization, and vascular regression to control tissue vascularization in normal versus disease states.

## 1.2 Concepts in Vascular Tube Morphogenesis in 3D Extracellular Matrices

### *1.2.1 Extracellular Matrix and Vascular Morphogenesis*

A critical regulator of vascular morphogenesis is the ECM which serves as a physical, mechanical, and agonistic substrate to affect survival, motility, invasion, and morphogenic events of both endothelial cells (ECs) and mural cells, including pericytes and vascular smooth muscle cells [1, 18, 34–36, 58, 81, 89, 92]. Interestingly, different types of ECM have distinct effects on the developing or mature vasculature, depending on the biologic context, with evidence for pro-morphogenic, pro-stabilization, or pro-regressive activities [31, 34, 35, 89]. Also, certain ECM environments may present quiescence signals to vascular cells that play an important role in vascular stabilization. Alteration in the ECM, through proteolysis or conformational changes, is known to generate matricryptic sites which activate cells, and thus, the ECM is a critical regulator of how cells perceive their environment and sense an injurious stimulus [24, 26]. Furthermore, the ECM is a scaffold that possesses adhesive signals for cells, by binding to both integrin and non-integrin surface receptors, but it also binds and presents specific growth factors to cells [34, 57, 58, 89]. The ECM also modulates the activation of specific growth factors and, thus, can modulate growth factor action to affect the vasculature [58]. It is clear that co-signaling between integrins and growth factor receptors is a critical regulator of vascularization events both during development and in postnatal life. The ECM is

also mechanosensitive and responds to mechanical forces generated by cells that exert tensional forces on this matrix. A key mechanosensitive ECM component is fibronectin which contains matricryptic sites that affect self-assembly reactions, particularly through the III-I domain [45, 77, 99, 106]. Interestingly, this domain is exposed in instances where fibronectin is absorbed into surfaces, such as cell surfaces or ECM [24, 26, 99], and appears to be particularly exposed when cells exert mechanical force on fibronectin through integrin-based interactions [106]. This facilitates fibronectin binding to itself which promotes the self-assembly reaction (i.e., including disulfide exchange to form covalent bonds between fibronectin molecules) necessary to form an insoluble matrix [77]. Since fibronectin is one of the few ECM proteins with clear mechanosensitive domains, it suggests that fibronectin may play a particularly important role in ECM assembly events that depend on mechanical forces, such as those observed during vascular morphogenic events in a variety of contexts [18, 94, 107].

### *1.2.2 Differential Effects of ECM Components on Vascular Tube Morphogenesis*

Certain ECM components are potent stimulators of vascular tube morphogenesis, while others appear inhibitory. Interestingly, collagen type I, the most abundant ECM component in adult animals, is a potent stimulator of vascular tube morphogenesis in 3D matrices [34, 100] (Fig. 1.1). An accumulating view is that fibrillar collagen matrices are potent ECM agonists for these events. Another strong ECM agonist for EC tube morphogenesis is fibrin [78, 91], a provisional matrix component that is deposited along with fibronectin during tissue injury [34]. Interestingly, the collagen-binding integrins, α2β1 and α1β1, have been shown to control EC tube morphogenic events in vitro and in vivo in collagenous matrices [9, 28, 34, 88], while the fibrin-/fibronectin-binding integrins, αvβ3 and α5β1, have been shown to control tube morphogenesis in fibrin matrices [9, 11]. Because of the strong pro-morphogenic influence of collagen and fibrin matrices, these have predominantly been used to establish 3D EC tube morphogenic models [68, 78] that have strongly enhanced our knowledge concerning the molecular basis for EC tubulogenesis, sprouting, and tube maturation events. Overall, the ECM and integrin data strongly suggests that vascular tube morphogenesis is connected to integrin-mediated recognition of different pro-morphogenic ECM components and that multiple members of the integrin family can participate in stimulating EC tubulogenesis in 3D matrix environments [27, 34]. It does not appear that any particular integrin family member is special in its ability to affect tube morphogenesis. Their influence is dictated by the ECM environment in which the morphogenic process takes place. In contrast, it appears that laminin-rich matrices are likely to present inhibitory signals to endothelial cells to interfere with morphogenic events [34, 35, 71]. As blood vessels mature, laminin matrix deposits as a component of the vascular basement

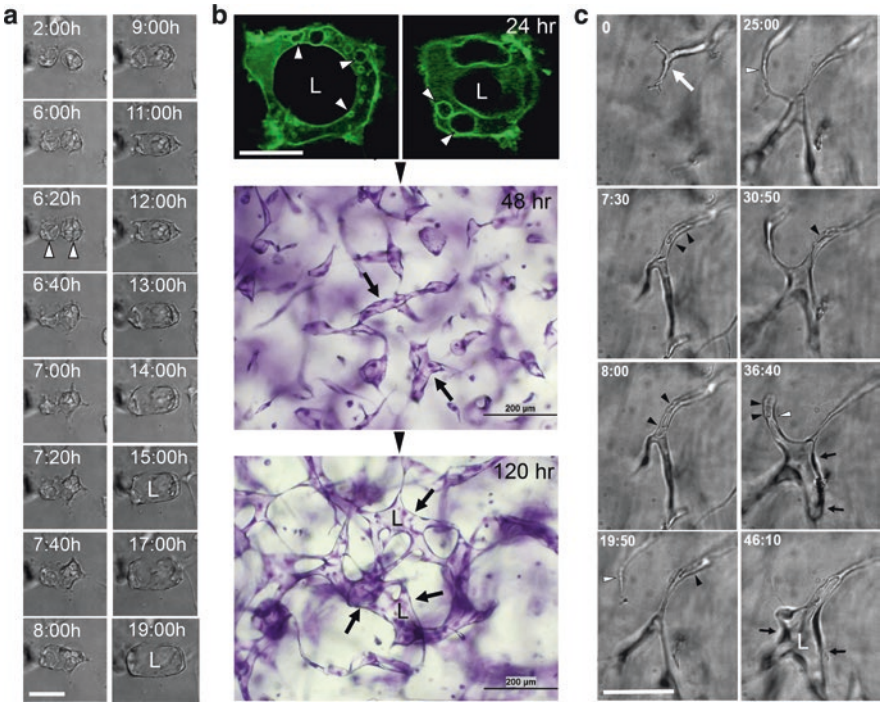


**Fig. 1.1** Temporal analysis of endothelial tube formation events in 3D collagen matrices during vasculogenesis and angiogenic sprouting. (**a**) A time-lapse series is shown whereby two ECs are shown to form intracellular vacuoles that eventually coalescence inside each cell and then following cell-cell contact with the neighboring cells form a lumenal space in between the two cells. Vacuole fusion events are observed while they are contacting each other. L indicates EC lumen; arrowheads indicate vacuolating ECs. Bar equals 25 µm. (**b**) Confocal images of ECs expressing GFP-Rac1 fusion proteins that label intracellular vacuoles, the developing lumenal membrane, as well as the plasma membrane during lumen formation events at 24 h of culture. Upper panels- L indicates EC lumenal space, and arrowheads indicate intracellular vacuoles. Bar equals 20 µm. Over time ECs form interconnecting networks of tubes which are illustrated using fixed and toluidine-blue stained cultures. Arrows indicate tube structures; L indicates EC lumen; Bar equals 200 µm. (**c**) EC sprouting was stimulated by the combination of sphingosine-1-phosphate (1 µm) and SDF-1α (200 ng/ml) which were mixed in the collagen matrix. A time-lapse series (over a 46 h period) was taken beneath the surface of the monolayer to examine sprouting events and lumen development in assays mimicking angiogenesis. White arrow and arrowheads indicate EC tip cells that are sprouting through the 3D collagen matrices; black arrowheads indicate intracellular vacuoles that are observed particularly in ECs directly trailing tip cells; black arrows indicate the cell border of EC tubes; L indicates EC lumen. Bar equals 50 µm

membrane, a key ECM remodeling process which occurs as a result of EC-pericyte interactions during capillary tube assembly [94]. Vascular basement membrane assembly generally is thought to be a tube maturation stimulus, and hence, decreased morphogenesis is coincident with its appearance around the abluminal surface of EC-lined tubes. This point needs to be investigated in more detail, but early

information suggests that some laminin isoforms have inhibitory activity toward ECs during morphogenic events [71]. The vasculature appears to predominantly express laminin-8 (α4,β1,γ1- 411), laminin-9 (α4,β2,γ1-421), laminin-10 (α5, β1,γ1- 511), and laminin-11 (α5,β2,γ1- 521) isoforms [34, 50, 76]. In the past and more recently, we have reported that these subunits are differentially expressed by both ECs and pericytes during vascular tube morphogenesis and maturation events [12, 94]. The biological role of each isoform has not yet been elucidated in sufficient detail during these processes.

One critical question that similarly has not been investigated in sufficient detail is the nature of the embryonic ECM environment where vascular development takes place [35, 57]. It is clear that there is much less fibrillar collagen during development, while the matrices are known to be rich in glycosaminoglycans, such as hyaluronic acid, proteoglycans, and fibronectin. It appears that developing embryos strongly depend on the presence of fibronectin (perhaps its importance relates to its mechanosensitive ability to self-assemble) [57]. Fibronectin knockout mice show severe defects in vascular development along with other abnormalities [6, 42]. Fibronectin is also alternatively spliced, and several splice isoforms (IIIA and IIIB) appear to play a critical functional role to promote vascular tube assembly and maturation during development [6]. One of the problems with investigating such issues in a more molecular detail is that there currently are no 3D systems available that mimic an embryonic ECM environment, an important future direction for in vitro model development using vascular or other cell types.

