

# Chapter 2

## Control of Vascular Tube Morphogenesis and Maturation in 3D Extracellular Matrices by Endothelial Cells and Pericytes

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### Abstract

An important advance using in vitro EC tube morphogenesis and maturation models has been the development of systems using serum-free defined media. Using this approach, the growth factors and cytokines which are actually necessary for these events can be determined. The first model developed by our laboratory was such a system where we showed that phorbol ester was needed in order to promote survival and tube morphogenesis in 3D collagen matrices. Recently, we have developed a new system in which the hematopoietic stem cell cytokines, stem cell factor (SCF), interleukin-3 (IL-3), and stromal derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) were added in conjunction with FGF-2 to promote human EC tube morphogenesis in 3D collagen matrices under serum-free defined conditions. This new model using SCF, IL-3, SDF-1 $\alpha$ , and FGF-2 also works well following the addition of pericytes where EC tube formation occurs, pericytes are recruited to the tubes, and vascular basement membrane matrix assembly occurs following EC-pericyte interactions. In this chapter, we describe several in vitro assay models that we routinely utilize to investigate the molecular requirements that are critical to EC tube formation and maturation events in 3D extracellular matrix environments.

**Key words** Endothelial cells, Pericytes, 3D matrices, Cell-cell interaction, Vascular tube morphogenesis

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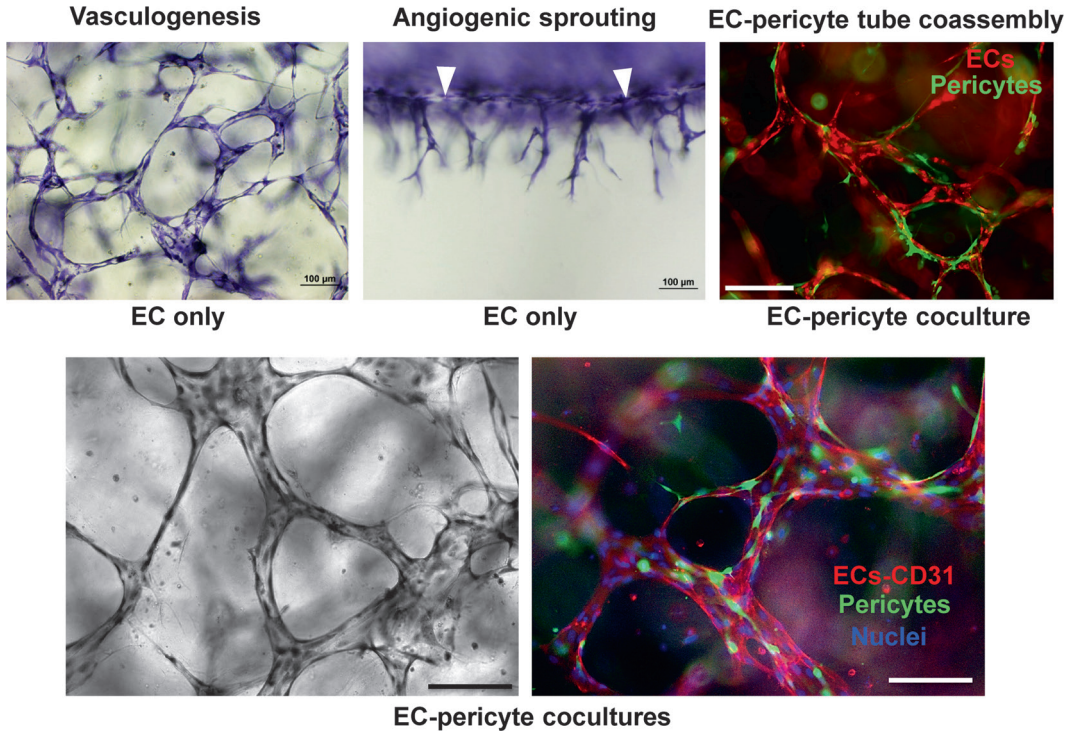
### 1 Introduction

Many recent studies have addressed the molecular basis for how blood vessels form and mature [1–7]. This work has progressed through the development of novel experimental approaches and the utilization of both in vivo and in vitro models. Animal models used to investigate tissue vascularization include those in mice, zebrafish, and avian species and also, sophisticated in vitro models have been developed to mimic vessel assembly including lumen and tube formation as well as sprouting behavior and maturation

events such as endothelial cell–mural cell interactions leading to vascular basement membrane matrix formation (Fig. 1). It is being increasingly appreciated that the parallel use of both approaches is leading to more rapid discoveries to elucidate the underlying mechanisms that control these fundamental cellular events. For example, blood vessel assembly involves important homotypic interactions between endothelial cells (ECs) as well as heterotypic EC interactions with mural cells such as pericytes and vascular smooth muscle cells and critical signal transduction cascades that result from such cell–cell contacts [1, 5, 7].

