PROTOCOL

VascuNet™ Pericyte Co-Culture Assay

INTRODUCTION

Vasculogenesis and angiogenesis are the mechanisms responsible for the development of a vascular network. Both endothelial cells and pericytes are key cell types in these processes. While endothelial cells form the lining of the vessels, pericytes are essential to the development of functional vasculature by stabilizing established vessel structures and facilitating local remodeling for network expansion. In addition to conveying structural support, pericytes are also integral in directing endothelial cells via cell-to-cell contact and paracrine signaling. Pericytes have been shown to co-localize with endothelial cells in both normal and abnormal vasculature, and have been implicated in playing a central role in numerous pathologies, including tumorigenesis, neurodegenerative disorders, and diabetic retinopathy.

The VascuNet Pericyte Co-Culture Assay combines human embryonic stem cell (ESI-017)-derived pericytes (PC-M cells) with primary human umbilical vein endothelial cells (HUVECs) in a co-culture system designed for a 96-well plate format. These unique PC-M cells display several key properties of pericytes, including expression of CD146, pro-angiogenic function, and effective stabilization of endothelial tube networks. The HUVECs and PC-M cell co-culture system supports vasculogenic tube assembly, resulting in the generation of extensive tube networks that persists at least 4 to 6 days in culture. Vasculogenic tube networks formed with the VascuNet Pericyte Co-Culture Assay persist over 4 times longer in culture than those formed by other assay systems, allowing researchers to study the relevant timing of delivery and long-term efficacy of pro- and anti-angiogenic compounds.

IMPORTANT TIPS

- All work with live cells should be completed in a sterile biological safety cabinet designated for tissue culture.
- Do not allow either PC-M cells or HUVECs to proliferate to more than 90% confluency during expansion. At high confluency, dense cell clusters may form, which can decrease vasculogenic tube assembly in mono- and co-cultures. Be sure to resuspend cells to single-cell suspension before plating for the angiogenic assay.
- Solvents used to dissolve test reagents, such as DMSO. may have inherent pro- or anti-angiogenic properties For this reason, all test reagents should be resuspended in diluents with no greater than 0.1% (v/v) of the solvent.
- Optimal vascular tube growth and stability is achieved when the HUVECs and PC-M cells are plated at a total of 42,000 cells per well in a ratio of 20:1 for HUVECs:PC-M cells, respectively.

REQUIRED MATERIALS

The VascuNet Pericyte Co-Culture Assay kit contains PC-M cells, HUVECs, and all media components for cell expansion and vasculogenic assay. Each kit undergoes extensive quality control to ensure reproducible vasculogenic tube assembly.

VascuNet™ Pericyte Co-Culture Assay Kit Contents and Storage Conditions

Kit Component	Quantity	Storage Condition
Cells:		
PC-M cells (ESI-017-derived pericytes) p19	1 vial $\geq 5.0 \times 10^5$ cells/vial	Liquid Nitrogen
HUVECs (secondary donor pool) p5	1 vial $\geq 1.0 \times 10^6$ cells/vial	Liquid Nitrogen
VascuNet Growth Medium Components:		
VascuNet Basal Medium	475 mL	2 to 8°C
Recombinant Human VEGF	0.5 mL	-20°C
Recombinant Human EGF	0.5 mL	-20°C
Recombinant Human IGF-1	0.5 mL	-20°C
Recombinant Human FGF basic	0.5 mL	-20°C
Ascorbic Acid	0.5 mL	-20°C
Heparin Sulfate	0.5 mL	-20°C
Hydrocotrisone Hemisuccinate	0.5 mL	-20°C
FBS	25 mL	-20°C
L-Glutamine	25 mL	-20°C
VascuNet Assay Medium Components:		
VascuNet Basal Assay Medium	95 mL	2 to 8°C
L-Glutamine	5 mL	-20°C
Negative Control:		
Suramin Hexasodium Salt	0.5 mL, 1 mM in H_2O	-20°C

