Mark Slevin · Garry McDowell *Editors*

Handbook of Vascular Biology Techniques

Springer

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ISBN 978-94-017-9715-3 ISBN 978-94-017-9716-0 (eBook)
DOI 10.1007/978-94-017-9716-0

Library of Congress Control Number: 2015936145

Springer Dordrecht Heidelberg New York London

Printed on acid-free paper

Springer Science+Business Media B.V. Dordrecht is part of Springer Science+Business Media (www.springer.com)

The authors would like to dedicate this book to:
(Mark-Asha Shakira Slevin)
(GMcD: To Christine and Amy)

Preface

Within this book, a variety of techniques are described in detail pertaining to methods used in both basic and advanced vascular biology. Methodologies range from in vitro cell culture to in vivo manipulations, through cell signalling proteomics and genomics to patient imaging in disease. A number of novel, state-of-the-art methodologies are also included. Each chapter is fully inclusive, containing sections on trouble shooting and additional notes/links thus ensuring the reader has sufficient information to carry out the protocol without additional requirements. This book should appeal to students, researchers and medical professionals working in all vascular-linked fields such as cardio- and cerebro-vascular, cancer and dementia.

Manchester, UK

Mark Slevin
Garry McDowell

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Part I
In Vitro Techniques

Chapter 1
In Vitro Angiogenesis Assay: Endothelial Migration, Proliferation, and Tube Formation

Kazuhide Hayakawa, Anna Chun-Ling Liang, Changhong Xing, Eng H. Lo, and Ken Arai

1.1 Introduction

Over the past four decades, angiogenesis has been well studied in both normal developmental processes and numerous pathologies including diabetes, cancer, myocardial infarction, brain injury and ischemic stroke [1–6]. Angiogenesis is a fundamental process to develop new blood vessels from pre-existing vessels and two different mechanisms have been described. One is an intussusceptive angiogenesis that is caused by the insertion of interstitial cellular columns into the lumen of pre-existing vessels [7]. The subsequent growth of these columns and their stabilization results in partitioning of the vessel and remodeling of the local vascular network [8]. The other is a sprouting angiogenesis induced by migration of endothelial cells toward the site of angiogenesis, proliferation of endothelial cells behind the front of migration, and lumen formation within the endothelial sprout and formation of loops by anastomoses of sprouts.

Most endothelial cells within the body are naturally quiescent and physiology of angiogenesis is well controlled to meet the tissue requirements. Under some pathological conditions, however, angiogenesis occurs in a disorderly sequence of cellular events. For example, moyamoya syndrome is an angiogenic disease caused by blocking blood flow through the vessel constriction, and blood clots. Newly formed brain vessels in moyamoya disease are small, weak and prone to hemorrhage, aneurysm and thrombosis [9]. Therefore, a greater understanding of the cellular/molecular mechanisms of angiogenesis in relevant diseases may be necessary to advanced therapeutic strategies. In this chapter, we will describe the methods to assess angiogenesis in vitro.

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M. Slevin, G. McDowell (eds.), *Handbook of Vascular Biology Techniques*,
DOI 10.1007/978-94-017-9716-0_1

1.2 Methodology

In vitro angiogenesis assays are well-established and useful tools to test efficacy of both pro- and anti-angiogenic agents. Major assessments of angiogenesis in vitro focus on endothelial migration, proliferation, and tubular- or capillary-like network formation on the biological materials including extracellular matrix. Here we will describe the endothelial scratch migration assay (wound healing assay), proliferation assay, and matrigel tube formation assay as principal methods commonly used in in vitro angiogenesis assays. Our protocol is outlined below.

1.3 Materials

1. Collagen I-coated culture plates (6 wells or 24 wells)
2. 5 % CO_2 incubator at 37 °C
3. EBM-2 basal medium (Lonza)
4. EGM-2 supplemental (Lonza)
5. Penicillin/streptomycin (GIBCO)
6. 0.05 % Trypsin-EDTA solution (GIBCO)
7. Phosphate buffer saline (GIBCO)
8. p200 or p1000 pipette tip
9. Cell counting kit-8 (WST) (Dojindo)
10. Growth factor reduced (GFR) Matrigel matrix (BD Bioscience)
11. Endothelial cells
    - Human brain microvascular endothelial cells (HBMEC, Cell Systems Corporation)
    - Rat brain endothelial cells (RBE.4)
    - Outgrowth endothelial progenitor cells (see the Sect. 1.4.4 in detail)
12. Two spleens isolated from 11 to 12 weeks old Sprague–Dawley (SD) rats.
13. Ficoll-Paque Plus (Amersham Biosciences Corp)
14. 40-μm nylon membrane
15. EPC lysis buffer (4 % BSA, 1 mM EDTA in PBS).
16. 4 % paraformaldehyde (PFA) (Wako)

