Lonza
Specialized Modalities

A quantitative, high-throughput, real-time angiogenic tube formation assay

Human umbilical vein endothelial cells and human dermal fibroblasts in co-culture

Paul Bridgers[1], Sarah Thacker[1], Scott Kelsey[1], Manisha Nautiyal[1], Kevin Grady[2], and Anshika Sharma[1]

1. Cell Biology Center of Excellence, Lonza, Durham, NC, USA; 2. Lonza, Walkersville, MD, USA

Quantifying results from angiogenic tube formation assays has historically presented a significant challenge in high-throughput settings. This study established and validated a physiologically relevant, scalable angiogenic tube formation assay that utilizes a co-culture of Lonza human umbilical vein endothelial cells (HUVECs) and Lonza normal human dermal fibroblasts. This approach allowed quantification of tube formation at multiple time points over 5 days, providing a wealth of data from multiple donor pools without having to repeatedly disturb or sacrifice cultures. This method allows researchers to incorporate a larger number of donors, replicates, and time points into their drug discovery screening assays, providing quantitative results with high physiological relevance while minimizing the amount of effort required.

Introduction

Angiogenesis is the process of blood vessel formation via the migration, growth, and differentiation of the endothelial cells of existing blood vessels. It is both a normal and vital process (e.g., for healthy growth and development, reproduction, wound healing, etc.), as well as a fundamental process underlying the pathology of some diseases (including cancer, retinopathy, and many others).[1–5]

Angiogenesis can be modeled *in vitro* using tube formation assays, which induce endothelial cells to form distinct tube-like structures. These assays can be used in the drug development process to test compounds that inhibit or promote angiogenesis; however, the quantification of results has historically presented a significant challenge in high-throughput settings. Interpretation relies either on qualitative visual measures that lack objectivity or labor-intensive manual counting. Plates can be imaged, but this forces a trade-off between replicates, time points, and donor availability.[6–8] Furthermore, choosing an assay that permits relevant endpoint analyses is critical. Assay design (e.g., use of gel matrix versus fibroblasts as the feeder layer), as well as the physiological outcome targeted by the intervention (e.g., proliferation vs. network formation), dictate both the physiological relevance of as well as the context under which assay results may be interpreted.[6,7,9] Angiogenic tube formation assays of physiological relevance that are both readily quantifiable as well as scalable to high-throughput are needed.

This study demonstrates and validates a high-throughput, quantitative angiogenic tube formation assay that utilizes a co-culture of human umbilical vein endothelial cells (HUVECs) and normal human dermal fibroblasts (“fibroblasts” hereafter).

Methods

This angiogenic co-culture assay was prepared in 96-well tissue culture plates according to the instructions found in the protocol published on the Lonza website.[10] The following steps briefly cover the optimized protocol developed in this study.

Thawing and plating fibroblasts

Fibroblasts were thawed and plated one hour prior to thawing and plating the HUVECs, as fibroblasts require an hour of incubation at room temperature to attach and acclimate. Lonza adult normal human dermal fibroblasts (part no. CC-2511) were thawed in a 37°C water bath one donor at a time. Seeding conditions require 30,000 cells/well, so the total number of cryovials needed per donor was calculated based on the number of viable cells. Amps were then thawed in groups of ≤4 cryovials at a time. Cells were then transferred to a single 15 mL conical tube containing 3 – 4 mL of room temperature Lonza FGM® 2 Medium. Cells were centrifuged and the supernatant was removed, then resuspended in FGM® 2 and counted using trypan blue and a hemocytometer. Fibroblasts were then plated in 96-well plates at a density of 30,000 cells/well in 200 µL room temperature FGM® 2 Medium for one hour. If the HUVEC cells were not going to be ready to seed within an hour, the fibroblasts were placed in the incubator at 37°C, 5% CO_2, and 90% humidity.