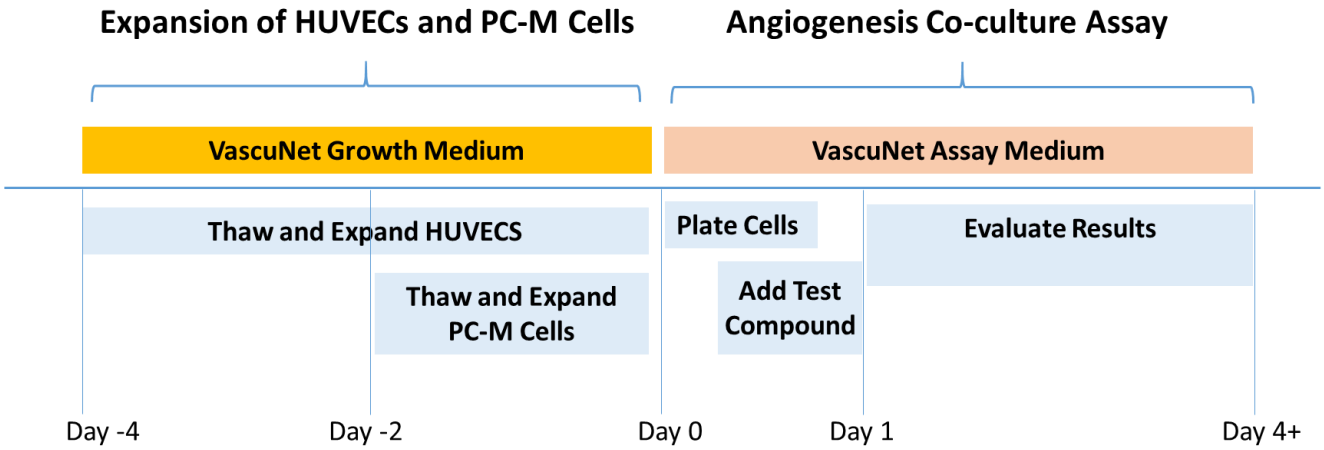
Ensure that kit components are stored at the indicated temperatures upon kit arrival. The VascuNet Pericyte Co-Culture Assay components are stable for a minimum of 3 months from date of receipt when stored as directed.

Additional Required Reagents and Materials

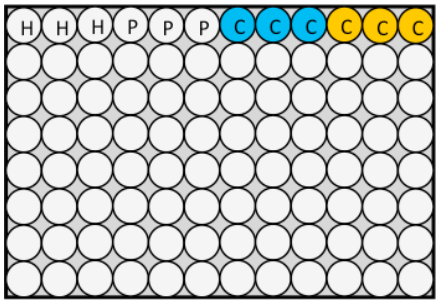
- Sample test compounds to assay
- Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, *LDEV-Free
- Phosphate Buffered Saline (PBS)
- Accutase® Cell Detachment Solution
- Trypan blue or alternative cell viability assay
- 0.22 µm sterile filtration unit, 500 mL
- 0.22 µm sterile filtration unit, 150 mL
- T150 tissue culture flasks
- T75 tissue culture flasks
- 96-well tissue culture plate

EXPERIMENTAL OVERVIEW

Experimental Timeline



Assay Set-up



Row 1: Control Row
H: HUVEC Monoculture Reference (40,000 cells/well)
P: PC-M Monoculture Reference (2,000 cells/well)
C: Co-Cultures (20:1 ratio of HUVEC:PC-M; 40,000 HUVECs and 2,000 PC-M cells/well)
Blue: HUVEC & PC-M Cell Co-Culture (Positive Control)
Yellow: HUVEC & PC-M Cell Co-Culture with 50 µM Suramin Hexasodium Salt (Negative Control)

Figure 1. Example assay set-up in a 96-well plate. The first row of the VascuNet Pericyte Co-Culture Assay is a control row, containing triplicate samples of monoculture wells and positive and negative control co-culture wells. The remaining 84 wells of the plate may be used for test compounds. Test compound assays may be performed following the same mono- and co-culture set-up as the control row, or simply as co-culture wells.

