

CellPlayer™ Kinetic Angiogenesis StemKit Assay

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The establishment and maintenance of the vascular system are required for the growth of both normal and pathogenic tissues (i.e. tumors) (1). Blood vessels form through one of two mechanisms, vasculogenesis and angiogenesis, resulting in the development of three layers: 1) the intima composed of endothelial cells, 2) the media composed of pericytes in microvessels, and 3) the adventitia composed mainly of connective tissue (1). Angiogenesis, the formation of new vessels from pre-existing vessels in the body, is essential in embryonic development, wound healing, and tissue remodeling (2). In healthy tissues under steady-state conditions, the angiogenic process, consisting of endothelial cell (EC) proliferation, EC migration, and vessel morphogenesis, is tightly regulated by physiologic control of growth factors and inhibitors (3,4). The formation of new tubes during angiogenesis is followed by recruitment of mural cell precursors, which, upon contact, differentiate into a pericyte fate and act to stabilize the newly formed endothelial tubes, regulating their survival, differentiation and branching (1,5-7). However, under pathological conditions such as tumor progression, diabetic retinopathy and neurodegenerative diseases, vessels become hemorrhagic and hyperdilated when they lose contact with the perivascular cells (8,9). Anti-angiogenic therapies have shown promise at slowing disease progression in certain clinical models, yet have proven transitory due to either inherent or acquired resistance. Current *in vitro* angiogenesis models have generally isolated the different component parts of the angiogenic process and have studied endothelial cell proliferation (10), endothelial cell migration (11) or the ability of endothelial cells to associate into tubules when in contact with various matrix proteins (12). Advancement of *in vitro* angiogenesis models to study drug resistance and more complex pharmacologic paradigms, i.e. combination regimens, are paramount to developing the next generation therapeutics (3).

It is difficult to reproducibly and quantitatively model the complex biologic processes of angiogenesis in a single *in vitro* approach. Essen BioScience previously developed a kinetic co-culture approach to studying angiogenesis *in vitro*. In this model, we utilized lentivirally-infected human umbilical vein endothelial cells (HUVEC) expressing GFP cultured with normal human dermal fibroblasts (NHDF). When co-cultured with NHDF, the HUVEC differentiate into large angiogenic

networks over the 8-10 day assay. The developed networks are very representative of *in vivo* angiogenesis, both phenotypically and pharmacologically. Further, the fluorescent nature of the GFP-infected HUVEC, when imaged with the IncuCyte FLR, enabled the visualization and quantification of tube formation over time to measure tube length, tube area, and branch point formation. However, while the NHDF are a sufficient stromal cell type to support HUVEC differentiation into tubes, they do not express pericyte markers or protect the HUVEC in tube regression models, such as late addition of anti-VEGF antibody therapy.

In the CellPlayer Angiogenesis *StemKit*, we developed a new, stem-cell based *in vitro* model, co-culturing human endothelial colony forming cells (ECFCs) and human adipose derived stem cells (ADSCs) in specially designed medium. In this model, similar to the HUVEC approach, the ECFCs differentiate into tubules when in close association with the ADSC. Additionally, the ADSCs in close association with the developing ECFC networks differentiate into pericyte-like cells, expressing pericyte markers such as α -smooth muscle actin, PDGFR- β , and desmin. The stem-cell nature of both cell types in the model primes them for differentiation and enables established network formation in a 3-5 day time course. The flexibility and kinetic nature of the Essen BioScience *StemKit*, when conducted in conjunction with the IncuCyte™ FLR and the accompanying angiogenesis algorithm, facilitates both a quantitative and a qualitative assessment of network development throughout the course of the assay. The *StemKit* assay is responsive to known inhibitors and stimulators of angiogenesis. In this application note, we provide evidence that the *StemKit* can be used to reproducibly and kinetically study complex processes and signaling pathways directly relevant to *in vivo* angiogenic pharmacology.