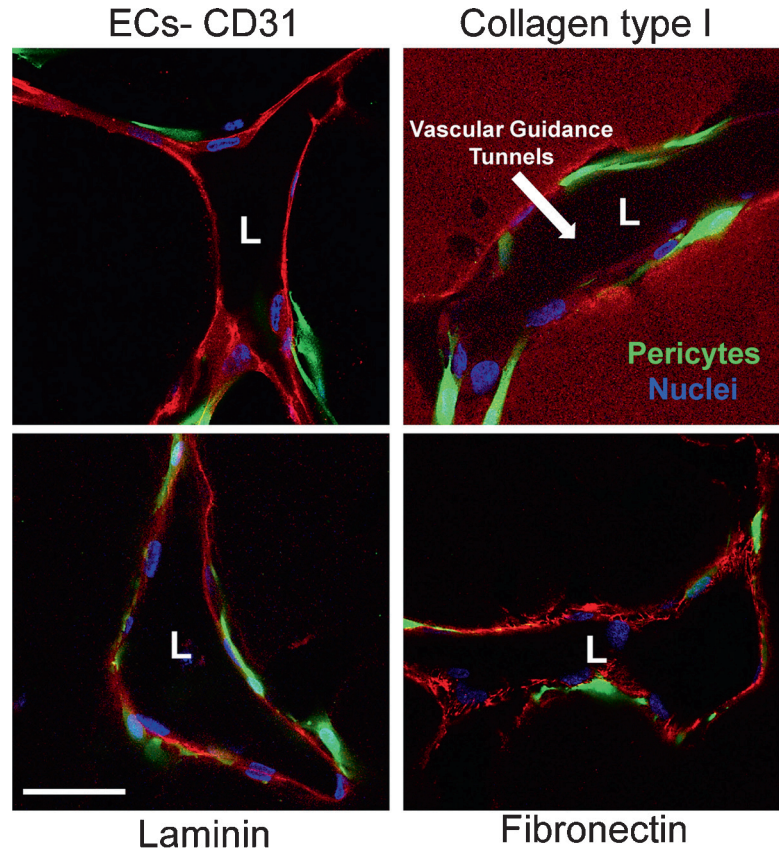
Major advances in recent years have occurred in our understanding of the molecular mechanisms underlying EC tube formation in 3D matrices and much of these advances have occurred due to the use of in vitro 3D morphogenesis assay systems in collagen or fibrin matrices [1, 6–9] (Fig. 1). Vascular tube formation occurs secondary to integrin signaling, cell surface proteolysis, and signaling through Rho GTPases and protein kinase cascades [1, 2, 6, 10]. Interestingly, a key step in EC tubulogenesis is the generation of vascular guidance tunnels [11] (Figs. 2 and 3) which are matrix-free spaces that are created as a result of MT1-MMP-mediated proteolysis. This proteolytic process occurs in coordination with integrin, Rho GTPase, as well as protein kinase C, Src family kinase, Pak kinase, Raf, and Erk kinase-dependent signaling [12–15]. Following creation of vascular guidance tunnels, which are matrix templates in 3D matrices, EC motility and tube remodeling events occur within these spaces and importantly, mural cells such as pericytes are recruited to EC-lined tubes on their abluminal surface [5, 11, 16, 17] (Figs. 2 and 3). Thus, after pericyte recruitment to tubes, both ECs and pericytes reside within tunnel spaces and both cell types work together to assemble the vascular basement membrane [5, 16] (Figs. 2 and 3). Disruption of pericyte recruitment to EC tubes in vitro or in vivo leads to markedly reduced basement membrane matrix deposition, showing that EC–mural cell interactions play a major role in stimulating this key extracellular matrix remodeling process [5, 17].