1.4 Basic Protocol

1.4.1 Scratch Migration Assay

1. Use endothelial cells fully confluent in the flask, wash twice with PBS, then add 3 mL cell detachment solution and incubate at 37 °C for 5 min.
2. Add fresh 3 mL EGM, gently draw up the cell suspension, and spin down to obtain cell pellet.

3. Resuspend cells with EGM, and plate 2×10^5 cells per well in 6-well plate pre-coated with collagen-I, as 5×10^4 cells per well for 24-well plate.
4. Return plates with cells to incubator at 37 °C and 5 % CO_2. Change to fresh EGM every 2–3 day until the cells are almost confluent and make a monolayer.
5. Change to working medium, and incubate overnight for serum starvation.
6. Scrape the cell monolayer in a straight line with p200 or p1000 pipette tip. Remove the debris by washing twice with warm working medium, and then replace with fresh working medium.
7. Mark the reference points to obtain the same field during the image acquisition with a razor blade on the outer bottom of the culture plate or with an ultrafine tip marker.
8. Return the cells back to culture incubator for 8–24 h.
9. After the incubation, wash the cells in each well twice with PBS gently to remove detached or dead cells, and fix cells by 4 % PFA for 10 min at 4 °C.
10. Place the culture plate under a phase-contrast microscope, match the reference point, and acquire images at least four random areas.
11. Count the cells that cross into the scratched area from their reference points, these cells are determined as migrated cells.

1.4.2 Proliferation Assay

1. Use endothelial cells fully confluent in the flask, wash twice with PBS, then add 3 mL cell detachment solution and incubate at 37 °C for 5 min.
2. Add fresh 3 mL EGM, gently pipette to get the cell suspension, and spin down to get cell pellet.
3. Resuspend cells with EGM, and plate 2×10^4 cells per well in 24-well plate pre-coated with collagen-I.
4. Return plates with cells to incubator at 37 °C and 5 % CO_2.
5. Wait until 50 % confluent and change to working medium, and incubate overnight for serum starvation.
6. Stimulate endothelial cells with reagents or test drugs for 24 h.
7. The cells are incubated with 10 % WST solution for 1 h at 37 °C. Then the absorbance of the culture medium was measured with a microplate reader at a test wavelength of 450 nm and a reference wavelength of 630 nm.

1.4.3 Matrigel Tube Formation

1. Thaw the matrigel on ice or in cold room completely and slowly. Keep it on ice until use.
2. Keep the multi-well plate on ice. Use pre-cooled tips to add 200 μl soluble matrix into wells of 24-well plate, make sure it cover the bottom evenly, and no bubbles in the gel mixture.

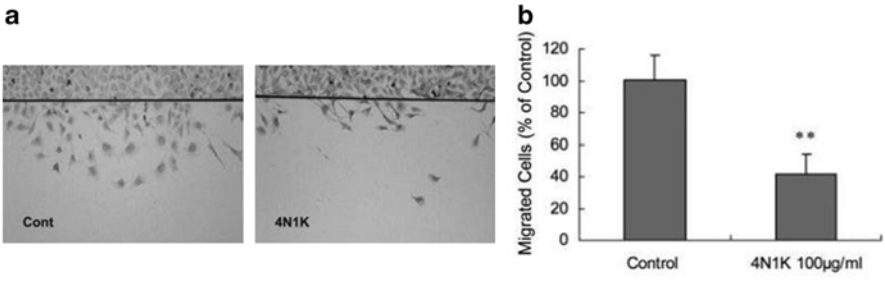
3. Incubate the plates with matrix at 37 °C for 30–60 min, the matrix will be polymerized to insoluble gels and ready to use.
4. Endothelial cells at 80–90 % confluency were trypsinized, collected and suspended in culture medium with reduced serum and growth factors.
5. Count the cells with hemocytometer, diluting the cells with enough medium to 2.5×10^5 per mL.
6. Seed the endothelial cells on the surface of Matrigel-coated wells at 5×10^4 in each well in 200 μl.
7. Incubate in the regular cell incubator for 6–18 h, at 37 °C and 5 % CO_2.
8. Cells are observed under phase-contrast microscope, and 2–3 images for each well are acquainted under light microscope.
9. Images are imported and counted for the complete rings in each well. More rings mean higher capability of tube formation.