Approach and Methods

Cell Culture

ECFCs are isolated from umbilical artery cord blood and expanded in modified EGM-2-MV growth media. During the expansion of the ECFC cell line, cells were infected with a



lentivirus expressing soluble GFP. ECFC are frozen at a concentration and passage (p7) optimized for *StemKit* performance. ADSC are isolated from adipose tissue isolated during surgical procedures (i.e. liposuction) and expanded in modified EGM-2 growth media. Similar to ECFC, ADSC are frozen at a concentration and passage (p4) that has been optimized for *StemKit* performance.

Assay Procedure

1. Two days (**Day -2**) prior to assay initiation, ADSCs were thawed and seeded into a 225cm² tissue culture treated flask (Corning Life Sciences, Corning, NY) containing **ADSC growth medium**. ADSCs were grown overnight at 37°C and 5% CO₂.
2. The following day (**Day -1**), ADSCs were harvested in **Assay medium** and seeded at 40,000 cells/well into a Corning 96-well plate. The ADSCs were then allowed to adhere at ambient temperature for 15 minutes prior to placing them at 37°C for overnight incubation.
3. The same day, ECFC were thawed and seeded in a collagen coated 75cm² tissue culture treated flask containing **ECFC growth medium**. ECFCs were grown overnight at 37°C and 5% CO₂.
4. The following day (**Day 0**), ECFCs were harvested in **Assay medium** and seeded at 5,750 cells/well on top of the ADSC monolayer. The co-culture was then allowed to adhere at ambient temperature for 15 minutes prior to placing the 96-well plate at 37°C for 3-4 hours.
5. After 3-4 hours at 37°C, the cells were treated with test reagents (growth factors ± compounds or antibodies), placed into the **IncuCyte FLR** for imaging, and allowed to form networks over the course of the 4 day experiment. The Stem Kit Assay forms stable networks at 48 hours and is able to maintain these networks at ≥ 96 hours without additional feedings (data not shown).
6. If running the assay in **Neoangiogenic mode**, looking at the inhibition of tube formation, the assay can be terminated at the 96 hour time point. If studying tube regression is desired, the assay can be run in **Established mode**. To do this, growth factor-driven networks are formed over the first 96 hours of the assay. At this point, a full media replacement occurs including fresh growth factor in the presence or absence of test agent. The assay is then imaged over the desired time frame to quantify regression of established networks.

Data Quantification and Analysis

Phase-contrast and fluorescent images were automatically collected every 6 hours in the **IncuCyte FLR** to detect network formation using the Tiled Field of View (FOV) mosaic imaging mode (Figure 1, A-D). The integrated Angiogenesis Analysis Module was used to identify the fluorescent signal from background in order to quantify multiple assay metrics, such as tube length and branch formation, for each time point (Figure 1, F-H). In the first step of the process, the angiogenesis algorithm analyzes each fluorescent image and assigns a segmentation mask that closely resembles the *in vitro* network. From here, the mask can be refined and filtered to exclude non-tube forming events, specifically measuring angiogenesis over time. Kinetic plots of the angiogenesis metrics can be generated using the IncuCyte software, allowing for a direct comparison of test agent treatments to validated control conditions (Figure 1E). Additionally, the ability to export images and movies using the IncuCyte software enables visual confirmation of quantified pharmacologic responses.