Thawing HUVECs and establishing co-culture

Pooled donor primary HUVECs that had been qualified for angiogenic markers (part no. C2519AS) were thawed one donor at a time. Seeding conditions require 10,000 HUVECs per well, so the total number of cryovials needed per donor was calculated based on the number of viable cells. Amps were thawed in groups of ≤3 cryovials at a time. After thawing, cells were gently resuspended, then transferred to a single 15 mL conical tube containing 2 – 3 mL of 37°C EBM® 2 basal medium (contains no fetal bovine serum [FBS]). Cells were counted with trypan blue and a hemocytometer, then stained with 1 µL of 10 mM Cell Tracker® Dye (Invitrogen™) and placed in a hula mixer in the incubator (37°C, 5% CO_2, and 90% humidity) for 45 minutes at low speed. After this, 10 mL of EGM® 2 without vascular endothelial growth factor (VEGF) supplement (all other EGM® 2 BulletKit® supplements were added, including FBS) was added to the 15 mL conical tube. Following this, cells were centrifuged, supernatant removed, and then resuspended in EGM® 2 without VEGF so that 100 µL of cell suspension contained an estimated 10,000 HUVECs (i.e., 1×10^5 cells/mL). The plate containing the fibroblasts was then retrieved and 100 µL of medium was carefully aspirated from each well. 100 µL of HUVEC cell suspension was then added to each well in the appropriate HUVEC-fibroblast donor combinations. The resulting optimized co-culture contained HUVECs and fibroblasts in a 1:3 ratio (~10,000 HUVECs and 30,000 fibroblasts). Plates were then placed in the incubator at 37°C, 5% CO_2, and 90% humidity overnight.

The following day, EGM® 2 Medium was prepared with a titration of custom VEGF concentrations. All medium was aspirated from all wells and replaced with 200 µL of EGM® 2 with VEGF without disturbing the cells. Plates were returned to the incubator and incubated for 5 days, with medium changes occurring every 2 – 3 days. Seeding densities and VEGF concentrations were optimized based on the rates of angiogenic tube formation as measured by live cell imaging, resulting in the final protocol.

Method validation: angiogenic tube formation assay with suramin inhibition

After optimizing the seeding densities and amount of VEGF to best promote angiogenesis, the co-culture was validated for use in an angiogenic assay using suramin, an angiogenesis inhibitor, as the compound of interest. Co-cultures were prepared as above, with the exception that only the optimal level of VEGF was used in the EGM® 2 Medium, and an 8-point titration of suramin (between 0 and 780 µL) was prepared using this optimized medium. Co-cultures were again incubated for 5 days, with medium changes (including suramin) performed every 2 – 3 days.

Endpoint measurement

We utilized the Sartorius Incucyte® SX5 Live Cell Analysis System combined with the Sartorius Incucyte® Angiogenesis Software Module to image and quantify angiogenesis in terms of network formation, calculated as the total number of network branch points. Plates were allowed to equilibrate for 30 minutes on the day of co-culture establishment before running the first scan. Images were captured at 4x magnification, which was found to be optimal for quantifying tube formation. Other live-cell imaging platforms can be used to carry out this protocol; however, each individual platform will require optimization of instrument and software parameters.

Results and discussion

In a previous study, Lonza developed a rapid, high-throughput angiogenic tube formation assay utilizing HUVECs plated with a Gibco™ Geltrex™ Matrix Membrane that permits live-cell imaging and provides qualitative results in ≤24 hours.[11] In that study, we found that HUVECs required plating, expansion, and passaging before plating with Geltrex™. Interestingly, when using the co-culture protocol, we were able to plate HUVECs with fibroblasts directly from cryopreservation, thus saving approximately a week's worth of time versus conventional methods.

After optimizing the cell densities to a ratio of 1 HUVEC:3 fibroblasts, the optimal VEGF concentration in the EGM® 2 Medium was found to be 313 pM. While other concentrations promoted adequate network formation (Figure 1), 313 pM VEGF promoted the most consistent tube formation across a longer period when comparing all three donor pools. Using the optimized protocol, all three HUVEC donor pools readily formed networks of branching tubes through 5 days in culture (Figure 2).

The protocol was then validated as a qualitative angiogenic inhibition assay by exposing each donor to an 8-point titration of the inhibitory drug suramin. Previous studies have shown that suramin doses around 20 µM are inhibitory for tube formation.[11,12] In this study, for Donors Pools 1 and 2, a 7.8 µM dose of suramin was found to be inhibitory for network branch formation (Figure 3).