An important advance using in vitro EC tube morphogenesis and maturation models has been the development of systems using serum-free defined media [2, 5, 7, 10, 18, 19]. Using this approach, the growth factors and cytokines which are actually necessary for these events can be determined. The first model developed by our laboratory was such a system where we showed that phorbol ester was needed in order to promote survival and tube morphogenesis in 3D collagen matrices [18]. We added both FGF-2 and VEGF in this model, but clearly phorbol ester addition was the major factor that allowed the system to function. More recently, we have developed a new system in which the hematopoietic stem cell cytokines, stem cell factor (SCF), interleukin-3 (IL-3), and stromal derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) were added in



**Fig. 1** Microassay systems of human EC tube morphogenesis, sprouting, and EC–pericyte tube co-assembly using serum-free defined media in 3D collagen matrices. In all cases, the hematopoietic stem cell cytokines, SCF, IL-3, and SDF-1 $\alpha$  were used in conjunction with FGF-2 to obtain these marked morphogenic responses and these factors were polymerized into the collagen matrix at the start of the assay. An assay is illustrated showing vasculogenic tube assembly (*upper left*) (ECs only) whereby single ECs are seeded together and over time assemble into tube networks in 3D collagen matrices. Cultures were fixed at 72 h, stained with toluidine blue, and photographed. An angiogenic sprouting assay is illustrated (*upper middle*) whereby the hematopoietic cytokines as well as FGF-2 and VEGF-165 were added into the matrix and ECs were seeded onto the gel surface. After 24 h, the culture was fixed, stained with toluidine (blue), cross-sectioned, and photographed. Arrow-heads indicate the monolayer surface. EC–pericyte tube co-assembly assays are illustrated in the *upper right panel* and *lower panels*. In the *upper right panel* experiment, mCherry-labeled ECs were seeded with GFP-labeled pericytes and after 72 h, the culture was fixed and photographed under fluorescence. In the *lower panel experiment*, unlabeled ECs were primed with VEGF-165 and FGF-2 and then were trypsinized and mixed with GFP-labeled pericytes in 3D collagen matrices which contained SCF, IL-3, SDF-1 $\alpha$ , and FGF-2. After 96 h, the cultures were fixed and stained with toluidine blue (*lower left panel*) or were immunostained with antibodies to CD31 (*lower right panel*) and then photographed. The latter culture was also stained with Hoechst dye to label nuclei. Bar = 100  $\mu$ m

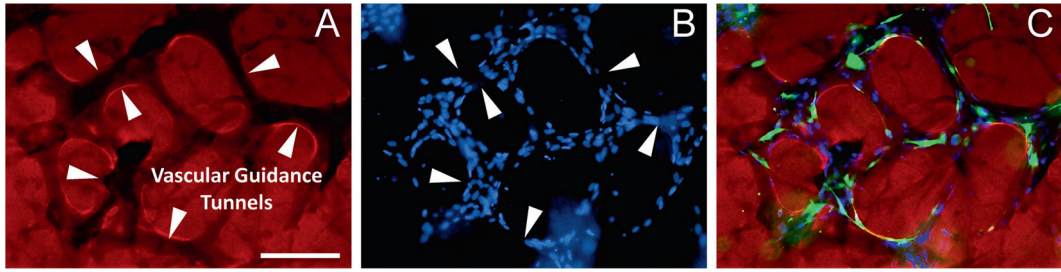
conjunction with FGF-2 to promote human EC tube morphogenesis in 3D collagen matrices under serum-free defined conditions [16, 20] (Fig. 1) (and in the absence of phorbol ester). Interestingly, the addition of VEGF and FGF-2 in combination fails to promote tube formation under these defined conditions [20]. This new model using SCF, IL-3, SDF-1 $\alpha$ , and FGF-2 also works well following the addition of pericytes where EC tube