## 1.3 Review of Work

### *1.3.1 Molecular Events Regulating Vascular Tube Morphogenesis and EC Sprouting in 3D Matrices*

A major effort of our laboratory has been to elucidate the molecule and signaling requirements for ECs to form tube networks when suspended within 3D matrices and to sprout and form tubes from a monolayer surface into 3D matrices [34, 36, 60, 84] (Fig. 1.2). To this end, we have developed 3D matrix microassay systems to assess both of these phenomena in assays that mimic vasculogenesis and angiogenic sprouting events, using either collagen or fibrin matrices [29, 68]. Other laboratories have developed related systems to investigate these events [3, 78]. The majority of our work has focused on models that mimic embryonic vasculogenesis, whereby human ECs are seeded as single cells within a 3D matrix [28, 29, 36, 68]. Using appropriate media conditions, ECs undergo dramatic morphologic changes that lead to the development of interconnecting networks of EC-lined tubes (Fig. 1.1). For example, two ECs are observed in a time-lapse series to form intracellular vacuoles, which fuse within each cell and then through exocytic events; the two cells then interconnect to form a multicellular lumen structure (Fig. 1.1a). There is no

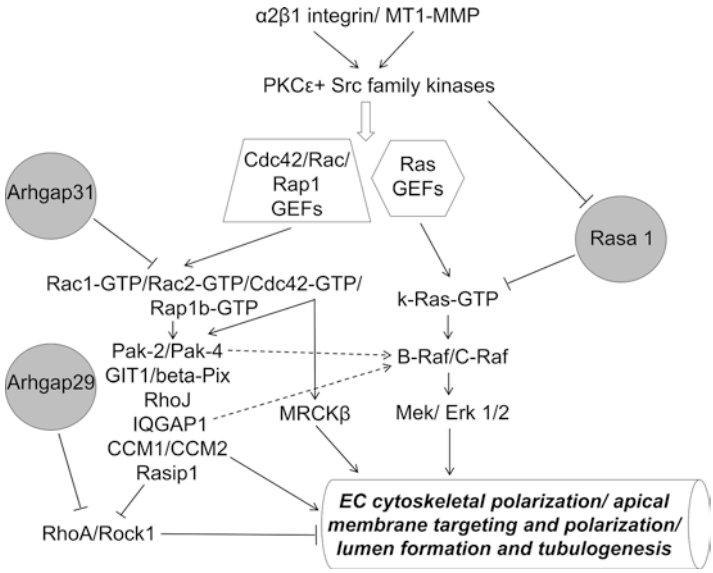


**Fig. 1.2** EC tubulogenesis in 3D matrices is controlled by activation of a Cdc42-, Rac-, k-Ras, and Rap1b-dependent signaling cascade: a process antagonized by RhoA, Arhgap31, and Rasa1. A schematic diagram is shown illustrating key molecules and signals that regulate how EC lumen and tube formation occurs in 3D collagen matrices. These molecules and signals control EC cytoskeletal polarization (subapically distributed acetylated and detyrosinated tubulin; basally distributed F-actin) and the generation of the apical membrane which is decorated with key small GTPases including Cdc42, Rac1, Rac2, k-Ras, and Rap1b and the effectors c-Raf and Rasip1. EC tubulogenesis also requires MT1-MMP-dependent matrix proteolysis, a step that is co-dependent and coordinated with the indicated GTPase-, effector-, integrin-, and kinase-dependent signaling cascades. Intracellular vacuoles and vesicles (strongly labeled with Rac1 and k-Ras) traffic along subapically oriented acetylated tubulin tracks and then fuse together in a polarized perinuclear region (where acetylated tubulin co-localizes with Cdc42) to generate and expand the EC apical surface. Another aspect of this EC lumen signaling cascade is to suppress RhoA signaling, and key molecules that participate in this suppression are Cdc42, Rac isoforms, Pak2, Pak4, Rasip1 and its associated Gap, Arhgap29, and the CCM proteins CCM1 and CCM2

intermixing of cytoplasmic contents when this occurs, so the tube consists of adjacent ECs which interact through cell-cell adhesive contacts. In addition, the ECs are attached to the ECM to form the wall of a luminal space and need to maintain these adhesive contacts (i.e., both cell-ECM and cell-cell adhesion) to remain stable on this luminal wall. Intracellular vacuoles can be observed to form through integrin- and cytoskeletal-dependent pinocytic events, and these vacuoles target to a pericentrosomal location in a polarized fashion and then move to fuse with the developing luminal membrane as shown in Fig. 1.1b (upper panel) [8, 28, 30, 62–64, 104]. In Fig. 1.1b, intracellular vacuoles are strongly labeled with a GFP-Rac1 construct [8]. We previously observed labeling of intracellular vacuoles with GFP-Rac1, GFP-Cdc42, and GFP-RalA [8, 30, 79]. This GFP-Cdc42 was expressed using an

EC-specific promoter in zebrafish, revealing that intracellular vacuoles were observed and participate in the lumen formation process of intersegmental vessels during vascular development [62, 104]. Additional work in vivo reveals the clear presence of intracellular vacuoles as a major determinant of EC lumen formation in other species, including mouse and quail [36, 110].

Both Cdc42 and Rac1 are required for intracellular vacuole formation, as well as EC lumen and tube formation [8, 30, 67]. We have performed these experiments using either dominant-negative mutants of Cdc42 and Rac1 or specific siRNAs to these GTPases. Developing multicellular lumenal structures then interconnect into more extensive networks over time (Fig. 1.1b, middle and lower panels). Using an angiogenic sprouting model [9, 68], time-lapse images are shown which reveal how invading ECs interact, develop intracellular vacuoles, and migrate toward each other to form multicellular lumen and tube structures over time (Fig. 1.1c).

After much effort over many years, we have elucidated the molecular requirements and signaling pathways that underlie the ability of human ECs to form lumen and tube structures in 3D matrices [8, 25, 27, 36, 60, 63, 64, 79, 84, 87, 89, 93, 95]. Most of our studies have focused on collagen matrices, and, thus, a major requirement for these events is the α2β1 integrin, a collagen-binding integrin [28] (Fig. 1.2). Blocking antibodies directed to α2β1 markedly block lumen formation, as do α2 integrin subunit siRNAs. Interestingly, blocking antibodies directed to many other integrin subunits, including α5β1, a fibronectin receptor, have no effect in this system. Also, considerable work has shown that α2β1 integrin is important for vascularization events in vivo and in both developmental and postnatal life contexts [85, 88]. In contrast, when our studies utilized fibrin matrices, we identified that both αvβ3 and α5β1 were required for EC lumen and tube formation [11], while α2β1 was not shown to be involved. Interestingly, the first fibrin system that we developed was performed in the presence of serum. More recently, we developed a serum-free defined system in fibrin matrices, where fibronectin was added in with the matrix when it is polymerized [91]. Under these conditions, α5β1 was involved in the tube formation process, not αvβ3. This result suggests that serum-derived vitronectin might have been the reason why αvβ3 played a role in this first system [11]. Nonetheless, both of these integrins have been shown to be involved during vessel formation during development and in postnatal mice [57, 97]. Thus, an important point is that the in vitro models by our laboratory and others have very accurately predicted in vivo findings made by other groups. Another key point is that the in vitro model systems demonstrated first that multiple integrin chains could be utilized by ECs to control tube morphogenesis and second that there is little evidence to suggest that any particular integrin is unique or special in this property to regulate the morphogenic cascade necessary to form new blood vessels. The role of particular integrins in morphogenesis appears to be directly linked to the ECM environment (and the predominant ECM components) that are in contact with the ECs.

### 1.3.2 Functional Role of the Rho GTPases, Cdc42 and Rac1, and the Effectors, Pak2 and Pak4, in EC Tube Morphogenesis

An important question raised by the above studies is which downstream signaling pathways are activated by integrins to control these morphogenic processes (Fig. 1.2). Integrins were known to activate Rho GTPases among other molecules [49] such as a variety of kinases, including Src and focal adhesion kinase [34, 57, 89]. Our laboratory reported that Cdc42 was a critical GTPase controlling EC lumen formation [8] (Fig. 1.2). This was also the first report from any system implicating Cdc42 and tube formation. Subsequent studies have revealed that Cdc42 is a critical regulator of lumen formation from both ECs and epithelial cells [8, 25, 66, 67, 75, 79, 83] (Fig. 1.2). Very recently, EC-specific knockout of Cdc42 in mice resulted in embryonic lethality due to lack of EC lumen and tube formation. We reported a role for Rac1 in EC tubulogenesis [8, 66], while RhoA had no ability to stimulate these events. In contrast, expression of constitutively active RhoA leads to marked inhibition of EC lumen formation [8]. Both Cdc42 and Rac1 were shown to be activated during the morphogenic cascade in 3D collagen matrices [66, 83]. To address the question of downstream effectors that are responsible for the influence of Cdc42 and Rac1, we screened a series of known effectors using siRNA treatment of ECs. Major blocking phenotypes were observed using siRNAs to p21-activated kinase (Pak)-2 and Pak-4 [66]. Both EC tube formation and EC sprouting into 3D collagen matrices were markedly inhibited by these siRNAs. We also demonstrated that a time course of Pak-2 and Pak-4 activation, as indicated by phosphorylation, directly correlated with the EC lumen formation process [66]. It was further demonstrated that activated Pak-2 and Pak-4 could be demonstrated to be associated with activated Cdc42 during these events [66]. Expression of a dominant-negative mutant of either Pak-2 or Pak-4 was shown to completely inhibit EC lumen formation [66]. Interestingly, both Cdc42 and Rac1 are able to activate Pak-2, while Cdc42 selectively activates Pak-4 [17, 44]. Recent experiments have revealed important roles for both Pak-2 and Pak-4 during vascular development [44, 70, 98], which again corroborate the in vitro findings.