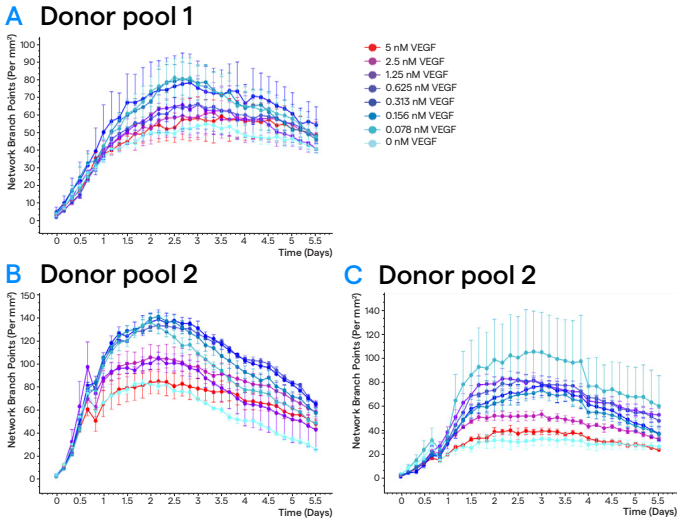

Figure 1.
Angiogenic tube formation (number of network branch points per mm² over time) as a function of medium VEGF concentration for the three donor pools (A – C). Error bars represent standard error (SE). N=2 for each donor.

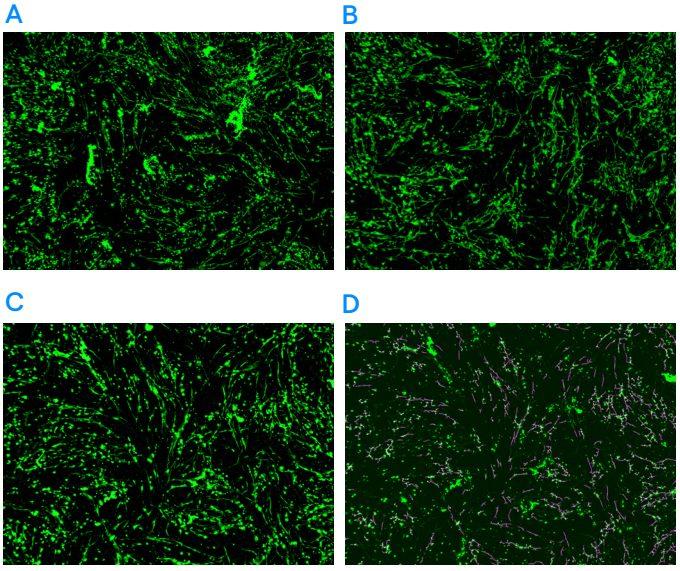

Figure 2.
Representative images of angiogenic tube formation taken on Day 5 for HUVEC Donor Pool 1 (A), Donor Pool 2 (B) and Donor Pool 3 (C) in co-culture with fibroblasts using the optimized protocol. HUVECs and fibroblasts were plated in a 1:3 ratio at a total density of 40,000 cells/well. VEGF was supplemented at 313 pM in the completed FGM® 2 Medium. Panel D shows the network branch skeleton overlay (pink lines) for Donor Pool 3 determined by the live cell imaging software and used to calculate angiogenic metrics found in Figures 1, 3 and 4. Images were edited using a secondary software (Paint.NET®) to increase visibility lost during image export from the Incucyte® software.

Less consistent results were obtained with Donor Pool 3, though suramin doses of ≥24.7 µM were clearly inhibitory. Mean network length was also assessed (Figure 4), which followed the same general patterns as network branch points. Interestingly, across all three donors, low doses of suramin (0.78 µM to as high as 7.8 µM in Donor Pool 3) actually increased network formation and length above the control.