1.4.4 Outgrowth Endothelial Progenitor Cells Isolation

1. Under the hood, spleens were mechanically minced, placed at 37 °C for 15 min in 20 ml EPC lysis buffer.
2. Then run through a 40-um nylon membrane to obtain cell suspension.
3. Add 4.5 ml cell suspension gently onto 5 ml Ficoll-Paque Plus and centrifuge 1,500 rpm for 25 min to obtain mononuclear cells (MNCs).
4. Isolated MNCs are gently washed twice with complete growth media EBM-2 plus supplement.
5. 3×10^7 MNCs per well are seeded on collagen I-coated six-well plates and incubated in a 5 % CO_2 incubator at 37 °C.
6. Outgrowth EPCs are obtained after culturing for 1–1.5 months.

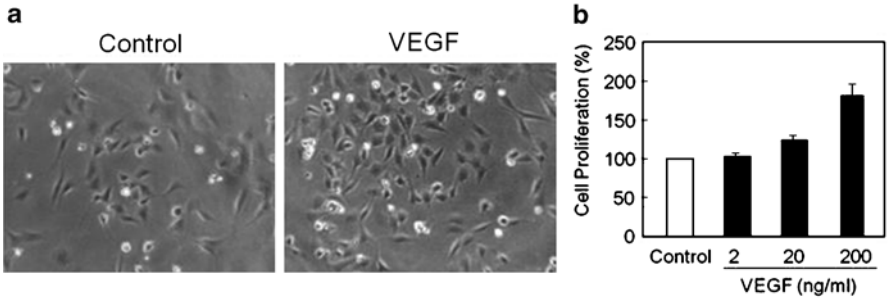
1.5 Sample Results

1.5.1 Scratch Migration Assay [10]



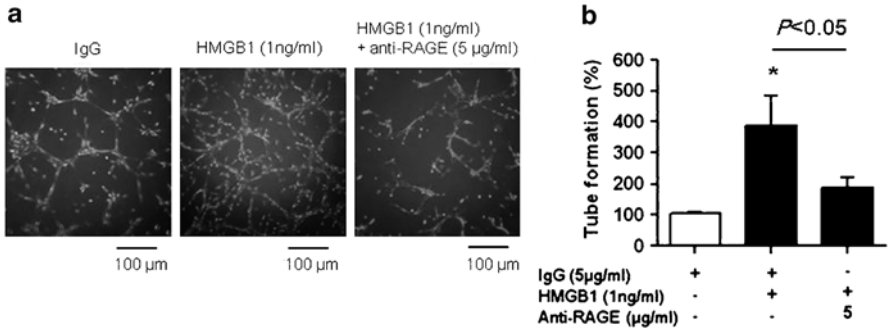
(**a**) Representative images showed decreased migration of human brain endothelial cells across a wound scratch line 24 h after treatment with a CD47-specific activator 4N1K (100 μg/ml) as compared to the control (untreated) cells. (**b**) Quantified cell counts showed a significant difference in cell migration between controls and 4N1K-treated cells (**$P<0.01$, $n=3$. Data are expressed as mean ± SD)