### 1.3.3 Functional Role for PKCε and Src in EC Tube Morphogenesis and Subsequent Pak Activation Events

Other known kinases that are activated by cell-ECM interactions include protein kinase C isoforms and Src family kinases. In our studies of EC lumen formation in 3D collagen matrices, we have shown that PKCε, but not PKCα or PKCδ, is involved in the process [66, 67]. siRNA suppression experiments or expression of

dominant-negative mutants of PKCε block lumen formation and downstream Src and Pak activation [67]. Interestingly, increased expression of PKCε strongly stimulates EC lumen and tube formation, and both increased Src, Pak-2, and Pak-4 phosphorylation events that directly correlate with its morphogenic influence [67] (Fig. 1.2). Our studies indicate that PKCε is upstream of Src activation, while Src activation is upstream of Pak activation [67]. Blockade of Src kinases by siRNA suppression; increased expression of the Src inhibitor, CSK (i.e., C-terminal Src kinase); or treatment with chemical inhibitors (e.g., PP2) completely interferes with EC tube formation. Expression of a dominant-negative Csk construct strongly increased lumen formation, again suggesting a positive role for Src in EC lumen formation [67]. Interestingly, Src and Pak kinases are known to active Raf kinases to affect processes such as cell survival which has previously been shown to influence angiogenesis in vivo [2], and we have recently shown that they are required for EC lumen formation [67]. Mouse knockout of B-Raf shows an embryonic lethal phenotype that is due to vascular abnormalities [43]. Of great interest is that we have shown that Raf kinase activation (of both C-Raf and B-Raf) occurs downstream of Src and Pak activation and controls EC tube morphogenic events along with survival [67] (Fig. 1.2). This is accompanied by Erk1/Erk2 activation which also directly correlates with the ability of these ECs to form tube networks. Interestingly, expression of a phosphatase, MKP-3, with selectivity for phospho-Erk1/Erk2, markedly decreases Erk phosphorylation and strongly blocks lumen formation [67]. A dominant-negative MEK kinase inhibitor also abrogates lumen formation and Erk1/Erk2 phosphorylation events. What is interesting about these results is that a known pathway to regulate both proliferation and survival is utilized by ECs to regulate a separate tubulogenic pathway in 3D matrices. In our systems there is little to no evidence for proliferation during these processes so the signaling cascade appears particularly focused on tube morphogenesis [12, 30]. Overall, this morphogenic pathway is coupled to cytoskeletal signaling (i.e., PKC, Src, Pak), survival (i.e., Raf), and transcriptional events (i.e., Erk) to coordinately control this process [30, 66, 67] (Fig. 1.2). Also, it is likely that these kinases are not limited to affecting only one of the critical functions during these events.

We have recently described a novel function for Src family kinases during EC lumen and tube assembly, which is to target and control the development of the apical membrane surface [64]. Marked intracellular vacuole membrane labeling with activated phospho-Src is observed which traffic along acetylated tubulin tracks to fuse in a subapical domain to create an apical membrane also decorated with activated Src isoforms [64]. Blockade of Src isoforms with PP2 completely blocks this process and siRNA suppression of Src, Fyn, and Yes, but not Lyn, interfere with EC lumen formation (Fig. 1.3). PP2 blocks lumen formation whether it is occurring during vasculogenic or angiogenic sprouting events, and interestingly, the addition of PP2 appears to markedly increase the number of EC tip cells as a result of this strong reduction in lumen formation [64].

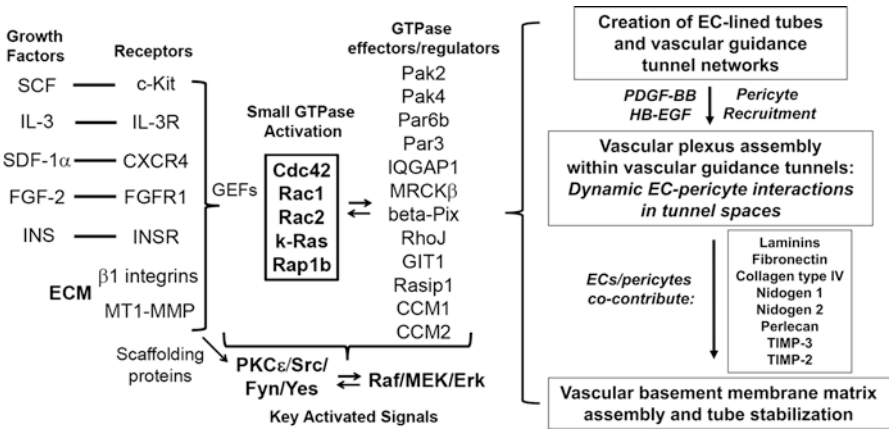


**Fig. 1.3** Fundamental signaling molecules, events, and requirements for the establishment of human capillary tube networks. Human EC tube assembly requires a combination of Factors which are SCF, IL-3, SDF-1α, FGF-2, and insulin, which act through their receptors to activate small GTPases, their effectors, and key kinase cascades. These signaling pathways lead to EC cytoskeletal polarization, vacuole formation, trafficking, and fusion to create a polarized apical membrane surface within the tube networks (which reside within vascular guidance tunnels which are created through MT1-MMP-dependent proteolysis). The tubulogenic signaling cascade leads to production and release of PDGF-BB and HB-EGF which facilitates the recruitment of pericytes to the abluminal surface of EC-lined tubes and within vascular guidance tunnels. Dynamic EC-pericyte motility within tunnel spaces results in the deposition of the capillary basement membrane matrix between the two cell types, a key step in capillary tube development and maturation

### *1.3.4 Identification of New Small GTPases and Their Effectors Controlling EC Tube Morphogenesis*

Recent studies have sought to identify additional small GTPases and downstream effectors that regulate the process of capillary tube morphogenesis. We demonstrated critical roles for k-Ras, Rac2, and Rap1b as well as a new series of effectors including Rasip1, IQGAP1, MRCKβ, GIT1, and beta-Pix (Fig. 1.2) [79]. siRNA suppression of these molecules individually or in combination resulted in marked defects in EC lumen and tube assembly [79]. In contrast, as in past studies, siRNA suppression of RhoA did not interfere with these processes. Interestingly, Rasip1 is known to interact with the RhoA-specific GTPase-activating protein (GAP), Arhgap29 [102], and siRNA suppression of this GAP lead to reduced lumen formation because of elevated RhoA activity [79]. Importantly, we also identified two additional GAPs that negatively regulate EC lumen formation, which are Arhgap31 (which inactivates Cdc42 and Rac1) and Rasa1 (which inactivates Ras isoforms) (Fig. 1.2). Combined siRNA suppression of Arhgap31 and Rasa1 led to marked increases in EC tube formation, suggesting an important combined role for Cdc42/Rac/k-Ras and possibly other Ras isoforms [79]. Combined siRNA suppression of Cdc42 with k-Ras leads to strong blockade of EC lumen and tube assembly [79].

Interestingly, previous collaborative studies revealed the role of another small GTPase, RhoJ, which also plays a role during these events [79, 105]. One of key remaining questions is to define the specific role for each of these individual GTPases. Together they appear to control membrane pinocytosis leading to intracellular vacuole formation, trafficking, and fusion of these vacuoles (and possibly other vesicular structures such as Weibel-Palade bodies) to contribute membrane to the developing apical surface. Interestingly, they appear to also regulate key cytoskeletal modifications such acetylation and detyrosination of tubulin which accumulates subapically to surround and regulate vacuole/vesicle transport (and thus direct new membrane) to the apical domain [79]. In addition, they may stimulate vesicle fusion and exocytic events to create this new polarized apical membrane. Clearly, more work is needed here to define the role of these GTPases and their downstream effectors at the different stages of this process. Of interest to this point is our finding that Rasip1, a Ras and Rap effector, strongly targets apically during the lumen formation process [79]. Apical targeting of Rac1, k-Ras, and Rap1b has also been observed, and interestingly, Cdc42 appears to accumulate predominantly subapically and can interestingly co-localize with acetylated tubulin in this subapical domain [79]. This co-localized region is where vacuole to vacuole fusion events occur to create and expand the apical membrane surface.

### *1.3.5 Cdc42 Coupling to Cell Polarity Pathways Controls EC Lumen and Tube Formation*

A major function of Cdc42 is its ability to affect cell polarity signaling, by interfacing with the polarity proteins Par6, Par3, and atypical PKC isoforms [40, 73]. Cell polarity signaling controls directional cell motility that involves Cdc42 [40]. In fact, active Cdc42 (i.e., Cdc42-GTP) binds directly to Par6 which then couples to Par3, a scaffold protein that also interacts with atypical protein kinase C isoforms, such as PKCζ [73]. We reported that Cdc42-dependent EC lumen and tube formation was dependent on Par6b, Par3, and PKCζ [66] (Fig. 1.3). Thus, this work reveals a fundamental role for Cdc42-dependent polarity signaling in EC tubulogenesis. Par3 is known to interact with a number of other cell surface proteins including members of the junction adhesion molecule (Jam) family (i.e., Jam-A, Jam-B, and Jam-C) [38, 39]. Our most recent work reveals that Jam-B and Jam-C associate with Par3 in ECs to control EC lumen formation in 3D collagen matrices [83]. Furthermore, these Jam proteins co-assemble into a defined EC lumen signaling complex consisting of α2β1, MT1-MMP, Jam-C, Jam-B, Par3, Par6b, and Cdc42-GTP that is responsible for the ability of ECs to form tubes in 3D collagen matrices [83] (see later on). Disruption of any member of this complex markedly interferes with the ability of ECs to form tubes [83]. These lumen signaling complexes are also directly coupled to the kinase cascade discussed earlier including PKCε, Src, Pak, Raf, and Erk1/Erk2, since blockade of these complexes completely interferes with the

downstream kinase signaling necessary to regulate vascular tube morphogenesis [83]. Cdc42 and Par3 have also been shown to control lumen formation in epithelial cells, and a recent study shows that Par3 and β1 integrins co-regulate arteriolar lumen formation in vivo using a conditional β1 integrin subunit knockout mouse system [110]. Thus, this latter work again confirms our prior conclusions obtained in vitro showing that β1 integrins, Cdc42, and polarity proteins control the lumen and tube formation process in 3D matrix environments [30, 36, 66, 67, 79, 83, 95].