EXPERIMENTAL PROTOCOL

EXPANSION OF HUVECs AND PC-M CELLS

The HUVECs and PC-M cells must be thawed and expanded individually for two days before they can be plated for the experimental set-up in a co-culture format.

Monitor cell proliferation, and do not allow either the HUVECs or PC-M cells to proliferate to more than 90% confluency during expansion. At high confluency dense cell clusters may form, which can decrease vasculogenic tube assembly in mono- and co-cultures.

DAY -4

Prepare VascuNet Growth Medium for HUVECs and PC-M Cell Expansion

1. Remove the following VascuNet Growth Medium supplements from -20°C storage and allow the following reagents to thaw at 2 to 8°C:

   rhVEGF, rhEGF, rhIGF-1, rhFGF basic, Ascorbic Acid, Heparin Sulfate, Hydrocortosone Hemisuccinate, FBS, L-Glutamine

2. Prepare VascuNet Growth Medium by combining all the reagents listed below.

VascuNet Basal Medium	475 mL
rhVEGF	0.5 mL
rhEGF	0.5 mL
rhIGF-1	0.5 mL
rhFGF basic	0.5 mL
Ascorbic Acid	0.5 mL
Heparin Sulfate	0.5 mL
Hydrocotrisone Hemisuccinate	0.5 mL
FBS	25 mL
L-Glutamine	25 mL

3. Filter sterilize the VascuNet Growth Medium using a 0.22 µm pore size, low protein-binding filter unit into a sterile 500 mL bottle.
4. Transfer 75 mL of the VascuNet Growth Medium to a sterile 100 mL bottle and place in a 37°C water bath for 30 minutes to warm.
5. Store the remaining VascuNet Growth Medium at 2 to 8°C for up to 2 weeks.

Thaw and Plate HUVECs for Expansion

6. Thaw the vial of HUVECs briefly in a 37°C water bath.
7. Transfer the entire volume of the vial into 4 mL of pre-warmed VascuNet Growth Medium in a 15 mL conical tube.
8. Centrifuge the cell suspension for 5 minutes at 200 x g.
9. Aspirate the supernatant and gently resuspend the cell pellet in 10 mL of VascuNet Growth Medium.

10. Count the total number of viable cells using Trypan blue.
11. Add 10 mL of VascuNet Growth Medium to each of two T150 flasks.
12. Divide the HUVEC suspension evenly between the two T150 flasks, such that each flask contains approximately 3.75 to 4.5 × 10^5 HUVECs at a density of 2.5 to 3.0 × 10^3 HUVECs/cm^2.
13. Add a sufficient volume of the VascuNet Growth Medium to each flask to bring the total volume to 30 mL per T150 flask.
14. Incubate the cells overnight at 37°C with 5% CO_2 humidified atmosphere.

DAY -2

Thaw and Plate PC-M Cells for Expansion

15. Transfer 45 mL of VascuNet Growth Medium to a sterile 50 mL conical tube and warm in a 37°C water bath for 30 minutes.
16. Thaw the vial of PC-M cells briefly in a 37°C water bath. Transfer the entire contents of the vial into 4 mL of pre-warmed VascuNet Growth Medium in a 15 mL conical tube.
17. Centrifuge the cell suspension for 5 minutes at 200 x g.
18. Aspirate the supernatant and gently resuspend the cell pellet in 10 mL of VascuNet Growth Medium.
19. Count the total number of viable cells using Trypan blue.
20. Add 10 mL of VascuNet Growth Medium to each of two T75 flasks.
21. Divide the PC-M cell suspension evenly between the two T75 flasks, such that each flask contains approximately 2.5 × 10^5 PC-M cells at a density of 1.0 × 10^4 PC-M cells/cm^2.