1.5.2 Proliferation Assay



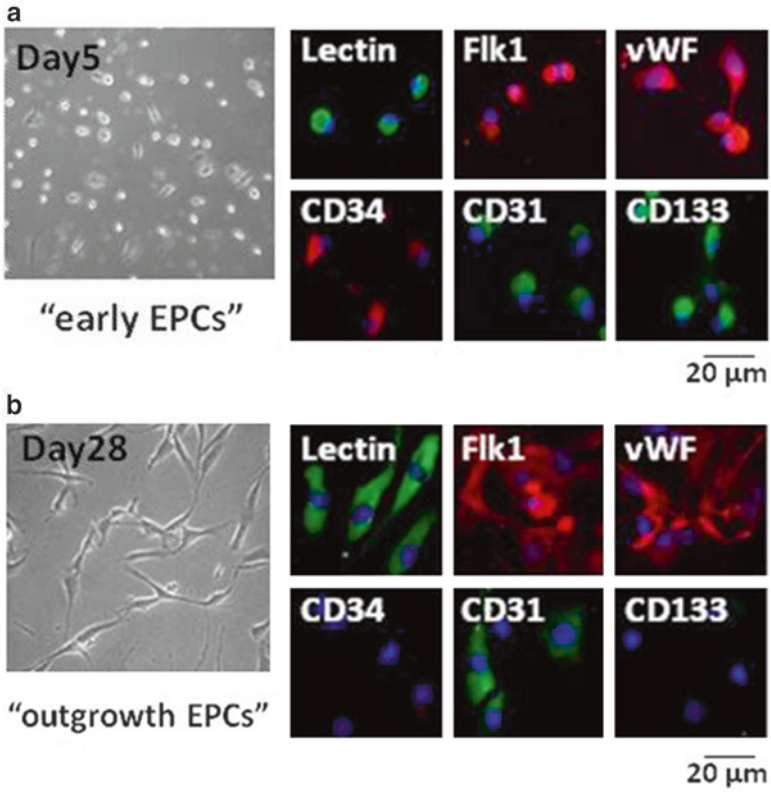
Rat brain endothelial cells were incubated with VEGF-A for 24 h. (**a**) Representative images of proliferation using rat brain endothelial cells. VEGF-A: 200 ng/ml (**b**) VEGF-A induced endothelial proliferation in a dose-dependent manner ($n=4$. Data are expressed as mean ± SEM)

1.5.3 Tube Formation Assay [11]



(**a**) Representative images of tube formation using outgrowth EPCs. HMGB1: 1 ng/mL, anti-RAGE: 5 ug/mL. (**b**) HMGB1 (1 ng/mL) significantly increased the number of tubes, and the HMGB1-induced tube formation was reduced by co-treatment with anti-RAGE neutralizing antibody (*$P<0.05$, $n=5$. Data are expressed as mean ± SEM)

1.5.4 Outgrowth Endothelial Progenitor Cells



Representative images of early EPCs and outgrowth EPCs. (**a**) Early EPCs at day 5 after seeding bind to lectin (Ulex Europaeus Aglutinin) and showed positive staining for Flk1, vWF, CD34, CD31, and CD133. (**b**) When allowed to mature into late-phase EPCs (28 days), these cells show endothelial morphology, and positive for lectin, Flk1, vWF and CD31, but dim stain for the immature markers CD34 or CD133 (All figures are counter-stained blue for DAPI)

1.6 Troubleshooting

1.6.1 Scratch Migration Assay

1. The endothelial cells should not be overgrown to keep monolayer. Overgrown cells could be multi-layers which causes higher amount of cell debris or free floating cells after scratching.
2. Serum starvation is an important step to minimize background signal before cell stimulation.

3. To create scratches of approximately similar size is critical to minimize any possible variation caused by the difference in the width of the scratches. For 24 well, the p200 pipette tip can be used easier than p1000 pipette tip.
4. Choose shorter incubation time under faster migrating conditions.
5. Before imaging, washing step is important. Wash gently and try to remove all debris and free-floating cells to distinguish real migrating cells. Fixing cells helps to keep cell morphology and allows longer imaging time.

1.6.2 Proliferation Assay

1. Serum starvation is an important step to minimize background signal before cell stimulation. Sensitive endothelial cells such as endothelial progenitor cells may need to be supplemented by adding 0.5 % FBS.
2. Percentage of cell confluence is important. Please use appropriate cell confluence around 50–60 %.

1.6.3 Tube Formation Assay

1. In the case of no tube formation is observed in the positive control group, there are two possibilities. One is that cells may not be healthy or cells are too old. Please use healthy cells or a lower passage. Secondly, the cell density may be too high or low. Please use optimal cell density (e.g., 2×10^4 cells/well for 24-well plate).
2. After getting solid matrigel, do not disturb the gel layer. If you need to change the culture medium during the experiment, use a pipette to gently remove the medium rather than using aspiration.
3. To avoid generating bubbles, do not mix the liquid matrigel solution or pipette the solution up and down before coating the plate. If bubbles are generated, a few seconds of centrifugation using ~10,000 g would be helpful to remove bubbles.
4. For the thin coating of Matrigel matrix described here, the surface is not flat but crescent, which may make it difficult to get the pictures with good focus. The μ-Slide Angiogenesis from ibidi GmbH (Planegg/Martinsried, Germany) is used with only 10 μL of matrix, and makes a flat surface and bring all cells in one focal plane. Please see the Method variations/alternative.