### *1.3.6 Key Role for Polarized Subapical Microtubule Modifications to Direct the Trafficking and Fusion of Pinocytic Vacuoles to Regulate Apical Membrane Development During EC Lumen Formation*

Recent studies indicate that an important step in EC lumen and tube assembly is for the increased accumulation of posttranslationally modified tubulins (i.e., acetylated and detyrosinated tubulin) [63] (Fig. 1.2). Both of these modifications, which are associated with increased tubulin polymer stability, are upregulation during EC lumen formation and correlate with the lumen formation process. We demonstrated that the microtubule tip complex proteins, EB1, p150 glued, and Clasp1, play a key role in regulating lumen formation as well as the accumulation of both acetylated and detyrosinated tubulin [63]. In addition, we identified HDAC6 and sirtuin2 as negative regulators of lumen formation due to their ability to reduce tubulin acetylation via their activity as tubulin deacetylases. siRNA suppression of these deacetylases increased lumen formation, while increased expression of them decreased it [63]. Further support for this conclusion is that addition of the HDAC6 inhibitor, tubacin, leads to increased EC lumen and tube formation [79]. Of great interest here is that these modified tubulins accumulate in a polarized manner subapically during EC lumen and tube assembly, while in contrast, filamentous actin (F-actin) accumulates in a distinct basal location [64, 79]. This subapical polarized region where acetylated and detyrosinated tubulin accumulates is precisely the location where vacuole and vesicle fusion events occur, to control the development of the apical membrane surface during this process. Interestingly, Cdc42 shows strong co-localization with acetylated tubulin in these regions of vacuole/vesicle fusion [79]. Thus, one of the central features of the EC lumen formation process is the creation of cytoskeletal asymmetry and tracks where vesicles can be trafficked to this subapical domain, to polarize the lumen formation process. Furthermore, our data suggests that this subapical membrane accumulation of modified tubulins is necessary to stabilize the apical membrane and maintain a stable tube structure [63, 64, 79]. We demonstrated in past studies that disruption of microtubules led to rapid collapse of tube networks [10].

### *1.3.7 Critical Functional Role for MT1-MMP in EC Lumen and Tube Formation in 3D Collagen Matrices*

Matrix metalloproteinases (MMPs) are a family of zinc-dependent metalloendopeptidases that degrade a variety of substrates, to affect the vasculature and other tissues [33, 47]. Their targets include the ECM, cytokines, and cell surface receptors, to affect vascularization as well as many other cellular responses [33, 47]. Our work and that of others have demonstrated a role for MT1-MMP (i.e., MMP-14) in EC morphogenic events in 3D matrix environments [21, 87, 95]. MT1-MMP is a transmembrane protein, and its cell surface expression is required for it to perform the localized ECM degradation necessary to control cell movement in 3D matrices. MT2-MMP is also able to participate in these types of events, while the function of MT3-MMP is less clear, although several studies show that it does not play a major role. Mouse knockout of MT1-MMP is compatible with embryogenesis, but the mice are small and ill and die within a month or two of birth [108]. Attempts to induce angiogenesis in these mice reveal that these responses do not occur [108]. Furthermore, aortic ring assays in 3D collagen matrices show no sprouting, in either 3D collagen or fibrin matrices using MT1-MMP knockout tissues compared to control [21]. Thus, this work demonstrates an important role for MT1-MMP in vascular morphogenic events in 3D matrices and during in vivo angiogenic responses (Fig. 1.4).

To elucidate the molecular mechanisms by which MT1-MMP controls vascular morphogenesis as well as cellular invasive events, our laboratory has examined this question using models of vasculogenesis and angiogenesis [9, 87, 95]. We have utilized protein and chemical MMP inhibitors as well as siRNA suppression approaches. In our first studies, we demonstrated that EC sprouting in response to sphingosine-1-phosphate (incorporated into the collagen matrices) was blocked by the broad spectrum inhibitor, GM6001, as well as tissue inhibitor of metalloproteinase (TIMP)-2, TIMP-3, and TIMP-4, but not TIMP-1 (Fig. 1.4a) [9]. Interestingly, MT-MMPs are insensitive to TIMP-1, but the other inhibitors utilized block their activity. When EC lumen and tube formation assays were performed, GM6001, TIMP-2, TIMP-3, and TIMP-4 blocked completely, while TIMP-1 had no influence. One important distinction among the TIMPs is that TIMP-3 is able to block the activity of MT-MMPs, but also many other members of the ADAM family of cell surface expressed metalloproteinases [7]. To functionally dissect which EC surface expressed metalloproteinases are relevant during EC sprouting and lumen formation, we performed siRNA suppression analysis. Our results suggest that the dominant metalloproteinase controlling these events is MT1-MMP (Fig. 1.4b), with a lesser influence of MT2-MMP during both sprouting and lumen formation [87, 95]. We observed a partial blocking effect of ADAM-15 siRNA knockdown in EC sprouting assays, using stromal-derived factor-1α as the invasion stimulus [87]. We did not observe an effect of either MT3-MMP or ADAM-17 siRNAs in our assays [87], although a recent study using a similar system revealed a potential role for ADAM-17 in modulating the invasion response [69]. The fact that TIMP-2 and

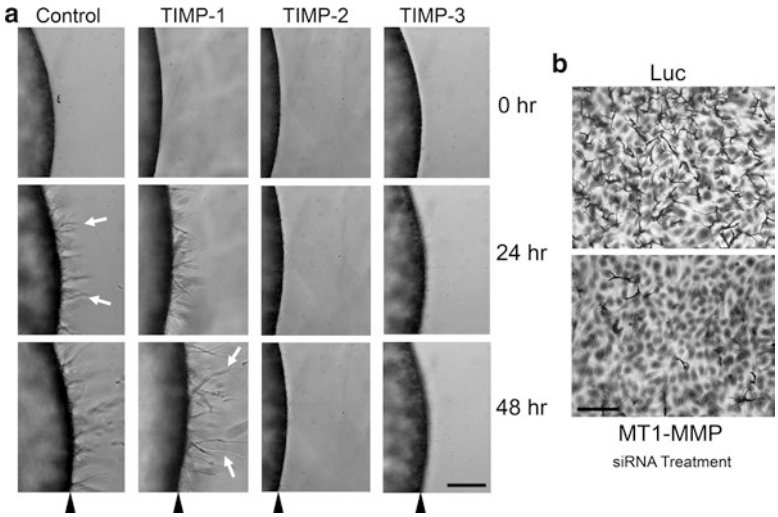


**Fig. 1.4** MT1-MMP plays a critical role in EC sprouting in 3D collagen matrices from a monolayer surface in sprouting angiogenesis bioassays. (**a**) A time-lapse series was performed of EC sprouting viewed from the side into 3D collagen matrices where either no addition or recombinant TIMPs were added. Sphingosine-1-phosphate (1 μm) was added into the collagen matrix. Arrowhead indicates EC monolayer surface; arrows indicate EC sprouts. TIMP-2 and TIMP-3 block sprouting in an equivalent manner, while TIMP-1 has no blocking influence relative to the control. Bar equals 100 μm. (**b**) An siRNA suppression experiment was performed to examine the influence of an MT1-MMP siRNA versus a luciferase control. Sprouting assays were performed using the treated cells, and they were seeded on collagen matrices containing 1 μm sphingosine-1-phosphate. Cultures were fixed, stained, and photographed after 24 h. Bar equals 100 μm

TIMP-4 have dramatic blocking effects on both sprouting and lumen formation is more supportive of a major role for MT-MMPs, rather than ADAMs, since there is currently no evidence to suggest that they can block ADAM proteinases [7].

A further important experiment which demonstrates a role for MT1-MMP during EC tubulogenesis is that increased expression of MT1-MMP using viral vectors leads to marked increases in lumen formation that depends on its MMP catalytic domain [95]. Furthermore, addition of GM6001 to block MT1-MMP completely inhibits the stimulatory influence of the recombinant protein. In support of this result is that a catalytically dead full-length MT1-MMP construct (EA mutant) has no ability to stimulate EC lumen formation [95], while interestingly, it does not exert an inhibitory influence. In very recent experiments, we have further shown that increasing the expression of wild-type, full-length MT1-MMP increases both the rate and extent of EC lumen formation in 3D collagen matrices [83]. Of great interest is that we have created a construct that appears to be a dominant-negative mutant of MT1-MMP in this system where we mutated the active site and at the same time deleted its cytoplasmic tail [83]. When expressed in ECs, the cells are completely unable to make lumens in 3D collagen matrices. An additional finding is that

expression of wild-type MT1-MMP without its cytoplasmic tail markedly stimulates the rate and extent of EC lumen formation compared to full-length wild-type MT1-MMP expression [83]. A number of studies suggest that its cytoplasmic tail plays a role in endocytic recycling, and thus, deleting the tail increases cell surface expression, which in our case leads to additional increases in EC lumen formation events. Overall, these results demonstrate that MT1-MMP is a major regulator of EC lumen and tube formation and that it works closely in conjunction with the α2β1 integrin as well as Cdc42, Rac1, and other small GTPases to control this process.

### *1.3.8 MT1-MMP-Dependent EC Lumen and Tube Formation Leads to the Formation of a Network of Physical Spaces Within the ECM Termed Vascular Guidance Tunnels*

During the course of the above studies, we made the novel observation that during EC lumen and tube formation, ECs are also creating a network of physical spaces that we term vascular guidance tunnels. These form as a result of MT1-MMP-mediated proteolysis of collagen matrices [95]. In every instance examined, there is a direct relationship between EC tube formation and the formation of vascular guidance tunnels [95]. Tunnels were first detected by staining the collagen type I matrix with a monoclonal antibody that recognizes native type I collagen and not denatured collagen (which is generated at 37 °C when it is cut with mammalian collagenases). The lumen and tube formation creates an extensive interconnecting network of these tunnel spaces within the 3D collagen matrices [95]. To further prove that these represent physical spaces in the ECM, they were microinjected with silicone oil [95]. Dramatic filling of networks was demonstrated showing that EC tube formation leads to the formation of interconnecting vascular guidance tunnel spaces. We further showed that the ECs produced twice as many tunnel spaces than were occupied by EC-lined tubes [95], raising the interesting possibility that vessel remodeling could occur through these pre-formed physical tunnel spaces. Although MT1-MMP was required for the formation of vascular guidance tunnel formation, once they were formed, blockade of MT1-MMP did not affect the ability of ECs to migrate within the spaces [95]. Thus, EC migration events which are necessary for EC tube formation are completely inhibited in 3D collagen matrices, if MT1-MMP is blocked from the beginning of culture. However, once vascular guidance tunnels have formed through MT1-MMP-mediated events, ECs are then able to migrate within these physical spaces in an MMP-independent manner [95]. Thus, vascular guidance tunnel spaces are similar to 2D matrix surfaces where EC motility is insensitive to MT1-MMP inhibition (siRNA or inhibitors) [95]. We also observed that while the creation of vascular guidance tunnels by ECs requires the α2β1 integrin, a native collagen-binding integrin, the motility of ECs within MT1-MMP-generated tunnels was not sensitive to inhibition with anti-α2 integrin subunit blocking antibodies. In contrast, EC motility was blocked using anti-αv subunit blocking antibodies [95] which are known to bind matricryptic RGD sites that are

present within unfolded collagen molecules following proteolysis [24, 26] (an event which controls the generation of the tunnel spaces).