**Fig. 2** EC-pericyte tube co-assembly occurs within vascular guidance tunnels and leads to vascular basement membrane matrix assembly between the ECs and pericytes. Unlabeled ECs and GFP-pericytes were cocultured in 3D collagen matrices for 96 h and were immunostained for the indicated molecules, CD31, collagen type I, laminin, and fibronectin (red staining). These cultures were also stained with Hoechst dye to stain nuclei. For the ECM protein stains, no detergent was added to ensure that only extracellular staining was being observed. Representative confocal sections are shown for each stain. *Arrow* indicates a vascular guidance tunnel space. *L* indicates lumen space. Bar = 25  $\mu$ m

formation occurs, pericytes are recruited to the tubes, and vascular basement membrane matrix assembly occurs following EC-pericyte interactions [16, 20] (Figs. 1 and 2). We also recently demonstrated that VEGF and FGF-2 (or their combination) can prime ECs for subsequent EC tube morphogenic responses to the hematopoietic cytokines [20] (Fig. 1, lower panels). This priming effect occurs in part secondary to upregulation of hematopoietic cytokine receptors on ECs including the SCF receptor, c-Kit, the IL-3 receptor (IL-3 receptor  $\alpha$ ), and the SDF-1 $\alpha$  receptor, CXCR4 [20]. Thus, in this defined model of human EC tube morphogenesis and sprouting, VEGF's primary action appears to be that of a





**Fig. 3** Vascular tube maturation events resulting from EC–pericyte interactions occur within vascular guidance tunnels in 3D extracellular matrices. ECs create vascular guidance tunnels secondary to MT1-MMP-mediated proteolysis in coordination with the EC lumen and tube formation process. Pericytes are recruited to EC-lined tubes and within vascular guidance tunnels as illustrated in this figure. Unlabeled ECs were seeded with GFP-pericytes in 3D collagen matrices and after 96 h of EC–pericyte tube co-assembly, cultures were fixed and stained with anti-collagen type I antibodies (*red*) to delineate vascular guidance tunnels and Hoechst dye to label nuclei (*blue*). Note that all of the cells were mixed randomly in the gel at the start of the assay, but after 96 h, all of the cells are present within vascular guidance tunnels as demonstrated by the nuclear stain showing that the ECs are assembled together in tube structures and that pericytes have all recruited to the abluminal surface of these tubes. *Arrowheads* indicate the borders of vascular guidance tunnels. Bar = 100  $\mu\text{m}$

primer which acts to prepare ECs for morphogenic responses to subsequent growth factor/cytokine and extracellular matrix signals [20]. In this chapter, we describe several *in vitro* assay models that we routinely utilize to investigate the molecular requirements that are critical to EC tube formation and maturation events in 3D extracellular matrix environments.

## 2 Materials

The key materials necessary to perform these morphogenic assays include human endothelial cells and human pericytes which are grown as described [8, 21]. Also, we can label these cells with fluorescent labels including green fluorescent protein (GFP) or monomeric Cherry (mCherry) that are introduced using recombinant lentiviruses. We perform morphogenic assays utilizing rat tail collagen type I 3D matrices and use recombinant growth factors and cytokines which are added either into the collagen matrix, the culture media, or both. All of the assays are performed in half-area 96-well plates. Immunostaining is performed by fixing the 3D collagen gels and staining the gels using methods similar to whole-mount staining of tissues or embryos. Cultures can also be stained with dyes such as toluidine blue which facilitates our ability to assess and quantitate lumen and tube formation by photography and tracing tube areas using Metamorph software [8].

## **2.1 Endothelial Cell and Pericyte Culture**

1. Human umbilical vein endothelial cells (HUVECs) are obtained from Lonza (Basel, Switzerland) and are grown from passages 2–6.
2. HUVEC medium: Medium 199 containing 20 % fetal calf serum (FCS), 400 µg/ml of bovine hypothalamic extract, and 100 µg/ml of heparin (Supermedia; *see* **Notes 1–3**).
3. Tissue culture flasks.
4. Phosphate-buffered solution (PBS).
5. 0.1 % Gelatin in PBS.
6. Human brain vascular pericytes (HBVP) are obtained from ScienCell (Carlsbad, CA, USA) and are grown from passages 2–10.
7. Bovine retinal pericytes are cultured from bovine retinas as previously described [15].
8. Pericyte medium: Dulbecco's modified Eagle's medium (DMEM) with 10 % FCS.
9. Collagen type I is purified from rat tails as described [8] and is lyophilized from a 0.1 % acetic acid solution in deionized water (sterile filtered). The collagen is then resuspended in 0.1 % acetic acid in water solution at a final concentration of 7.1 mg/ml. We obtain approximately 100 mg of collagen type I from one rat tail (*see* **Note 1**).
10. Reduced serum supplement II (RSII): This supplement is made by mixing a combination of insulin, transferrin, selenium, and fatty acid-free bovine serum albumin with added C18 oleic acid which is prepared as described [8]. The mixture is frozen and stored at –20 °C.
11. Recombinant growth factors, cytokines, and other medium additives: Recombinant FGF-2 (EMD Millipore, Billerica, MA, USA), SCF, IL-3, and SDF-1α (R&D Systems, Minneapolis, MN, USA) and ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA).
12. Half-area 96-well tissue culture plates (A/2) (Costar, Corning, Tewksbury, MA, USA).