1.7 Method Variations/Alternative

1.7.1 Transwell Migration Assay

Using a modified Boyden chamber [12], endothelial migration from upper chamber to lower part of membrane could be counted for migrated cells.

1. Use endothelial cells fully confluent in the flask and culture overnight with serum starvation, wash twice with PBS, then add 3 mL cell detachment solution and incubate at 37 °C for 5 min.
2. Add fresh 3 mL EGM, gently pipette to get the cell suspension, and spin down to obtain cell pellet.
3. Resuspend cells with EBM-2 basal media, and plate 2×10^4 cells/100 μl per upper chamber for 24-well plate.
4. Carefully place the upper chamber onto the lower wells with 600 μl EBM-2 basal media and test compounds.
5. Return plates with cells to incubator at 37 °C and 5 % CO_2.
6. Wait for 4 h, and gently wash upper chamber three times with PBS.
7. Incubate the upper chamber in 4 % PFA for 15 min to fix migrated cells.
8. Rinse in PBS again, cut the membrane, and place the membrane onto glass slides, mount onto glass slides with mounting media, cover with cover slips, and allow to dry overnight.
9. Image using a microscope to quantify the number of cells that have physically migrated from one side of the membrane to the other side.

1.7.2 BrdU Cell Proliferation Assay

5-bromo-2'-deoxyuridine (BrdU), a thymidine analog, enables detection of DNA replication in actively proliferating cells using a monoclonal antibody directed against BrdU and a fluorophore-conjugated secondary antibody. BrdU staining facilitates the identification of cells that have progressed through the S phase of the cell cycle during the BrdU-labeling period.

1. Use endothelial cells fully confluent in the flask, wash twice with PBS, then add 3 mL cell detachment solution and incubate at 37 °C for 5 min.
2. Add fresh 3 mL EGM, gently pipette to get the cell suspension, and spin down to obtain cell pellet.
3. Resuspend cells with EGM, and plate 2×10^4 cells per well in 24-well plate pre-coated with collagen-I.
4. Return plates with cells to incubator at 37 °C and 5 % CO_2.
5. Wait until 50 % confluent and change to working medium, and incubate overnight for serum starvation.
6. Stimulate endothelial cells with reagents or test drugs for 24 h.
7. Dilute BrdU with EBM-2 basal media to a final concentration 160 μM.
8. Incubate endothelial cells with BrdU solution for 60 min at 37 °C and 5 % CO_2.
9. Aspirate culture medium and add 100 μl of 70–80 % methanol for 15 min.
10. Aspirate fixation solution and wash plate twice with PBS.
11. Proceed with immunocytochemical staining to detect BrdU.

1.8 Additional Note

1.8.1 Scratch Migration Assay

1. RBE.4 cells could migrate into the scratched area under basal condition (with working medium). Four random areas from both edges of the scratched area could be photographed, providing large sample sizes for statistical analysis.
2. Acquired images could be further analyzed quantitatively with other indexes, such as the migrating distance from reference point from at least 100 cells for each condition, or the area occupied by migrated cells. Some free software, such as Image J (http://rsb.info.nih.gov/ij/) could be used for the measuring.

1.8.2 Tube Formation Assay

1. Recommended protein concentration of BD Matrigel matrix is 10 mg/ml or higher. The effect of Matrigel from a different lot on tube formation might be different, so it is better to use the Matrigel from same lot number for your whole experiment.
2. Thaw the soluble matrix slowly on ice, usually overnight. Once thawed, swirl the bottle slowly on ice to make the solution leveled. Try to avoid repeatedly freezing and thawing. The new bottle of Matrigel matrix could be dispensed into appropriate aliquots, and refreeze immediately.
3. The cells could elongate and align to one another as early as 3 h after seeding. The images can be acquired at early time (~6 h incubation time) or late time (~18 h incubation time) for analysis. After longer incubation time, the proteinases secreted from cells might digest the gels and destroy the supports for the tubes.
4. The parameters for quantitative image analysis could be numbers, areas or perimeters of the rings (tubes), or the numbers of branching points, and cell-covered areas.

1.9 Applications and Discussion

Angiogenesis plays important roles in tissue healing and in recovery from ischemic incidents, including heart attack or stroke. On the other hand, angiogenesis is also triggered in cancer, and solid tumors promote new vessel formation that allows supplying nutrients and oxygen to the growing mass. Thus, understanding cellular/molecular mechanisms of angiogenesis in relevant diseases may be critical to establish advanced therapeutic strategies.