Another important finding from this work is that inhibitors of EC lumen and tube formation, including anti-α2 and anti-β1 integrin blocking antibodies, chemical inhibitors of PKC and Src, as well as MT1-MMP inhibitors, completely abrogate the formation of vascular guidance tunnels [95]. Thus, the formation of EC tubes is an obligate step in the formation of vascular guidance tunnels, and thus, these processes are directly linked in some fundamental manner. Several critical questions arise from these studies, including how the lumen and tube formation processes are functionally connected with the cell surface proteolytic machinery to create vascular guidance tunnels. Very recent work, described below, provides some insights into these questions.

### *1.3.9 Cdc42 and MT1-MMP Are Functionally Interdependent Signaling Molecules Which Are Components of an EC Lumen Signaling Complex that Controls EC Tubulogenesis in 3D Extracellular Matrices*

Several newer findings begin to shed light into how Cdc42-dependent signaling events, which activate kinase cascades and interact with cell polarity machinery (i.e., Par3, Par6, atypical PKC), intersect with MT1-MMP proteolysis to create EC lumens, tubes, and vascular guidance tunnels [66, 67, 83, 95]. One important point is that the EC lumen and tube formation is a 3D matrix-specific process [27], in that tubulogenesis does not occur on a 2D matrix surface. In contrast, EC motility can occur quite readily on a 2D matrix surface, while it also occurs in 3D matrices in a manner that depends on MT1-MMP proteolytic events. Importantly, MT1-MMP activity is not required for EC motility on a 2D matrix surface, as discussed above. With this introduction, our findings show that blockade of MT1-MMP activity using siRNA suppression or MT1-MMP inhibitors leads to marked interference with Cdc42 activation (a critical step necessary for activation of effectors such as Pak2, Pak4, and Par6 that leads to EC tubulogenesis) in 3D collagen matrices [83]. However, this blockade of MT1-MMP does not affect Cdc42 activation of ECs when they are seeded on 2D collagen surfaces, and coincidently, their motility is also not affected [83]. Expression of the dominant-negative MT1-MMP construct also markedly blocks lumen formation and Cdc42 activation [83]. Interestingly, the activation of RhoA, which is not involved in EC lumen and tube formation, is not affected by blockade of Cdc42 or MT1-MMP, nor is it affected by 2D vs. 3D collagen matrices. This data shows that MT1-MMP activity is directly coupled to Cdc42 activation in 3D, but not 2D, matrices to control the tube formation process [83]. The reverse is also true, in that blockade of Cdc42 using siRNA suppression leads to marked decreases in vascular guidance tunnel formation, a consequence of inactivation of MT1-MMP-dependent proteolysis [83]. Thus, this work suggests a

new hypothesis which states that Cdc42 and MT1-MMP are interdependent signaling molecules that control vascular morphogenic events specifically in a 3D matrix environment.

### *1.3.10 Critical Role for MMPs in the Molecular Control of Vascular Tube Regression Responses in 3D Collagen Matrices*

A critically important direction of research is to understand how blood vessels regress under physiologic and pathophysiologic situations. Physiologic regression occurs with hyaloid vessels in the developing eye, in both the endometrium and ovaries during the menstrual cycle, and during vascular remodeling events in development [33]. Pathophysiologic regression characteristically occurs during wound repair and during disease processes such as hypertension and diabetes where vessel densities can decrease particularly in the distal limbs. Also, there have been considerable efforts to induce vascular tube regression responses, in the context of the tumor vasculature by disrupting VEGF and PDGF signaling [15]. An important point here is that it is critical to understand how vascular regression is controlled at the molecular level, in much the same way that the studies described earlier have been performed to determine how blood vessels form.

A variety of studies have identified MMPs that regulate vascular tube regression events [32, 86, 109]. A number of years ago, we identified the secreted MMPs MMP-1 and MMP-10 as being involved in vascular tube regression responses in vitro [32, 33, 35, 86]. We showed that these enzymes were secreted as proenzymes and that they need to be activated by serine proteases, such as plasminogen/plasmin or plasma kallikrein, in order to cause vascular tube collapse and regression [86]. Disruption of their activity by TIMP-1 (which blocks both MMP-1 and MMP-10) or blockade of serine protease activity leads to inhibition of the MMP-1- and MMP-10-dependent regression response. Other studies using the aortic ring model reached similar conclusions, with the exception that MT1-MMP was also found to be involved in both tube formation as well as regression [4]. We also identified ADAM-15 as being involved in the vascular regression response in a manner similar to that of MMP-1 and MMP-10 [87]. Interestingly, siRNA suppression of either MMP-1 or MMP-10 did not affect tube formation but markedly blocked tube regression, following addition of plasminogen or plasma kallikrein to the serum-free media system [86]. Also of note, a mouse knockout of histone deacetylase 7 (HDAC7) caused a vascular hemorrhage phenotype in vivo, during vascular development, which leads to embryonic lethality [20]. siRNA suppression of HDAC7 resulted in marked increases in MMP-10 and marked decreases in TIMP-1 expression which lead to the vascular developmental regression phenomenon [20]. Other work reveals that EC-specific knockout of the chromatin remodeling enzyme,

CHD4, leads to plasmin-dependent disruption of vascular integrity in both blood-derived and lymphatic EC-lined vessels during vascular development [22, 59].

Of great interest is that the MMP-1 and MMP-10 regression phenomena are strongly abrogated in our in vitro model when pericytes are added along with the ECs [87]. Pericyte recruitment to the tubes occurs, and they become much more resistant to pro-regressive stimuli. Thus, our model mimics that observed in vivo where EC tubes without pericytes are much more susceptible to regression [5, 13, 14]. Interestingly, tumor vessels have associated pericytes, but the interactions are abnormal. Despite these abnormalities the tumor vessels persist, which in this case may relate to the fact that many aggressive tumors overproduce TIMP-1 [101], which can interfere with the MMP-1 and MMP-10-dependent regression system [33]. We also observed that EC-pericyte interactions upregulate the production of EC TIMP-2 and pericyte TIMP-3 which together control how EC-pericyte interactions protect against pro-regressive MMP-1 and MMP-10, and also ADAM-15 [87]. Pericytes are a rich source of TIMP-3 [87] which is interesting because of its ECM-binding ability (i.e., ability to bind cell surfaces and basement membrane matrices) and its dual ability to inhibit soluble and membrane MMPs, as well as membrane ADAM proteinases [7]. In addition to interfering with pro-regressive stimuli, TIMP-3 and TIMP-2 block MT1-MMP, to interfere with further EC tube formation. Thus, these TIMPs contribute to vascular tube stabilization by inhibiting both vascular tube regression phenomena and further vascular tube morphogenesis. Additional support for this their latter influence, both TIMP-2 and TIMP-3 are antagonists of VEGFR2 [80, 90], an important EC signaling receptor. Interestingly, we have described the concept that VEGF does not directly stimulate vascular tube morphogenesis but that it primes ECs so that they respond in a more robust manner to a distinct set of downstream growth factors (see later on). Using this reasoning, TIMP-2 and TIMP-3 might be capable of suppressing EC priming and, thus, enhance EC tube stability in this manner.

### *1.3.11 Critical Functional Role for EC-Generated Vascular Guidance Tunnels During Blood Vessel Assembly in 3D Matrices*

As shown in Fig. 1.2, ECs utilize a lumen signaling complex to form tubes in 3D matrices while at the same time generating networks of vascular guidance tunnel spaces. ECs are able to migrate through these spaces in an MMP-independent manner. We have also shown that ECs can regrow within these spaces following tube collapse. EC-lined tubes were treated with thrombin which reversibly causes tube collapse, leaving rounded up ECs within tunnel spaces [95]. After inhibition of thrombin with the thrombin inhibitor hirudin, the ECs regrow within tunnels to reassemble the collapsed tube [95]. Thus, pre-existing tunnel spaces are matrix conduits that allow for rearrangement of tubes and migration of ECs, and, thus, they are used

for tube remodeling events. In early vascular development, there is considerable evidence for dramatic tube network remodeling that occurs following the onset of flow [23, 72, 82], and we hypothesize that this is possible in large part due to the presence of vascular guidance tunnels which allows ECs to rapidly rearrange to accommodate the flows and pressure forces that are applied to the network. Also, at this stage of development, the ECM is likely to be elastic, and, thus, the forces generated may be able to expand lumen or tunnel width by mechanical distension. We have also shown that groups of cells comprising a tube structure can migrate together through tunnel spaces, to move and connect with adjacent EC tubes to regulate such vascular remodeling events [95]. As mentioned earlier, the EC lumen and tube formation process generates more vascular guidance tunnels than are utilized at any given time, which further suggests that this occurs to accommodate the necessary vascular remodeling events involved in generating a proper microcirculatory network.