## **2.2 Lentivirus Production**

1. Lentiviruses are generated using the ViraPower Lentiviral Expression system (Invitrogen, Grand Island, NY, USA).
2. We utilize a lentiviral construct (pLenti6/V5 TOPO) with a blasticidin resistance gene or we use the Lenti-X system (Clontech, Mountain View, CA, USA) that uses either the lentiviral vector pLVX-IRES-Neo (G418 selection) or pLVX-IRES-Puro (puromycin selection).
3. A lentiviral vector packaging system (Clontech) is utilized along with 293FT cells (grown in DMEM with 10 % FCS) to make recombinant lentiviruses as described by the manufacturer.

### 3 Methods

#### **3.1 EC Tube Morphogenesis Assay in 3D Collagen Matrices**

1. Trypsinize confluent human ECs, wash 1× with 10 ml of M199, and then resuspend at  $1 \times 10^7$  cells/ml (gently mix cells with a P200 tip to break up small clumps).
2. Seed cells at  $2 \times 10^6$  cells/ml in 2.5 mg/ml of collagen type I suspended in M199. To make 1 ml of collagen gel, 350  $\mu$ l of 7.1 mg/ml type I collagen in 0.1 % acetic acid, 39  $\mu$ l of  $10 \times$  M199, 2.1  $\mu$ l of 5 N NaOH, and 409  $\mu$ l of  $1 \times$  M199 are mixed together thoroughly.
3. Add 200  $\mu$ l of ECs to the collagen gel mixture (**step 2**), which is then swirled and placed on ice.
4. The recombinant growth factors, SCF, IL-3, SDF-1 $\alpha$ , and FGF-2, are all added at 200 ng/ml within the collagen matrices.
5. Add 28  $\mu$ l of gel per well and periodically tap the plates on each edge to make certain that the gels are evenly distributed in each well prior to polymerization.
6. Allow the plates to equilibrate in a CO<sub>2</sub> incubator for 30 min and then add 100  $\mu$ l of culture medium to each well. The culture medium is M199 which contains a 1:250 dilution of RSII supplement, 40 ng/ml of FGF-2, and 50  $\mu$ g/ml of ascorbic acid.
7. We add 125  $\mu$ l of water in every well surrounding the gelatin-containing wells and we also add 150  $\mu$ l of water in non-well areas that surround each of the wells to maintain humidity and to reduce potential dehydration of culture wells over time.
8. Allow assays to proceed for 1, 3, or 5 days and at these time points, cultures can be fixed with either 2 % paraformaldehyde in PBS or 3 % glutaraldehyde in PBS. Paraformaldehyde-fixed gels can be utilized to perform immunostaining using various antibodies such as CD31 (Figs. 1 and 2) or extracellular matrix proteins (Fig. 2). Glutaraldehyde-fixed gels are typically stained with 0.1 % toluidine blue in water, which is an excellent stain to visualize tubes (Fig. 1).

#### **3.2 EC Tube Sprouting Assay in 3D Collagen Matrices**

1. Collagen gels (2.5 mg/ml) are prepared as above (Subheading 3.1) and contain 200 ng/ml of recombinant IL-3, SDF-1 $\alpha$ , SCF, FGF-2, and VEGF-165.
2. Add 28  $\mu$ l of gel to each well in 96-well plates.
3. Allow gels to polymerize and equilibrate the pH by placing them in a CO<sub>2</sub> incubator for 30–60 min.
4. Seed ECs at 50,000 cells/well in M199 culture medium (100  $\mu$ l/well) that contains a 1:250 dilution of RSII, as well as FGF-2 at 40 ng/ml and ascorbic acid at 50  $\mu$ g/ml.

5. Allow cultures to incubate for 1, 3, or 5 days of culture and after this time, fix the cultures with 3 % glutaraldehyde in PBS (140  $\mu$ l per well). For the 5-day culture, 60  $\mu$ l of medium is removed and replaced with fresh medium at 3 days of culture and it is prepared as described above. Fixed cultures are stained with 0.1 % toluidine blue in water. Gels can be bisected with a clean razor blade to visualize a cross section of EC sprouting and tube morphogenesis (Fig. 1).