In vitro angiogenesis assays are useful for screening potential targets and valuable tools to resemble the in vivo conditions, especially with supporting materials such as extracellular matrix. However, in vitro assays are only best viewed as inceptive data. In order to fully understand the effects of the test compounds in angiogenesis, multiple tests should be used to obtain maximum interpretations from in vitro tests. Also, angiogenesis pathways with either the relevant endothelial cells or multiple endothelial cell types are necessary to organ-specificity. Finally, combination assessments with in vivo models are required to fully investigate disease-related angiogenic mechanisms under pathological conditions.

Acknowledgments and Funding Supported in part by the National Institutes of Health. Materials including figures in this chapter have been extensively drawn from our previously published papers including: Xing et al., J Neurosci Res, 2009; Hayakawa et al., J Neurochem, 2012; Hayakawa et al., PNAS, 2012.

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Chapter 2
Endothelial Cell Tube Formation on Basement Membrane to Study Cancer Neoangiogenesis

Amelia Casamassimi, Filomena de Nigris, Concetta Schiano, and Claudio Napoli

2.1 Introduction

During angiogenesis, endothelial cells (ECs) undergo activation after binding of angiogenic factors to their receptors, release of proteases to dissolve the basement membrane, migration towards an angiogenic signal, proliferation, and an increase in cell number for new blood vessel formation. Finally, reorganization of ECs forms the three-dimensional vasculature. Tube-formation assay is one of the simple, but well-established in vitro angiogenesis assays based on the ability of ECs to form three-dimensional capillary-like tubular structures, when cultured on a gel of growth factor-reduced basement membrane extracts. During the assay, ECs differentiate, directionally migrate to align, branch, and form the tubular polygonal networks of blood vessels.

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M. Slevin, G. McDowell (eds.), *Handbook of Vascular Biology Techniques*,
DOI 10.1007/978-94-017-9716-0_2

The in vitro formation of capillary-like tubes by endothelial cells on a basement membrane matrix is a powerful in vitro method to screen for pro-angiogenic and anti-angiogenic factors. This assay can be used as a first screen before in vivo models and can be done also as a high throughput procedure. Since the first description in 1988, this assay have been used for many purposes both in cancer and cardiovascular fields, including analysis of pro-angiogenic or anti-angiogenic factors, definition of the signaling pathways involved in angiogenesis, identification of the genes regulating angiogenesis and endothelial progenitor cell characterization.

In our experimental settings we used in a model of osteosarcoma this assay to analyze the role of YY1 transcription factor and some cyclin-dependent kinases (CDK) on angiogenesis; to this aim, we performed tube formation assay to test both the effect of YY1 silencing and some inhibitors, like T22, a peptide affecting the chemokine receptor CXCR4 and roscovitine, a CDK inhibitor [1, 2].

2.2 Methodology

This angiogenesis assay consists of a quick measurement of the ability of ECs to form three-dimensional structures (tube formation) in vitro when they are plated on an appropriate extracellular matrix support. Endothelial tube formation on ECM gel mimics the in vivo environment and may be employed to test angiogenesis stimulators or inhibitors before in vivo analysis. Factors to be tested can be added exogenously to the medium, or they can be transfected or knocked down in the endothelial cells to determine their effects on angiogenesis.

As extracellular matrix support, the basement membrane extract obtained from a murine tumor rich in extracellular matrix proteins is generally used for this assay. The extract is in a liquid state at 4 °C or lower temperatures whereas it forms a gel at 16–37 °C. The routinely used basement membrane extracts are sold commercially; examples are BD Matrigel, Cultrex BME and other Engelbreth–Holm–Swarm (EHS) extracts. It is generally recommended the use of growth factor-reduced material especially to study stimulators of angiogenesis. Importantly, variability in the tube-formation activity of different preparations purchased from different vendors has also been observed. Thus, when possible, the same preparation should be used for the entire study.

Endothelial cells (ECs) typically used for this assay are obtained from human umbilical vein (HUVECs) or human aorta (HAECs), but other cells, such as lines established from mice (SVEC4-10) and humans (HMEC-1) also work well. It should be considered that ECs have considerable organ- and tissue-specific heterogeneity that may affect their response to specific factors and the time required for tube formation in vitro. The cells should be of low passages if they are primary; for instance, HUVECs should not be passaged more than 10 times, HAECs work well until the 7th–8th passage. The cells should be 80% confluent and passaged the day

before the assay for optimum and consistent tube formation. The seeding density is another important point to consider with about 50,000 cells per cm^2 as recommended density for HUVECs and HAECs, but this number may vary depending on the cell source. Cells initially attach to the matrix, then migrate toward each other, align and finally form tubes. The time of the assay is very short for transformed cells compared to primary cells (3 h versus 16–20 h for primary cells), but should be determined for each EC type. Moreover, time is also dependent on the utilized matrix.