Vascular guidance tunnels are also important to consider in the context of vascular tube regression and regrowth of vessels. One of the ways to eliminate the possibility of vascular regrowth following regression events would be to induce regression of both vascular tubes and vascular guidance tunnels. In fact, the MMP-1 and MMP-10 regression mechanism discussed earlier does cause the collapse of both structures. The presence of pericytes, which block the regression event, can thus protect not only the vascular tube structure but also the integrity of the vascular guidance tunnels. Of interest here is that tumor vessels are highly resistant to vascular regression, due to their production of regression inhibitors such as TIMP-1. Again, TIMP-1 is capable of protecting both the vessels and the tunnel spaces. Also, when tumors are treated with vascular regression agents, such as VEGF or VEGFR2 antagonists, vessels regress, but they can rapidly regrow (following withdrawal of the regression agent), in a similar fashion, to recapitulate the original pattern of vessels [74]. This appears to occur through the vascular guidance tunnels that were generated during initial tumor vessel formation. So an important therapeutic consideration here would be to devise approaches to induce vessel and vascular guidance tunnel regression. In this way vessel regrowth is less likely to occur, allowing for a better therapeutic opportunity to treat the tumors, their vascular supplies, and existing matrix conduits which facilitate vascular regrowth.

It is also important to consider how events such as arteriovenous identity might be regulated by vascular guidance tunnels. The tunnels represent a 2D matrix surface in a 3D matrix environment [95]. There is important data showing that ephrinB2 (an arterial marker) and EphB4 (a venous marker) represent a repulsive signaling pair, which appears early in development to control the development of A-V identity [46]. These repulsive interactions allow differential cell sorting, and, early in development, ECs expressing these markers are intermixed. Over time they sort out and become segregated to either the arterial or venous side [46, 53, 65]. They also appear to sort very early, even at the level of initial cardinal vein formation, due to sprouting from the developing aorta. This process is very analogous to what has been described for lymphatic sprouting and development from the cardinal vein [103]. Notch signaling appears to control this phenomenon, and, when

overactive Notch4 is produced in ECs, venous ECs inappropriately express ephrinB2, contributing to the development of arteriovenous malformations [65]. Of great interest is that many of these lesions will regress following withdrawal of the overactive Notch4. The important point to be made is that the repulsive ephrinB2-EphB4 interactions are occurring within vascular guidance tunnels (formed as a result of EC tube assembly), and the ability to sort following such interactions requires the ECs to move around on 2D matrices at the vessel wall surface. These interactions are secondary to ECs contacting each other.

However, the concept of vascular guidance tunnels extends to recruitment and sorting of mural cells within the vessel wall as well. EphrinB2 is expressed on vascular smooth muscle cells, with selectivity in arteries so similar repulsive interactions are likely to control this cell distribution as well [41]. What is interesting here is that this type of interaction would require that mural cell recruitment, to EC-lined tubes and within vascular tunnel spaces, occur in a polarized fashion exclusively on the EC abluminal surface. In fact, we have recently discovered that this is precisely what occurs during pericyte recruitment to EC-lined tubes. Within vascular guidance tunnels, they recruit to, and are localized only on, the EC tube abluminal surface [94]. Thus, mural cells could sort through repulsive interactions with each other but also secondary to repulsive interactions with ECs. The vascular guidance tunnel matrix conduit is a critical ECM structure necessary for these EC-EC, mural cell-mural cell, and EC-mural cell interactions to occur and to also allow the motility events required for proper sorting. In support of this possibility is that ECs and mural cells are highly dynamic during vascular tube assembly, and in fact, we have shown that both ECs and pericytes rapidly migrate in the ECM, as well as within vascular guidance tunnels during tube co-assembly and maturation events [94]. ECs have also been shown by a number of groups to be rapidly migrating in vivo during vascular development, to regulate both tube assembly and vascular remodeling [23, 72].

### *1.3.12 Pericyte Recruitment to Vascular Guidance Tunnels Induces Vascular Tube Stabilization*

Many studies indicate that microvessels covered with pericytes are more stable to pro-regressive stimuli but also show reduced vascular permeability indicative of tube stabilization. Different vascular beds have varying pericyte numbers covering capillary networks, although many have approximately 20–25% coverage of pericytes relative to ECs. Individual pericytes can span across multiple ECs, resembling other types of supporting cells such as glia in the nervous system interacting with multiple neurons. Tissues such as the central nervous system, including the retina, have a very high pericyte to EC ratio which approaches 1:1. Thus, these interactions account in part for the blood-brain barrier with strongly increased permeability barrier functions relative to other vascular beds. Considerable work suggests that the

high VEGF environment of tumors is one reason why pericyte coverage is decreased compared to normal vascular beds [48, 61]. Treatment with VEGF antagonists has led to the finding that pericyte coverage increases, which results in improved microcirculatory function (i.e., vascular normalization) [61]. This approach represented a new strategy to improve drug delivery into the tumor microenvironment, since poor perfusion exists due to the abnormal microcirculatory network that is present.

### *1.3.13 Molecular Mechanisms Underlying Why Pericytes Are Able to Stabilize EC-Lined Tube Networks*

A major question that has not been sufficiently addressed is why pericyte coverage stabilizes vessels and what their functions are when they arrive at the EC abluminal surface. To address this question, we established novel EC-pericyte coculture models in 3D collagen matrices (Fig. 1.5). We developed systems using either bovine retinal pericytes or human brain pericytes. In each case, the pericyte populations express the pericyte markers NG2 proteoglycan, 3G5 ganglioside, smooth muscle actin, and desmin. Perhaps the most important function of pericytes is to recruit to microvascular capillary beds. Using our model of EC vasculogenic tube assembly, we developed a system whereby we randomly mix together ECs and pericytes at a 5:1 or 5:1.25 ratio (i.e., 20–25% pericytes compared to 100% of ECs). Remarkably, the ECs form tube networks, and then, pericytes are recruited to these tubes [94] (Fig. 1.5). This ratio of ECs to pericytes is particularly optimal, and the reasons for this are currently not clear. It may be that too many pericytes (through their production of TIMP-3) [87] interfere with morphogenesis, by inhibiting MT1-MMP-dependent signaling, or that they are physically in the way and counteract the ability of ECs to find neighbors to properly form multicellular tubes. It is clear that too many pericytes can disrupt EC-pericyte tube co-assembly.

We further made the observation that EC tubes, from EC-only cultures, eventually became much wider than EC tubes from EC-pericyte cocultures. We examined this issue over time and observed that vascular diameters reached a range of 20–25 μm in EC-pericyte cocultures, which are vessel diameters observed in vivo during vasculogenesis, while EC-only cultures' diameters can reach 80–100 μm over a 5-day period [94]. Thus, pericytes have a marked ability to negatively regulate vascular tube diameters, which may have to do with the induction of TIMP-2 and TIMP-3. As discussed earlier, they are induced in EC-pericyte cocultures [87] and can inhibit and restrict EC lumen diameters. A number of studies indicate that vascular diameters are greater when pericyte recruitment is reduced or when ECM components such as fibronectin are knocked out of ECs during vasculogenesis in vivo [6, 42].

The mechanisms whereby pericytes are recruited to EC-lined tubes are still being investigated although past data supports the concept that PDGF-BB plays a role [5, 16, 54]. In past and ongoing studies from our laboratory, we have shown that pericyte recruitment is dependent on signals derived from the combined action

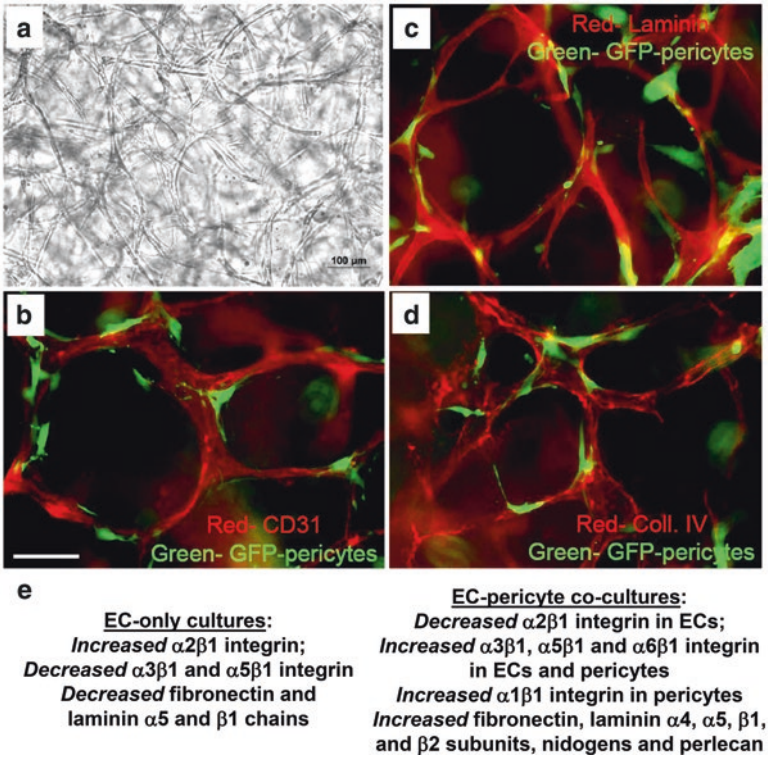


**Fig. 1.5** EC-pericyte tube co-assembly in 3D collagen matrices leads to vascular basement membrane matrix deposition and tube stabilization. ECs were cocultured with bovine retinal pericytes (20% pericytes relative to 100% ECs) in 3D collagen matrices and after 5 days of culture were fixed and photographed (**a**) or were processed for immunofluorescence microscopy. The pericytes were labeled with GFP, while the ECs and extracellular matrix were stained with the indicated antibodies. Fluorescent images were overlaid to assess the relationship of the ECs and matrices with the presence of pericytes. (**b**) CD31 staining to detect EC-lined tubes. Bar equals 50 µm. (**c**) Laminin and (**d**) collagen type IV staining to detect vascular basement membrane matrix assembly. In the latter two cases, no detergent was utilized so that only extracellular antigens would be detected. (**e**) RT-PCR and Western blot analyses of EC-only versus EC-pericyte cocultures demonstrate marked changes in integrin and basement membrane matrix protein expressed during this process

of PDGF-BB and HB-EGF [96]. Blockade of these growth factors using blocking antibodies, receptor traps, or chemical disruption of their receptors revealed their involvement [96]. Importantly, we assessed pericyte motility and invasion under our serum-free media conditions and demonstrated that they failed to move, when cultured by themselves. In contrast, when pericytes were cocultured with ECs, pericyte motility ensued allowing for recruitment to EC-lined tubes [96]. This EC-dependent pericyte motility response was strongly abrogated by the combined blocking reagents directed at both PDGF-BB and HB-EGF [96]. These findings are consistent with work showing that EC-specific knockout of PDGF-BB leads to

about a 50% decrease in pericyte coverage of vessels [16]. Of interest is that these mice show primary defects in microvessel beds (where pericyte coverage is present), while larger vessels are much less affected. This microvascular deficiency phenotype manifests particularly in the kidney and central nervous system which strongly resembles that observed in diabetic microangiopathy [16]. Loss of pericytes is a major pathogenic cause of this type of microvascular disease [51]. It is important to further understand the signaling mechanisms which underlie pericyte recruitment to EC-lined tubes, to both identify other factors that regulate this recruitment as well as understand how pericytes invade 3D matrices to recruit to these tubes. This is currently a major research direction of our laboratory.