    Note: The PC-M cells are plated at a higher density than the HUVECs.
22. Add a sufficient volume of the VascuNet Growth Medium to each flask to bring the total volume to 15 mL per T75 flask.
23. Incubate the cells overnight at 37°C with 5% CO_2 humidified atmosphere.

Exchange Medium in HUVEC Culture

24. Warm 60 mL of VascuNet Growth Medium at 37°C.
25. Observe HUVEC cultures under microscope to assess cell proliferation, confluency, and overall health.
26. Aspirate the medium in both of the T150 culture flasks, and replace with 30 mL of fresh VascuNet Growth Medium per flask.
27. Incubate cells at 37°C with 5% CO_2 humidified atmosphere.

ANGIOGENESIS ASSAY

Once the HUVECs and PC-M cells have been expanded, the HUVEC and PC-M cells are plated in co-cultures and monoculture wells of a 96-well plate for the angiogenesis assay. Experimental and control assay wells are established to determine the effects of test compounds on angiogenesis.

DAY 0

Prepare 96-well Assay Plate

28. Thaw sufficient amounts of Growth Factor Reduced (GFR) Matrigel aliquot(s) on ice at 2 to 8°C.

    Note: Thaw a sufficient amount of GFR Matrigel to coat the total amount of wells used for the experiment, including the control row (see Fig. 1). Each well used in the assay will require 50 µL of Matrigel solution.

29. Place a 96-well tissue culture plate and P100 pipet tips at -20°C for 1 hour to chill.
30. Transfer the chilled 96-well plate, chilled pipet tips, and thawed GFR Matrigel bottle to an ice bucket in the tissue culture hood.

    Note: The GFR Matrigel will solidify rapidly when warmed above 2 to 8°C. To ensure even coating and distribution of the matrix, keep all of the reagents, tips, and plate on ice.

31. Add 50 µL of GFR Matrigel to each well of the 96-well plate, changing tips often for even plating.
32. Incubate the coated 96-well plate at room temperature for 1 hour, then transfer to a 37°C incubator with 5% CO_2 humidified atmosphere for 1 to 2 hours prior to plating cells.

Prepare VascuNet Assay Medium

33. Thaw the L-Glutamine (for assay medium) at 2 to 8°C. Allow the VascuNet Basal Assay Medium to warm to room temperature.
34. Prepare the VascuNet Assay Medium by combining the reagents below.

VascuNet Basal Assay Medium	95 mL
L-Glutamine	5 mL

35. Filter sterilize the VascuNet Assay Medium using a 0.22 µm pore size, low protein-binding filter and a sterile 100 mL bottle.
36. Transfer 50 mL of VascuNet Assay Medium to a 50 mL conical tube. Warm this aliquot in a 37°C water bath for 30 minutes. The required amount of medium may vary depending on the number of test component wells.
37. Store the remaining VascuNet Assay Medium at 2 to 8°C for up to 2 weeks.

Harvest HUVECs

38. Aspirate the culture medium from each of the T150 flasks, and add 10 mL of PBS per flask to wash. Aspirate the PBS wash.
39. Add 5 mL of Accutase Cell Detachment Solution to each flask and incubate at room temperature for 5 minutes, or until cells appear rounded.
40. Add 5 mL of VascuNet Assay Medium to each flask. Firmly tap the side of the flask to release the cells from the culture surface.
41. Collect the HUVECs from each flask and transfer the cells within each flask to a 15 mL conical tube. Pipet gently to dissociate any remaining cell clumps.
42. Centrifuge the cells for 5 minutes at 250 x g.
43. Aspirate the supernatant from each tube and resuspend the cell pellets to a single cell suspension in 5 mL of VascuNet Assay Medium per tube.

    Note: It is essential to thoroughly resuspend the cells to single-cell suspension before plating for the angiogenic assay.