2.3 Materials (and Company Name)

Endothelial cell lines can be those with and without drug treatment or expressing the gene of interest.

2.3.1 Reagents

Growth factor-reduced BD Matrigel (BD Biosciences 354230)
Primary Human Aortic Endothelial Cell (HAEC) (Lonza CC2535)
Trypsin-EDTA
Endothelial Basal Medium-2 (EBM-2; Lonza, CC-3156)
EGM-2 SingleQuot BulleKit (Endothelial cell growth medium-2; Lonza, CC-3162)
Dulbecco's Phosphate-Buffered Saline, 1x
OPTI-MEM (Life technologies, Gibco 31985-047)
Cultrex Cell Staining Solution (Trevigen, 3437-100-01)
Methanol (Sigma, M3641)
96-well cell culture plates
15 ml conical centrifuge tubes-sterile
Tissue culture flasks, 25 cm^2, filter cap, 50 ml
Disposable sterile plastic pipettes
ImageJ software downloaded from the NIH website.

2.3.2 Equipment

Tissue culture setup
Inverted microscope with digital camera
Cell culture incubator (humidified, 5 % CO_2)
Biological hood with laminar flow and UV light
Pipette aid

Sterile micropipette
37 °C water bath
Centrifuge with a swing-bucket rotor, refrigerated
Hemocytometer (Burker chamber)

2.4 Basic Protocol

All procedures should be performed under sterile conditions in a biological safety cabinet using aseptic technique to prevent contamination. The procedure is standardized for HAEC (Lonza) as ECs and Matrigel (BD Biosciences) as matrix; however, other ECs or matrix extracts can also be used in substitution; in this case technique requires optimization. See also additional notes for reagent preparation.

2.4.1 Thawing and Passaging of Human Aortic Endothelial Cells (HAEC)

1. Prepare a bottle of complete EGM-2 as indicated. Note: the supplemented medium should be stored in the dark at 4 °C and should not be frozen. When stored in these conditions it is stable for 1 month.
2. Seed cryopreserved endothelial cells at 2.5×10^5 viable cells per a 25-cm^2 tissue-culture flask using 5mL EGM-2.
3. Change culture medium 24–36 h after seeding.
4. Change the medium every other day thereafter, until the culture is approximately 80 % confluent (5–6 days).
5. Using standard procedures to passage endothelial cells, split them when they are 80 % confluent. Usually, plating 5×10^5 to 1×10^6 cells in a 25-cm^2 flask works well.

The cells should be passaged at least twice after thawing before being used in the tube formation assay. However, the HAECs should not exceed passage 8–10.

2.4.2 Coating Plates with Matrigel

6. Remove Growth factor-reduced BD Matrigel from the freezer (−20 °C) and thaw in an ice bath in a refrigerator (4 °C) overnight. As other extracellular matrix preparations, Matrigel gels very easily; thus, it is important not to warm it during the thawing process and always to keep it on ice and to pre-chilled pipet-tips and plates.

7. Add 150–200 µl of Matrigel per cm^2 growth area and incubate plate for 30–60 min at 37 °C on a level surface to allow the gel to solidify. Note that 100 µl is necessary for each well in 96-well plates. It is very important to avoid bubble formation. If air bubbles get trapped in the wells, centrifuge the plate at 300xg for 10 min at 4 °C.

2.4.3 HAEC Preparation for the Assay

8. Starve cells with non-supplemented EBM-2 for 3–6 h prior to performing the assay. Then, harvest cells according to the producer instructions (Lonza).
9. Determine cell number and viability by counting cells in a Burker chamber.
10. Collect cells by centrifugation at 220xg for 5 min.
11. Aspirate supernatant and resuspend cell pellet in non-supplemented EBM-2 at a concentration of 2×10^5 cells/ml by gently pipetting up and down a few times to obtain a homogeneous single cell suspension. Be sure that cells are well mixed since cell density has an effect on tube formation (see also the point below and sect. 2.8).
12. To set the experimental points, dilute cells in non-supplemented EBM-2 in the presence or absence of angiogenesis inducers and inhibitors to be tested. Use appropriate negative and positive control samples (non-supplemented EBM-2 and complete EGM-2, respectively).