A cursory search of the primary literature did not turn up any similar studies for comparison of results, though studies using HUVEC fibroblasts co-cultures with suramin[6] or quantitative tube formation assessments[9] do exist. No previous data were found to establish whether the observed increase in network branch points seen at low doses of suramin is inherent to HUVEC cells, is dependent on co-culture with fibroblasts and therefore physiologically accurate, or is an artifact of *in vitro* co-culture with fibroblasts and is not found *in vivo*. Further experimentation is necessary to establish the root cause of this finding.

This study builds upon previous works by establishing a high-throughput co-culture that can be imaged and quantitatively assessed in real-time. This allows for a much higher degree of granularity in the data, as both a greater number of doses can be used and a greater number of time points can be measured without sacrificing cultures, thus providing far greater context than methods using sacrificial methods at single time points.

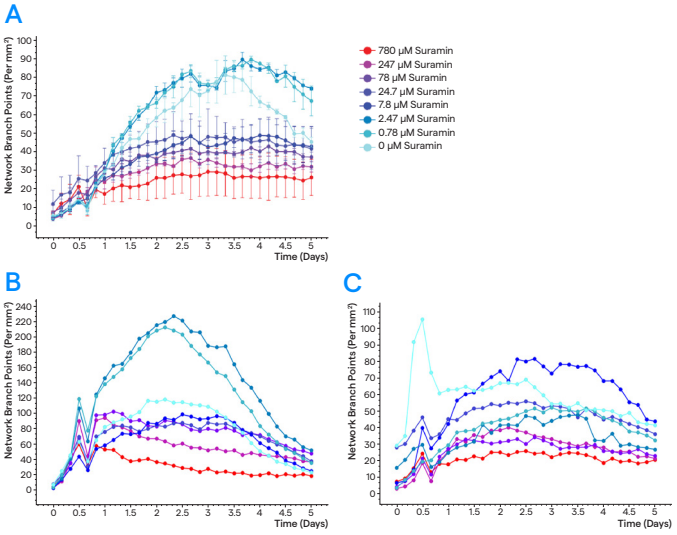

Figure 3.
Network branch points per mm² for Donor Pool 1 (A), Donor Pool 2 (B), and Donor Pool 3 (C) HUVECs in co-culture with fibroblasts across an 8-point titration of suramin. Inhibition is indicated where network branch point formation falls lower than the control (0 µM suramin) condition. N=2 replicates for Donor Pool 1 (A), while N=1 for Donor Pools 2 (B) and 3 (C).

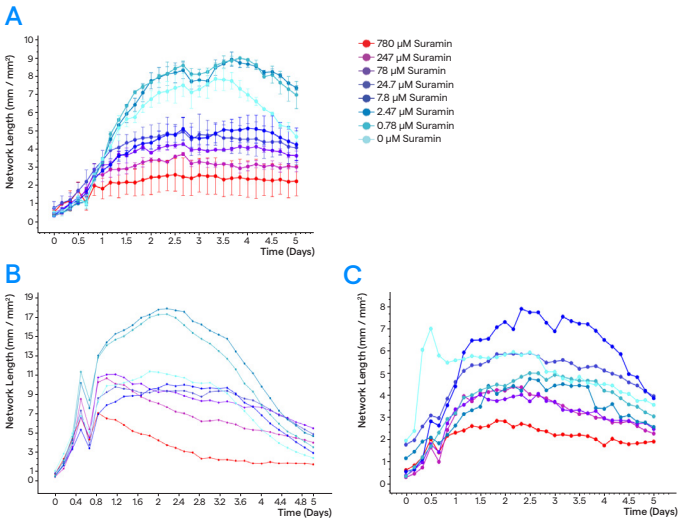

Figure 4.
Network branch length (mm) for Donor Pool 1 (A), Donor Pool 2 (B), and Donor Pool 3 (C) HUVECs in co-culture with fibroblasts across an 8-point titration of suramin. Inhibition is indicated where network branch point length falls lower than the control (0 µM suramin) condition. N=2 replicates for Donor Pool 1 (A), while N=1 for Donor Pools 2 (B) and 3 (C).