44. Combine single cell suspensions (10 mL total volume) and pipet to mix.
45. Count the total number of viable cells in the combined cell suspension using Trypan blue.
46. Dilute the HUVECs in VascuNet Assay Medium to a concentration of 5×10^5 viable cells/mL.

Harvest PC-M Cells

47. Aspirate the culture medium from each of the T75 flasks, and add 5 mL of PBS per flask to wash. Aspirate the PBS wash.
48. Add 2.5 mL of Accutase Cell Detachment Solution to each flask and incubate at room temperature for 5 minutes, or until cells appear rounded.
49. Add 2.5 mL of VascuNet Assay Medium to each flask. Firmly tap the side of the flask to release the cells from the culture surface.
50. Collect the PC-M cells from each flask and transfer to a single 15 mL conical tube. Pipet gently to dissociate any remaining cell clumps.
51. Centrifuge cells for 5 minutes at 250 x g.
52. Aspirate the supernatant and resuspend the cell pellet to a single cell suspension in 10 mL of VascuNet Assay Medium.

    Note: It is essential to thoroughly resuspend the cells to single-cell suspension before plating for the angiogenic assay.

53. Measure the total number of viable cells in the sample by performing a cell count using Trypan blue.
54. Dilute the PC-M cells in VascuNet Assay Medium to a concentration of 1×10^5 viable cells/mL.

Plate HUVECs and PC-M Cells

Refer to sample plate set-up diagram (Fig. 1). In addition to the experimental wells, one row of the 96-well plate will be utilized for control wells. Determine the total number of cells needed, in addition to the control row, based on the number of test samples.

55. **For each set of triplicate HUVEC monoculture samples**, combine 1.32 x 10^5 HUVECs with a sufficient volume of VascuNet Assay Medium to bring the total volume to 495 µL per triplicate set.
56. Dispense 150 µL of the HUVEC cell suspension into each well of the assay, for a total of 4.0 x 10^4 HUVECs per monoculture well.
57. **For each set of triplicate PC-M monoculture samples**, combine 6.6 x 10^3 PC-M cells with a sufficient volume of VascuNet Assay Medium to bring the total volume to 495 µL per triplicate set.
58. Dispense 150 µL of the PC-M cell suspension into each well of the assay, for a total of 0.2 x 10^4 PC-M cells per monoculture well.
59. **For each set of triplicate co-culture samples**, combine 1.32 x 10^5 HUVECs with 6.6 x 10^3 PC-M cells. Add a sufficient volume of VascuNet Assay Medium to bring the total volume to 495 µL per triplicate set.

    Note: This will make a 20:1 ratio of HUVEC:PC-M cells.
60. Dispense 150 µL of the combined cell suspension into each well of the assay, for a total of 4.2 x 10^4 cells per co-culture well.
61. Incubate the plate at 37°C with 5% CO_2 humidified atmosphere undisturbed for 4 to 6 hours to allow cells to adhere and initial tube networks to form.

Addition of the Test Compounds

Following the 4 to 6 hour incubation period, visually inspect the wells to confirm attachment and tube formation in both HUVEC monocultures and co-culture conditions (see Fig. 2). After confirmation of initial tube formation, the cells are ready to be treated with the test reagents. For best results, this is done 4 to 6 hours after plating, but must be completed within 24 hours after cells are plated.

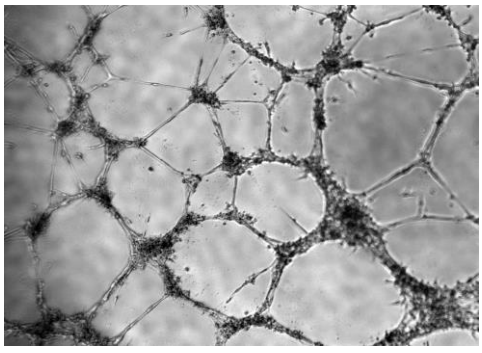


Figure 2. VascuNet HUVEC and PC-M Cell Co-Culture at 4 hours. Co-Culture of HUVEC and PC-M cells seeded at a 20:1 ratio begin formation of tube networks as early as 4 hours.