### *1.3.14 Pericyte Recruitment to EC-Lined Tubes Stimulates ECM Remodeling Events and Vascular Basement Membrane Matrix Assembly*

Using our new model of EC-pericyte tube co-assembly, we sought to identify how pericytes contribute to vascular tube maturation and stabilization events. At different time points of tube co-assembly, we performed transmission electron microscopy and immunofluorescence microscopy to examine if basement membrane matrix assembly occurred [94]. In Fig. 1.5b, we show immunostaining for the EC marker CD31, while the pericytes stably express green fluorescent protein (GFP). This image shows EC tube networks that have associated pericytes at day 5 of culture. We also show a light microscopy image of the coculture system in Fig. 1.5a. As shown in Fig. 1.5c, d, there is marked deposition of laminin and collagen type IV, two critical basement membrane matrix components. In addition, we reported that fibronectin, nidogen-1, nidogen-2, and perlecan were also deposited around EC-lined tubes only when pericytes were cocultured with ECs [94] (Fig. 1.5e). We demonstrated that basement membrane matrices were observed by transmission electron microscopy, only when EC-pericytes were cocultured [94], and over many years we have never observed basement membrane deposition in the absence of pericytes in electron microscopic studies. Further, we confirmed our results in vivo and demonstrated that pericyte recruitment to developing quail EC tubes directly correlates with vascular basement membrane assembly, at day 7 of embryonic development [94]. Prior to pericyte recruitment, no vascular basement membranes around EC tubes were observed in vivo [94].

To perform the immunostaining experiments, we utilized detergent-free conditions so that we are examining only ECM that is deposited extracellularly [94] and not intracellular ECM molecules. We utilized this approach in our in vitro 3D cultures but also stained an in vivo tissue, the quail chorioallantoic membrane, in the same manner [94]. This is key point because we have shown that extracellular deposition of vascular basement membrane matrix is markedly stimulated by pericyte

recruitment [94]. Although increased production of individual basement membrane components was observed, this upregulation was not as marked as that observed in the immunostaining experiments. We utilized our human EC and bovine pericyte coculture system to determine which cell type produces particular ECM components over time, to regulate basement membrane matrix assembly (using species-specific RT-PCR primer sets) [94]. Major findings were that ECs increased the production of fibronectin selectively in the presence of pericytes (and not in their absence) and nidogen-1 was induced in pericytes that occurred selectively in the presence of ECs [94]. We also observed induction of particular laminin isoforms as well as perlecan at the mRNA level, which occurred through EC-pericyte interactions. Thus, EC-pericyte contacts during tube co-assembly events affected mRNA and protein levels for key basement membrane matrix molecules [94]. Interestingly, both fibronectin and nidogen-1 are known to bridge key molecules that compose the basement membrane matrix [34, 76, 89]. Fibronectin shows affinity for collagen type IV and perlecan, while nidogen-1 binds collagen type IV and laminin isoforms. It is possible that these ECM components initiate a nidus which leads to the assembly of the insoluble matrix surrounding the EC-lined tubes that control basement membrane deposition, as observed by electron microscopy. Most ECM proteins have self-assembly functions, but they need to interact with each other to create the complex meshwork that is characteristic of fully assembled basement membrane matrices. It is also intriguing that collagen type IV, a fundamental basement membrane component greatly responsible for its structural integrity, shows affinity for both fibronectin and nidogen-1, which are selectively affected by EC-pericyte interactions [94].

One question that is of great interest is how continuous basement membrane assembly is accomplished along EC-lined tubes, despite the fact that pericytes are only one-fifth to one-fourth of the total number of ECs. We believe that this occurs due to the motility of pericytes along the abluminal EC tube surface which scans along the tubes to stimulate the deposition of the basement membrane in a continuous manner [94]. Furthermore, the movement of both pericytes and ECs along each other, within vascular guidance tunnels, will almost certainly exert mechanical stress on the newly deposited ECM to facilitate basement membrane assembly. Thus, it is intriguing that fibronectin, a mechanosensitive ECM component whose assembly is facilitated by cell-exerted tensional forces, is a critical protein that only strongly deposits around EC-lined tubes when pericytes are present along the tube surface [94]. Another interesting possibility is that the presence of pericytes, along the EC abluminal surface (and in a polarized fashion) [94], may stimulate the directional secretion and deposition of basement membrane components from both cell types toward each other. Thus, both mechanical forces and vectorial secretion mechanisms may play a fundamental role in how pericyte recruitment to EC-lined tubes leads to vascular basement membrane matrix assembly, a major step toward further tube maturation and stabilization. As discussed above, the deposition of laminin isoforms may represent stimuli for ECs to stop undergoing morphogenesis and become a stable tube structure with a quiescent layer of ECs. Another molecule, TIMP-3, whose deposition in the basement membrane would lead to a similar

phenotype, binds basement membrane perlecan as well as other components, to suppress vascular morphogenesis [87]. The roles of these individual components need to be investigated in more detail in future studies.

### *1.3.15 Critical Functional Role for Fibronectin Matrix Assembly During Vascular Development*

A series of studies indicate that fibronectin gene knockouts result in an embryonic lethal phenotype during vasculogenesis [42, 57]. Also, evidence has been presented that fibronectin alternative splicing (IIIA and IIIB isoforms) is important during these events [6]. Further work will be necessary to elucidate why these particular fibronectin isoforms are playing a role during these processes. Vessel diameters from these animals are extremely wide which, as discussed above, may be secondary to defects in proper EC-pericyte interactions causing abnormal basement membrane formation or reduced adhesiveness to these remodeled, but abnormal, matrices. EC-specific knockout of the α5 integrin also shows phenotypes that are manifested in a wider vessel phenotype, which appears to be further enhanced by knockout of αv integrins, another class of fibronectin receptors [57].

Since we observed strong fibronectin upregulation in ECs, as well as deposition selectively in EC-pericyte cocultures, we performed additional experiments to determine if fibronectin matrix assembly affected EC tube maturation events in this system. We incorporated a 70 kDa N-terminal fragment of fibronectin, which is known to block fibronectin matrix assembly [106], to assess if it had any influence during these events. Our work shows that disruption of fibronectin matrix assembly affects EC tube width by significantly increasing it [94], suggesting that deposited fibronectin may play a role in restricting vascular tube diameter. Interestingly, this treatment also markedly disrupted collagen type IV matrix deposition [94] while having a lesser influence on laminin assembly. In support of these findings are experiments showing that selective blockade of EC α5β1 integrin, a fibronectin receptor, also significantly increases vascular tube width in the EC-pericyte cocultures, but not in the EC-only cultures, where this receptor appears to play little role [94].

### *1.3.16 Important Functional Role for Collagen Type IV in EC-Pericyte Tube Co-Assembly and Maturation Events*

In addition to the critical roles for fibronectin and nidogen-1, as bridging proteins for ECM assembly, collagen type IV is another key basement membrane component with affinity for both of these bridging molecules. Interestingly, pericyte-induced fibronectin assembly around developing tubes appears to be involved in collagen type IV assembly [94]. To assess which cell types contributed the collagen type IV

that was deposited extracellularly during these events, we performed siRNA suppression experiments revealing that ECs were the predominant source of collagen type IV [94]. Knockdown of collagen type IV in ECs strongly decreased collagen type IV assembly around tubes and resulting again in increased vascular tube width, an indicator of dysfunctional interactions between ECs and pericytes [94]. Knockdown of collagen type IV in pericytes had lesser, but nonetheless significant, inhibitory effects on both collagen type IV deposition and vessel tube width [94]. This suggests that both ECs and pericytes contribute collagen type IV during basement membrane assembly.

### *1.3.17 Pericyte TIMP-3 Contributes to Vascular Basement Membrane Matrix Assembly by Increasing Collagen Type IV Deposition or Stability*

Another contributing role of pericytes during this process is the delivery of TIMP-3, a basement membrane and ECM-binding protein. As discussed earlier, TIMP-3 plays a critical role in pericyte-induced tube stabilization by blocking MMP-1, MMP-10, and ADAM-15, which promote vascular regression events as well as inhibiting further morphogenic events by blocking MT1-MMP [87]. In this work, we show that TIMP-3 plays yet another role, by facilitating collagen type IV assembly in EC-pericyte cocultures. siRNA suppression of pericyte TIMP-3 results in markedly decreased collagen type IV assembly [94], which may be due to less deposition or increased turnover due to lack of inhibition of MT1-MMP (which degrades type IV collagen). With decreased collagen type IV assembly around EC tubes, there was a significant increase in vessel diameter [94]. Thus, collagen type IV assembly may be a primary determinant of vascular tube diameter. It is particularly intriguing to consider that EC-only tubes, which are not surrounded by basement membranes, become very wide during morphogenic events. This suggests a lack of inhibitory signals. EC-pericyte co-assembled tubes are much narrower, suggesting that the inhibitory signals are delivered to ECs through interactions with the assembled basement membrane to suppress further morphogenesis and promote maturation. The marked differences in vessel diameter in these two situations demonstrate functional evidence for both the production and deposition of basement membrane matrices but also reveal that ECs recognize the proteins and respond by restricting tube diameter. Decreased vessel diameter and tube network areas are measurements that reflect the ability of pericytes to negatively regulate vascular tube morphogenesis while at the same time preventing pro-regressive stimuli from acting. Thus, EC tube diameter is also an important indicator of dysfunctional EC-pericyte interactions that lead to a variety of vessel abnormalities (which frequently show increased vessel diameter). In addition, it is well known that basement membranes can facilitate cell polarity functions, by enhancing cell-cell contacts mediated through junctional contacts, such as through adherens and tight junctional

proteins. Although the EC apical membrane domain has been difficult to define in molecular terms, the clear evidence of EC polarization in our coculture model is the deposition of basement membrane matrices specifically to the abluminal surface and the prior recruitment of pericytes to these same abluminal membranes [94].