### **3.3 EC-Pericyte Tube Co-assembly Assay in 3D Collagen Matrices**

1. Plate ECs in collagen gels (2.5 mg/ml) at  $2 \times 10^6$  cells/ml, and GFP-labeled pericytes at  $0.4 \times 10^6$  cells/ml.
2. The gels also contain 200 ng/ml of SCF, IL-3, SDF-1 $\alpha$ , and FGF-2 and add 28  $\mu$ l of the cell-gel mixture to the A/2 microwells.
3. After polymerization and equilibration in a CO<sub>2</sub> incubator for 30–60 min, add M199 culture medium (100  $\mu$ l/well), which also contains a 1:250 dilution of RSII, 40 ng/ml of FGF-2, and 50  $\mu$ g/ml of ascorbic acid.
4. Allow cultures to proceed for 3 or 5 days of culture. For the 5-day culture time point, cultures are fed on day 3 by removing and replacing 60  $\mu$ l of medium with fresh medium.
5. Cultures are fixed with either 2 % paraformaldehyde in PBS for immunostaining and fluorescence microscopy or 3 % glutaraldehyde in PBS to then stain with 0.1 % toluidine blue in water.

### **3.4 Priming of ECs with VEGF Isoforms to Activate EC Morphogenic Responses**

1. Culture ECs in T25 or T75 cm<sup>2</sup> flasks to confluence in Supermedia as described above (Subheading 2.1) and then wash 2 $\times$  with 5 or 15 ml of media, respectively.
2. Culture the cells for 16–20 h in M199 medium containing a 1:250 dilution of RSII, VEGF-165 at 40 ng/ml, and FGF-2 at 40 ng/ml.
3. ECs are then trypsinized and used for either the EC-only or EC-pericyte coculture assays as described above (Subheading 3.3). In all cases, EC morphogenic responses are strongly enhanced using this VEGF priming protocol. Our work suggests that a major morphogenic influence of VEGF is to prime or prepare ECs for morphogenic responses that are stimulated by the hematopoietic stem cell cytokines, SCF, IL-3, and SDF-1 $\alpha$  [20].



### **3.5 Analysis of Vascular Basement Membrane Deposition Resulting from EC–Pericyte Interactions During Tube Co-assembly in 3D Collagen Matrices**

We have shown that a key consequence of pericyte recruitment to EC-lined tubes is the deposition of vascular basement membrane matrices [5, 16]. We have demonstrated that both cell types directly contribute to the deposition process and have further shown that EC-only cultures fail to deposit a vascular basement membrane matrix under our defined media conditions in 3D collagen matrices [16]. For this analysis, we have utilized either EC–pericyte cocultures with human or bovine pericytes and both cell types work very well for this analysis.

EC–pericyte cocultures are established in 3D collagen gels as described above (Subheading 3.3) and after 3 or 5 days of culture, cultures are fixed with 2 % paraformaldehyde (for immunofluorescence staining) or 3 % electron microscopy-grade glutaraldehyde in culture media (for transmission electron microscopy). To fully demonstrate that basement membrane matrix assembly has occurred, both immunofluorescence microscopy and transmission electron microscopy need to be performed. Another critical point is that the immunostaining protocol for vascular basement membrane matrix assembly is performed in the absence of detergent such that only extracellular staining is observed [16]. We typically utilize unlabeled ECs and GFP-pericytes and thus stain for the vascular basement membrane components, laminin, fibronectin, collagen type IV, nidogen 1, nidogen 2, and perlecan, using AlexaFluor 594-conjugated secondary antibodies (Molecular Probes) so that they are red in color (Fig. 2). To stain ECs, we typically utilize antibodies to CD31 and utilize the AlexaFluor-conjugated secondary antibodies as illustrated in Fig. 2. Nuclei are stained with Hoechst dye (Figs. 2 and 3).