Positive Control Wells

Positive control wells contain cells in co-culture at a 20:1 (HUVEC:PC-M cell) ratio in VascuNet Assay Medium. No additional compounds are added to these wells.

Negative Control Wells

The negative control samples are run in triplicate co-culture conditions with 50 µM Suramin Hexasodium Salt.

62. Thaw the vial of Suramin Hexasodium Salt for 1 hour at room temperature.
63. Add 25 µL of 1 mM Suramin Hexasodium Salt to 475 µL of VascuNet Assay Medium, for a final Suramin Hexasodium Salt concentration of 50 µM. Warm the 50 µM Suramin Hexasodium Salt solution to 37°C before use.
64. Aspirate the VascuNet Assay Medium from each well of cells and replace with 150 µL per well of the 50 µM Suramin Hexasodium Salt solution.

Experimental Wells

Experimental wells should be tested in co-culture wells in triplicates. Triplicate monoculture samples can also be run for comparison.

65. Prepare additional pro- or anti-angiogenic test compounds by diluting with VascuNet Assay Medium to desired concentrations. Warm diluted test compound solutions to 37°C before use.
66. Aspirate VascuNet Assay Medium from each well to be assayed and replace with 150 µL per well of VascuNet Assay Medium containing the appropriate test compound in solution.

Day 1 to Day 4+

67. Observe and image the cells every 4 to 24 hours to monitor vasculogenic tube assembly and stability.

    Note: Vascular tube formations in co-cultures containing both HUVECs and PC-M cells will be stable for at least 4 days in culture without the need to replace media or add exogenous factors.

EXPECTED RESULTS

The following data describes the expected results for co-culture and monoculture conditions at the recommended cell seeding density of 42,000 cells per well containing a 20:1 ratio of HUVECs to PC-M cells (40,000 HUVECs and 2,000 PC-M cells).

- HUVEC monocultures will begin to form tube networks as early as 4 hours. A complete tube network can be observed at 24 hours. HUVEC tubes lacking PC-M cell support should destabilize by Day 2 of the angiogenesis assay and do not re-assemble.
- PC-M cell monocultures do not form complete tube networks. Minimal branching may be observed.
- Co-cultures form tube networks within four days and are maintained for up to 6 days.
- The addition of 50 µM Suramin Hexasodium Salt (negative control) to both monocultures and co-cultures will reduce tube formation by > 90% within 4 to 24 hours after treatment. The presence of Suramin Hexasodium Salt will prevent tube re-assembly for at least 4 days.