Results and Discussion

Growth Factor Responses: VEGF and bFGF

Multiple growth factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), play an integral role in the stimulation, differentiation, and development of endothelial and mural cells into angiogenic networks *in vivo* (4). As VEGF is one of the major growth factors in this process, inhibition of VEGF signaling is a prevalent therapeutic target in such diseases as cancer, macular degeneration, rheumatoid arthritis, and other pathologies (4,5). More recently, pro-angiogenic therapies are also being developed to treat critical limb ischemia and cardiovascular diseases (4,5). As such, we investigated the VEGF and bFGF responses in the *StemKit*, to characterize the role of these growth factors in ECFC tube development and to establish the *StemKit* as a robust *in vitro* angiogenesis model. VEGF and bFGF were added on Day 0 at the indicated concentrations and allowed to form networks over four days. As seen in Figure 2, cultures responded to VEGF and bFGF in a concentration dependent manner, as quantified using the Essen BioScience automated angiogenesis algorithm. The measured tube length is dependent on the growth factor concentrations, with both VEGF and bFGF showing a maximal response at 25 ng/mL and a 50% response at 1.56 ng/mL. The kinetic curves reveal that the concentration response curves are similar for VEGF- and bFGF-mediated

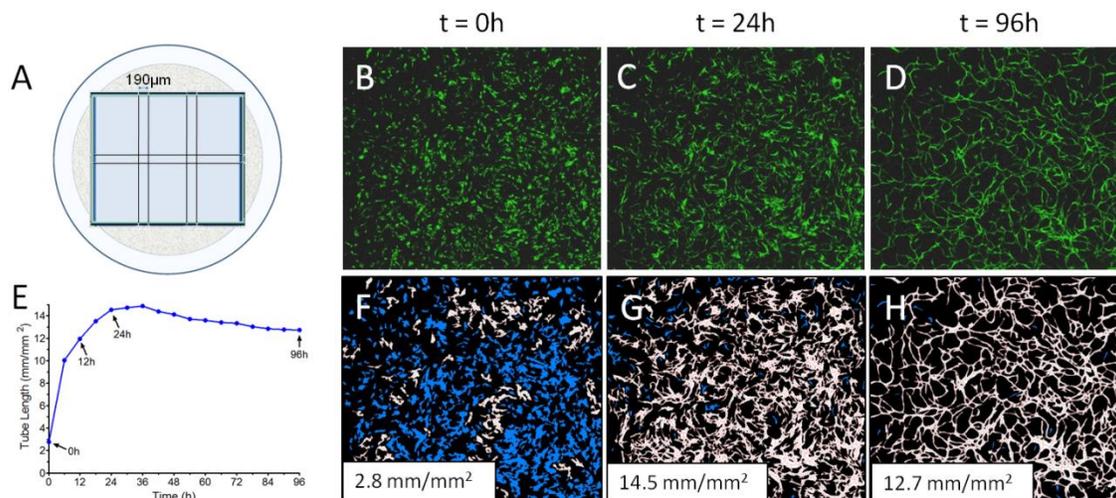


Figure 1: Imaging and quantification of GFP labeled ECFCs. A, A single well of a 96-well plate overlaid with a tiled FOV image demonstrating the 3 x 2 tiled images with ~190 µm overlap. Tiled FOV with overlap allows for a single mosaic image of 3.37 x 3.92 mm. B-D, Phase-contrast and fluorescent, kinetic live-cell images (tiled FOV) showing the development of tube formation at the indicated time points. E, Quantitation of the VEGF response for the images shown in B-D. Networks develop in the first 24 hours after VEGF initiation (t = 0h) and refine into established networks for the next 48-72h. F-H, Computational analysis of tube formation (white mask) using the IncuCyte™ Angiogenesis Analysis Module. Blue "cells" indicate events that were filtered out and not included in calculated tube length.

angiogenesis (Figure 2, A-B). However, the IncuCyte FLR images for these two growth factors demonstrate that differences between the growth factor responses can be observed. Qualitatively, the areas where tubes come together in a "nodal junction" appear thicker in the bFGF-treated wells compared to the VEGF response (Figure 2, C-D). Together, these data highlight the importance of both imaging and quantitation to accurately and more completely define the pharmacologic response of the test agent.