### *1.3.18 Specific Upregulation of EC and Pericyte Integrins Recognizing Basement Membrane Matrices During EC-Pericyte Tube Co-Assembly in 3D Collagen Matrices*

Again, using our coculture system with human ECs and bovine pericytes, we assessed how EC vs. pericyte integrins were regulated during this process. We assessed mRNA levels and performed function blocking experiments with anti-integrin monoclonal antibodies. As discussed above, blocking antibodies to the α5β1 integrin had function blocking effects that selectively occurred in the EC-pericyte cocultures but not the EC-only cultures [94]. Interestingly, the EC α5 integrin subunit was induced at the mRNA level in EC-pericyte cocultures, but not in EC-only cultures where it was downregulated. An important theme which emerged from these studies is that integrins which recognize the newly remodeled ECM assembling between ECs and pericytes were induced, while others that recognized collagen type I matrices, such as the α2 integrin subunit from ECs, were downregulated [94] (Fig. 1.5e). Thus, as basement membranes assemble around EC tubes, the direct interaction of ECs with collagen type I decreases, while their contact with basement membrane matrices increases. Concomitantly, we observed increases in the expression of integrin α5, α3, and α6 from ECs, which can recognize fibronectin, nidogens, and laminin isoforms, while α5, α3, α6, and α1 integrin subunits were increased, from pericytes which recognize fibronectin, nidogens, laminin isoforms, and collagen type IV [94]. We also observed functional effects of these integrins, since blocking antibodies to the α5, α3, α6, and α1 integrin all caused abnormalities in the tube maturation process, by significantly increasing tube width [94]. None of these antibodies have any influence on EC-only cultures, which are solely dependent on the collagen-binding integrin, α2β1 [28, 94]. This data strongly indicates that the purpose of the multiple β1 integrins, on the EC cell surface, is to recognize key ECM components they encounter at different stages of the tube morphogenic and maturation process. When they are exposed to collagen type I matrices, which serves as a strong agonist for tubulogenesis, they utilize collagen-binding integrins such as α2β1. However, when EC-lined tubes attract pericytes, ECM remodeling occurs that induces deposition of basement membrane matrices that are recognized by different sets of integrins such as α5β1 (a fibronectin receptor), α3β1 (a nidogen and laminin isoform receptor), α6β1 (a laminin isoform receptor), and α1β1 (a collagen type IV, collagen type I, and laminin receptor) [94]. Interestingly, α1β1 appears to be predominantly pericyte-derived during the

EC-pericyte tube co-assembly process. Thus, the effects of blocking antibodies that have been observed may be due to an inhibitory influence on pericyte recognition of basement membrane matrices during these events [94]. EC-dependence on α2β1, which is continuously observed over time in EC-only cultures is lost with time in EC-pericyte cocultures, as basement membrane matrix assembly occurs and exposure of ECs to collagen type I is strongly diminished. In conclusion, our findings show that EC-pericyte interactions control vascular basement membrane matrix assembly and that concomitant changes in EC and pericyte integrins occurs to recognize this newly remodeled matrix to facilitate further tube maturation and stabilization events.

### *1.3.19 Defining the Critical Growth Factors that Control Human Vascular Tube Morphogenesis and Pericyte Recruitment to EC-Lined Tubes*

A central question in vascular biology is to elucidate the nature of the growth factors that are necessary to assemble the developing vasculature. Considerable data, particularly obtained from knockout mice or zebrafish, suggested a major role for VEGF [1]. Using serum-free defined models of vascular tube morphogenesis, we have assessed a role for VEGF and FGF-2, singly or in combination, in directly regulating EC tube assembly. Neither factor (alone or in combination) (i.e., VEGF+FGF) was able to support EC tubulogenesis [93]. Using a broad screen for growth factors, peptides, and other small molecules in a 96-well plate microassay format, we identified a single combination of five growth factors that leads to human EC lumen and tube network assembly. The five Factors are stem cell factor (SCF), interleukin-3 (IL-3), stromal-derived factor-1 alpha (SDF-1α), FGF-2, and insulin [93] (Fig. 1.3). Detailed screening of hundreds of additional combinations of molecules failed to identify any other mixture that supported this process [18]. Adding pericytes to ECs does not substitute for the Factor requirements [93]. Importantly, admixing pericytes with ECs under these Factor-driven defined conditions leads to EC tubulogenesis and marked pericyte recruitment as well as proliferation [93]. Of great interest is that pericytes proliferate, but ECs do not. In addition, this stimulated EC-pericyte tube co-assembly results in capillary maturation events, including basement membrane matrix assembly [93, 94]. Pericyte recruitment and basement membrane formation lead to narrower tube diameters, compared to EC-only cultures [94]. Using this Factor-driven system, we identified a key role for EC-derived PDGF-BB and HB-EGF, in pericyte recruitment and proliferation, during EC-pericyte tube co-assembly [96]. Disruption of recruitment using antagonists of PDGF-BB and HB-EGF (using blocking antibodies or receptor traps) leads to wider tubes, fewer pericytes, and markedly reduced basement membrane deposition [96]. Overall, these data strongly indicate that we have identified a key combination of five Factors that are necessary to stimulate the process of human capillary tube

assembly [18, 93]. These Factors, by acting on ECs, are also critical in facilitating pericyte recruitment (through PDGF-BB and HB-EGF) which is required for capillary basement membrane matrix assembly, a fundamental step in vessel maturation.

Finally, we have addressed a functional role for VEGF during these events, by acting as an upstream primer of EC responses to the Factors [93]. VEGF pretreatment leads to upregulation of c-Kit, IL-3Rα, and CXCR4, which are the key receptors necessary to act in conjunction with FGF and insulin receptors [93]. The addition of a combination of FGF and insulin together fails to support EC tubulogenesis. In contrast, addition of all five Factors leads to marked EC tubulogenesis. Importantly, VEGF pretreatment of ECs for 8 h or more leads to significant increases in their response to the downstream Factors [93]. Furthermore, VEGF priming also enhances pericyte recruitment responses, during EC-pericyte tube co-assembly [93]. This work strongly suggests that VEGF's functional role needs to be reinterpreted, in that it fails to directly stimulate EC tubulogenesis but can clearly act as an upstream EC primer/activator, which allows for enhanced responses to downstream Factors. The unique signaling features of VEGF priming must be investigated and distinguished from those supplied by the Factors, which separately leads to the major EC tubulogenic process. Thus, these unique signaling events control distinct steps in the process of EC tube morphogenesis which then leads to pericyte recruitment to establish capillary networks that mature over time.

## 1.4 Future Directions

It is clear that major advances have occurred over the past two decades in elucidating molecular mechanisms that underlie the ability of vessels to form, mature, and regress. In our view, it is this type of mechanistic research that will most likely lead to the generation of novel therapeutic strategies to manipulate blood vessels in the context of disease. It is also critical that both in vitro and in vivo approaches be continued and appreciated by individuals who focus on either side of these strategies. As the in vitro models and experimental strategies have evolved, it is more and more evident that very rapid advances are occurring in this area. Particular assay systems have repeatedly been shown to accurately reflect the biology of developing and postnatal vessels in vivo, and thus, these systems represent a critical experimental approach to rapidly advance the field.

In terms of key future directions, it is clear that more cytokine and growth factor research needs to be coupled with signal transduction studies to elucidate when and where particular molecules act to control the development, maturation, and stability of the vasculature. A key aspect of this question is how distinct growth factors or combinations (and their unique downstream signals) are necessary to create and maintain arteries, capillaries, veins, and the lymphatic vasculature. Our recent findings showing that five growth factors, SCF, IL-3, SDF-1α, FGF-2, and insulin, are required in combination to support human capillary tube assembly is an example of

this type of advance. The EC tubulogenic signaling cascade is highly complex and needs to be investigated in considerably more detail. A fundamental question, which remains unanswered, is what allows ECs to form lumen and tubes, while cells such as pericytes and fibroblasts cannot perform this function. In attempts to create the EC lineage from any other cell type, it is essential to determine whether the cells can form tubes in a 3D matrix environment, which is one of primary functional roles of ECs. It is likely that a detailed understanding of these processes will lead to important new opportunities to treat disease. Many of the most important acute and chronic diseases include a major component involving the dysfunction or breakdown of the vasculature (e.g., cancer, diabetes, tissue fibrosis, hypertension, atherosclerosis). Another important concept that needs to be stressed is that molecules (growth factors, ECM, receptors, MMPs) and downstream signaling molecules work together (i.e., multiprotein complexes), and it is critical to understand how such molecules and pathways temporally coordinate to control the observed biological responses. The single molecule analysis and approach that is inherent to many studies can be quite misleading in terms of our understanding of complex biological events. Systems approaches (i.e., RNAseq, non-coding RNA regulation, and proteomics analyzing protein-protein interactions with identification of key phosphorylation sites) are important directions in future work to identify new critical regulators of the pathways that control vascularization responses and to assess how these are altered in the context of vascular disease.

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