Image Timeline

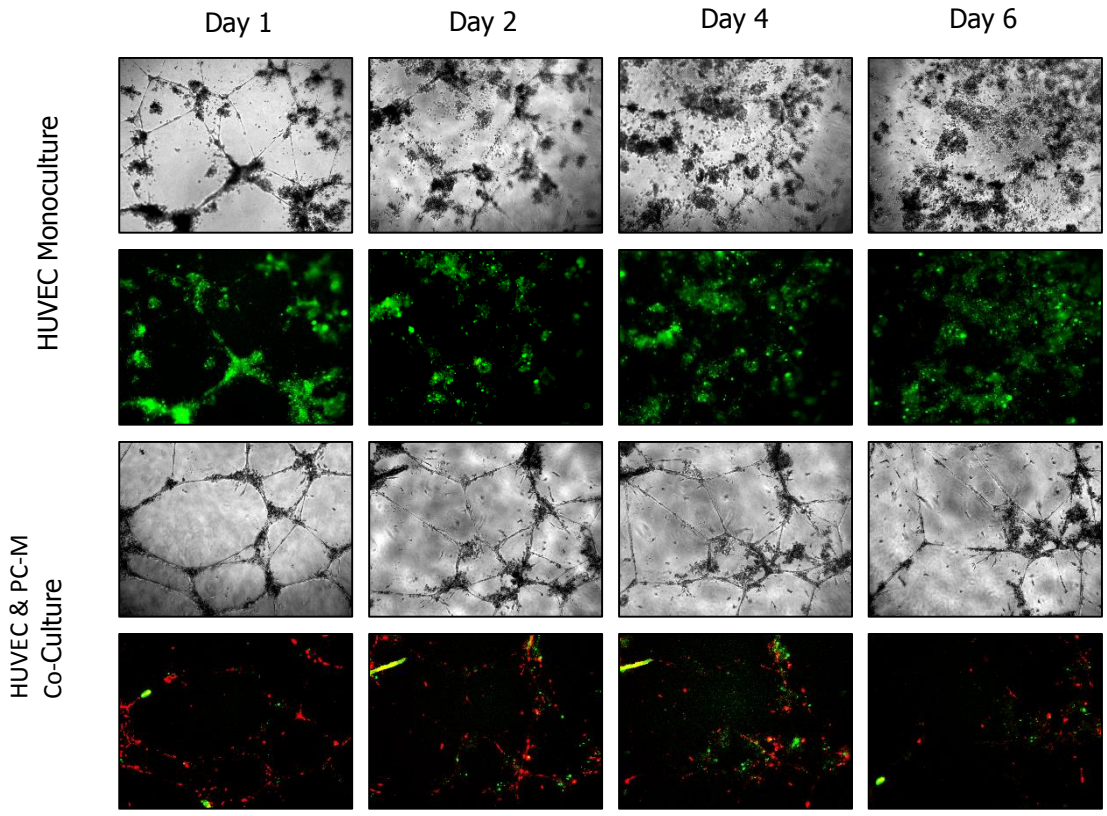


Figure 3. Stability of tube structures over time. HUVEC monocultures (rows 1 and 2) seeded at 120,000 cells/cm^2 and stained with Vybrant DiO (green), show tube formation on Day 1 post seeding. By day 2, degredation of the vessels is already observed. In contrast, tube structures with multiple branching points are visible from Day 1 and are still stable at Day 6 in the HUVEC and PC-M cell co-cultured wells plated at a 20:1 ratio (rows 3 and 4). PC-M cells are stained red with Vybrant DiI. Images were taken at 4X magnification.

APPENDIX

COMMERCIAL SOURCES:

Reagent	Source	Catalog Number
VascuNet Pericyte Co-Culture Assay	ESI BIO	EM-2202
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, *LDEV-Free	Corning	354230
Accutase® Cell Detachment Solution	Innovative Cell Technologies	AT104 – 100 mL

REFERENCES

Armulik, A., et al. (2005) Endothelial/pericyte interactions. Circ Res 97: 512–523.

Benjamin, L.E., et al. (1998) A plasticity window for blood vessel remodeling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 125: 1591–1598.

Bergers, G., et al. (2003) Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 111: 1287–1295.

Blocki, A., et al. (2013) Not all MSCs can act as pericytes: functional in vitro assays to distinguish pericytes from other mesenchymal stem cells in angiogenesis. Stem Cells Dev 22: 2347–2355.

Gerhardt, H., and Betsholtz, C. (2003) Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res. 314: 15–23.

Hamilton, N.B., et al. (2010) Pericyte-mediated regulation of capillary diameter: a component of neurovascular coupling in health and disease. Front Neuroenergetics 2: 5.

Stratman, A.N., et al. (2009) Pericyte recruitment during vasculogenic tube assembly stimulates endothelial basement membrane matrix formation. Blood 114: 5091–5101.

von Tell, D., et al. (2006) Pericytes and vascular stability. Exp Cell Res 312: 623–629.

Zimmerlin, L., et al. (2010) Stromal vascular progenitors in adult human adipose tissue. Cytometry A 77A: 22–30.