Immunocytochemistry: Molecular Markers of Pericytes

Recent data suggests that ADSCs, similar to those in the *StemKit*, are able to differentiate into multiple cell types, including peri-endothelial cells that are functionally and phenotypically equivalent to pericytes (13,14). Further, when ADSC and ECFC are implanted into mice in a Matrigel plug model, the ECFC differentiate and anastomose with the existing mouse vasculature to form functional vasculature supporting blood flow through the tubes (15). During the ECFC differentiation and network formation, the ADSC also differentiate into pericytes and act to stabilize the ECFC-formed tubes (15). To confirm that the same process occurs *in vitro*, we characterized the ADSC in the *StemKit* to demonstrate that they were differentiating into pericytes upon growth factor addition. Although no proteins are known to be exclusively expressed in pericytes, there are a few dynamic markers commonly used for their characterization. These markers include contractile filament proteins, α -smooth muscle actin (α -SMA) and desmin, which are dependent on

the developmental or angiogenic stage of the microvessel (8). In order to evaluate the differentiation of ADSCs into perivascular cells we fixed 96-well co-cultures plates at 96 hours after growth factor initiation, permeabilized the cells with 0.1% Triton X-100, and stained for pericytes (α -SMA and desmin), or tubes (PECAM). As seen in Figure 3, the

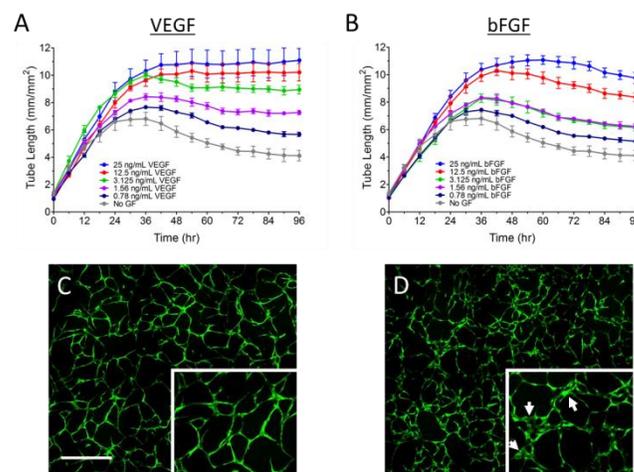


Figure 2. Growth Factor Response. A-B, ADSC and ECFC co-cultures were treated with the indicated concentrations of VEGF (A) and bFGF (B) to induce tube formation. Graphs illustrate live-cell kinetic data showing dose-dependent tube formation when treated with either VEGF or bFGF. C-D, Representative images of VEGF (C) and bFGF(D) mediated tube formation. Images are shown at 96 hours after growth factor initiation. Insets are zoomed regions showing "nodal junctions". Arrows indicate where bFGF treatment results in a thickening of these junctions (inset D). Scale bar, 800µm.

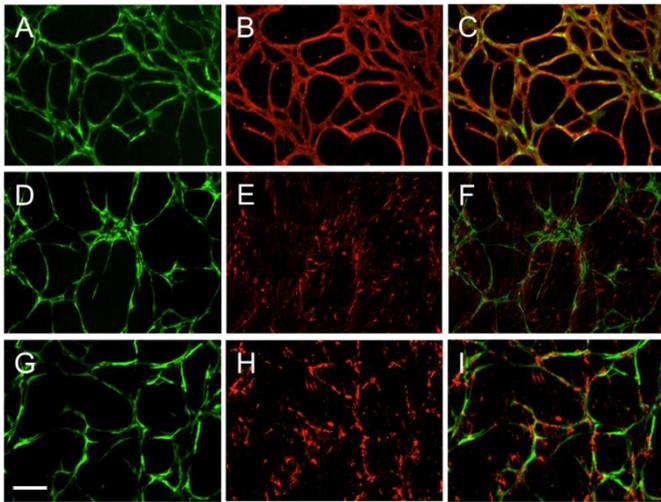


Figure 3: Expression of angiogenic markers in both ECFC and ADSC cell lines. *StemKit* co-cultures were treated with 20ng/mL VEGF for 96 hours prior to fixation and staining for angiogenic markers. (A, D, and G) Cytolight Green™ expression in ECFCs demonstrates extensive network formation by day 5. (B) Staining with PECAM marks endothelial network formed by ECFC as seen in merged image (C). ADSCs differentiate into pericyte-like phenotype, expressing α -smooth muscle actin (E) and desmin (H) that is in close proximity to angiogenic networks (F and I). Scale bar, 300 μ m.