2.4.4 Starting the Assay and Analyzing Data

13. Prepare the appropriate number of HAEC cells in 1.5 ml tubes, according to the number of target cell lines to be used. Note: Per each test, one tube of HAEC will be suspended with a sufficient amount of cells to be dispensed in three wells. Thus, a triplicate will be done per each experimental point. Note that each well requires 100 µl of the HAEC cell suspension (corresponding to 2×10^4 cells/well).
14. Dispense 100 µl of the HAEC cell suspension obtained thorough mixing into the labeled wells of a 96-well plate. Be careful not to touch the surface of the gel when adding the cells to avoid injuring the gel. Incubate the plate at 37 °C, 5 % CO_2 for a period of 4–16 h, or until the desired result is achieved.
15. Visualize the cells every hour for tube formation under an inverted light microscope with 4× or 10× objective.
16. Photograph the capillary network in the wells using a digital camera attached to the inverted microscope (see also paragraph 8).
17. Analyze data using the ImageJ software (Fig. 2.1).

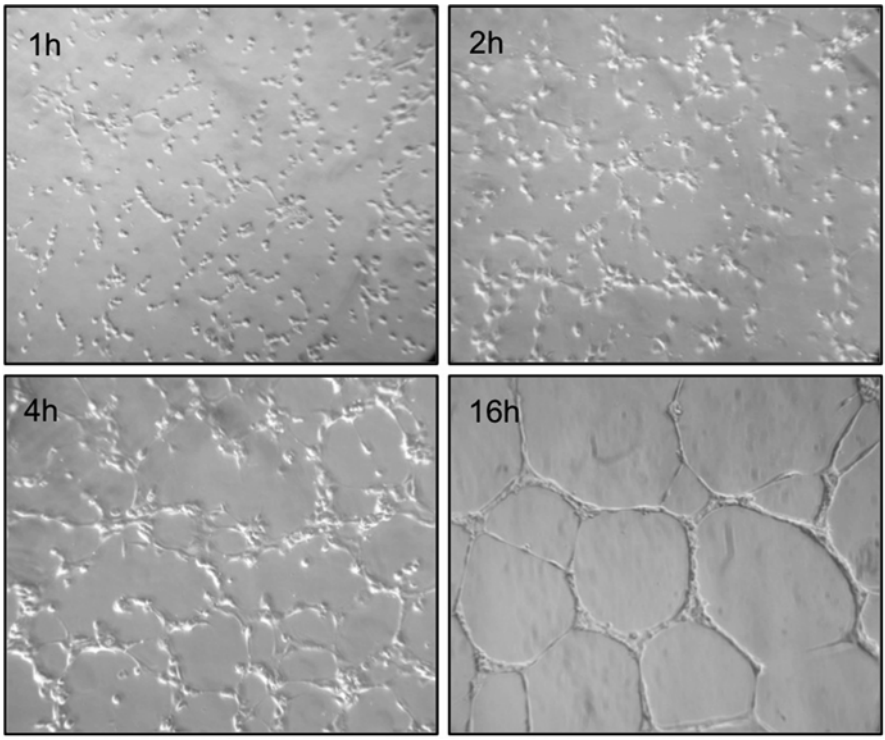

Fig. 2.1 Representative images of tube formation assay on the growth factor-reduced Matrigel by HAEC after incubation with supplemented EGM-2 for 1, 2, 4 and 16 h

2.5 Examples of Experimental Plans

This assay can be used to test in vitro a novel molecule and verify its potential as pro-angiogenic or anti-angiogenic factor. The angiogenic activity of one molecule at different concentrations or of more molecules can be easily tested simultaneously. To this purpose, cells (HAECs or alternatively HUVEC or other ECs) are diluted in non-supplemented EBM-2 medium in the presence or absence of the molecule to be assayed.

Alternatively, you can use conditioned medium from a cancer cell line to study its potential to activate angiogenesis through the release of angiogenic factors in the medium. In this setting you can test the cancer cell line under different experimental conditions. For instance, you can study the involvement of a particular gene in this process by utilizing the cancer cell line transfected to overexpress or silence that particular gene. Indeed, in our studies we have used an osteosarcoma cell line (SaOS) both un-transfected and silenced for the transcription factor YY1 [1, 2].