Conclusions

This study demonstrates a high-throughput angiogenic tube formation assay protocol that can be used in conjunction with live cell imaging technology to screen compounds that promote or inhibit angiogenesis. It is both physiologically relevant[9] and quantitative, and could serve as an excellent tool for use in drug development workflows to determine the efficacy of pro- or anti-angiogenic compounds. It should be noted that tube formation assays provide only one specific means of assessing angiogenesis. The addition of other analyses (e.g., percent well coverage by cells) would enhance interpretation by addressing additional factors (e.g., inhibition of cell proliferation), which could be easily integrated into a live-cell analysis workflow to provide comprehensive data on drug targets and modes of action.[6,12]

Ordering information

Part code	Description	Size
C2519AS	Human Umbilical Vein Endothelial Cells, Angiogenesis Qualified	≥500,000 cells/amp
CC-2511	BHuman Normal Dermal Fibroblast Cells - Adult	≥500,000 cells/amp
CC-3156	EBM® 2 Basal Medium	500 mL bottle
CC-4176	EGM® 2 SingleQuots® Supplement Kit	1 kit
CC-3162	EGM® 2 BulletKit®	500 mL basal medium + supplement kit
CC-3131	FBM® Basal Medium	500 mL bottle
CC-4126	FGM® 2 SingleQuots® Supplement Kit	1 kit
CC-3132	FGM® 2 BulletKit®	500 mL basal medium + supplement kit

References:

1. Rizov, M., Andreeva, P. and Dimova, I. (2017). Molecular regulation and role of angiogenesis in reproduction. *Taiwanese Journal of Obstetrics and Gynecology* 56: 127 – 132.
2. Kaufmann, P., Mayhew, T. M. and Charnock-Jones, D.S. (2004). Aspects of human fetoplacental vasculogenesis and angiogenesis. II. Changes during normal pregnancy. *Placenta* 25: 114 – 126.
3. Veith, A.P., *et al.* (2019). Therapeutic strategies for enhancing angiogenesis in wound healing. *Advanced Drug Delivery Reviews* 146: 92 – 125.
4. Aguilar-Cazares, D., *et al.* (2019). Contribution of angiogenesis to inflammation and cancer. *Frontiers in Oncology* 9: article 1399.
5. Tímár, J., *et al.* (2001). Angiogenesis-dependent diseases and angiogenesis therapy. *Pathology Oncology Research* 7: 85 – 94.
6. Ljoki, A., *et al.* (2022). *In vitro* angiogenesis inhibition and endothelial cell growth and morphology. *International Journal of Molecular Sciences* 23: 4277.
7. Staton, C. A., *et al.* (2004). Current methods for assaying angiogenesis *in vitro* and *in vivo*. *International Journal of Experimental Pathology* 85: 233 – 248.
8. Sanz, L., *et al.* (2002). Development of a computer-assisted high-throughput screening platform for anti-angiogenic testing. *Microvascular Research* 63: 335 – 339.
9. Donovan, D., *et al.* (2001). Comparison of three *in vitro* human 'angiogenesis' assays with capillaries formed *in vivo*. *Angiogenesis* 4(2): 113 – 121.
10. Lonza Walkersville, Inc. Human umbilical vein endothelial cell and normal human dermal fibroblast co-culture and angiogenesis assay. Instructions for use. Link.
11. Lonza Walkersville, Inc. Primary human umbilical vein endothelial cells: a qualitative, real-time, *in vitro* tube formation assay. Link.
12. Bishop, E.T., *et al.* (1999). An *in vitro* model of angiogenesis: basic features. *Angiogenesis* 3(4): 335 – 344.

Contact Us

North America
Customer Service: + 1 800 638 8174 (toll free)
order.us@lonza.com
Scientific Support: + 1 800 521 0390 (toll free)
scientific.support@lonza.com

Europe
Customer Service: + 32 87 321 611
order.europe@lonza.com
Scientific Support: + 49 221 99199 400
scientific.support.eu@lonza.com

International
Contact your local Lonza Distributor
Customer Service: + 1 301 898 7025
Fax: + 1 301 845 8291
scientific.support@lonza.com

Lonza Walkersville, Inc. – Walkersville, MD 21793

Learn more.

CD-DS055 08/25

bioscience.lonza.com/endothelial-cells