### **3.6 Labeling ECs or Pericytes with Membrane-Targeted GFP or mCherry Facilitates Visualization of EC Tube Morphogenesis and EC–Pericyte Tube Co-assembly in 3D Collagen Matrices**

An important advance in our ability to image vessel tube morphogenesis and maturation events is to label ECs or pericytes with fluorescent markers. A requirement for such labels is that they should have no deleterious influence on their functional ability to participate in morphogenic events in 3D matrices. This technology has been particularly useful in real-time imaging of EC–pericyte tube co-assembly, where we demonstrated for the first time how the two cells interact with each other during these events in 3D extracellular matrices [5, 16, 17]. We have successfully labeled pericytes with enhanced GFP and these cells appear to be functionally normal in our assay models allowing us to readily quantitate pericyte motility and recruitment to EC-lined tubes in 3D matrices. More recently, we have labeled them with mCherry constructs and they also appear to be functionally normal. For ECs, we have found that membrane-targeted GFP or mCherry constructs work best in the assays described above. For this purpose, we have utilized a Ras membrane-targeting sequence that is fused to the C-terminus of AcGFP (Ac-GFP-F) (Clontech) and made a recombinant lentivirus. We have also created an mCherry-F lentiviral

construct that has the same membrane targeting motif fused to the C-terminus of mCherry. The sequences of the primers used for this construction are:

AcGFP-F Upstream-NotI

5'-AGGCGGCCGCACCATGGTGAGCAAGGGCGCCG  
AGCTGTTAC-3'

AcGFP-F Downstream-BamHI

5'-AGGGATCCTCAGGAGAGCACACACTTGCAGCTC-3'

mCherry-F Upstream-NotI

5'-AGGCGGCCGCACCATGGTGAGCAAGGGCGAG  
GAGG-3'

mCherry-F Downstream-BamHI, 5'-AGGGATCCTCAGGAGA  
GCACACACTTGCAGCTCATGCAGCCGGGG  
CCACTCTCATCAGGAGGGTTTCAGCT  
TCTTGACAGCTCGTCCATGCC-3'

We have cloned these constructs into a pLVX-IRES-Neo lenti-viral vector (Clontech), infected ECs or pericytes, and then selected for the cells carrying the vector using G418 at 200 µg/ml. Assays with fluorescent ECs or pericytes are established in the same manner as with unlabeled cells.

### **3.7 Real-Time Imaging of EC Tubulogenesis and EC-Pericyte Tube Co-assembly in 3D Collagen Matrices**

1. Establish EC-only or EC-pericyte cocultures in 3D collagen matrices in half-area 96-well microwell plates as described above (Subheading 3.3).
2. Place a glass plate with the dimensions of 75 mm × 50 mm on the surface of the 96-well plates and then replace the lid to the plate. We have found that the glass plate strongly decreases potential condensation on the top of the plates which can interfere with imaging.
3. We utilize two independent microscopy systems for our real-time imaging, which are the Nikon Eclipse TE2000-E with Photometrics CoolSNAP-HQ2 camera and the Leica DMI6000B with Hamamatsu ORCA-ER camera which are each equipped with an incubator that surrounds the microscopic stage to control the temperature of the cultures to 37 °C. In addition, there is a CO<sub>2</sub> controller that is placed on the surface of the 96-well plates that delivers CO<sub>2</sub> to a level of 5 % on the cultures.
4. Image cultures at different starting times and collect images every 10 min for 24, 48, or 72 h. This can be adjusted to individual experiments. We can acquire light, fluorescent, or both images depending on the particular purpose of the experiment and movie. We usually acquire images every 10 min and after this time, files are converted into movies using Metamorph

software. In many cases, we overlay these images with others obtained at a different fluorescent wavelength (i.e., ECs carry mCherry while pericytes carry GFP) (Fig. 1). After this step and construction of movies using Metamorph, they are converted into Windows Media Player or Quicktime files for routine viewing and publication purposes.

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## 4 Notes

1. Two key reasons for our success over the years in performing these EC tube morphogenesis assays is that we make our own bovine brain extract as well as rat tail collagen type I preparations. In this way, we have an internal consistency (for more than 15 years) that is not dependent on variable commercial sources or availability issues.
2. For our assays to be optimal, the HUVEC cells should be of passages 2–6 and the growth media needs to be utilized exactly as we describe. Because of different additives that are used in commercially available media, they may adversely impact the performance of the assays that we describe. This issue has not been assessed in detail; however, our assays are highly reproducible and if performed as we describe, they should work well each time an assay is established.
3. Our assay systems are highly compatible with siRNA suppression protocols for either ECs or pericytes which we have described previously in detail [8].

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Cell-Cell Interactions

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