GFP-infected ECFC are identifying the entire angiogenic network in the image (compare Figure 3, A-C), indicating high transduction efficiency during lentiviral GFP delivery. Moreover, upon VEGF stimulation, the ADSC differentiate into pericyte-like phenotype, expressing both α -SMA and desmin in close proximity to the angiogenic networks (Figure 3, D-I). Interestingly, the pericyte markers were not expressed in ADSC upon bFGF-stimulation, again indicating phenotypic differences between the two growth factors (data not shown).

Mechanism Studies: Proliferation

To further characterize the angiogenic processes occurring in the *StemKit* assay, we analyzed the rate of proliferation using nuclear localized GFP expressing ECFCs in conjunction with the IncuCyte FLR fluorescent object counting algorithm. Proliferation is one of the early phases of the angiogenic process, and has shown to occur in the HUVEC/NHDF angiogenesis kit that we have developed previously. To study this in the *StemKit*, we used two independent methods. First, ECFCs were lentivirally infected with NuLight Green, a nuclear localized GFP construct. ADSC and NuLight Green-ECFC co-cultures were set-up according to standard assay procedure, followed by treatment with either 20 ng/mL VEGF (N=32 wells), 20 ng/mL VEGF + 100 μ M Suramin (N= 32

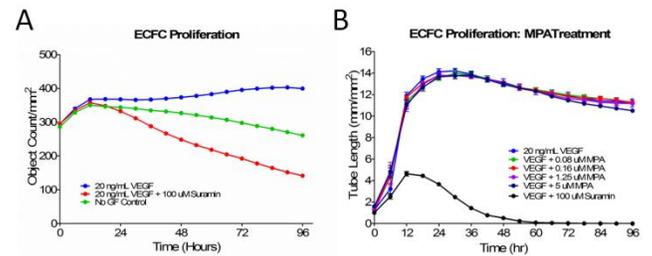


Figure 4: Proliferation Studies. (A) ECFCs were lentivirally infected with NuLight Green™, nuclear expressing GFP construct. Co-Cultures were left untreated or treated with 20 ng/mL VEGF \pm 100 μ M Suramin. Isolated fluorescent nuclear signal shows no significant proliferation. (B) Treatment with multiple concentrations of mycophenolic acid (MPA) demonstrate no effect on ECFC proliferation, based on tube network formation.

wells), or no growth factor (N=32 wells). The IncuCyte integrated fluorescent object counting algorithm was used to quantify the number of fluorescent nuclei per-area (1/mm²) for each time point. As seen in Figure 4A, the ECFCs only show a minimal increase in nuclear count upon VEGF treatment during the early stages of the assay, followed by a prolonged stabilization of nuclear count. The decrease object count observed when the co-culture is treated with Suramin is due to the combination of ECFC death and the compact clustering of viable cells. As a second approach, we used pharmacologic intervention, measuring the response of the *StemKit* assay to an anti-proliferative agent, mycophenolic acid (MPA). MPA is a well-characterized immunosuppressive agent, but has also been shown to be a potent inhibitor of endothelial cell proliferation. Figure 4B, illustrates that co-cultures treated with MPA showed no significant reduction in tube length when compared to the 20 ng/mL VEGF control treatments. Together these data suggests that the ECFCs do not proliferate during the course of the Stem Kit Assay, rather rapidly migrate and differentiate into angiogenic networks.

Investigating pharmacologic response in the *StemKit*: neoangiogenic vs. established mode

Having more fully characterized the nature of the differentiated cell-types, we next developed different strategies for measuring pharmacologic responses using the *StemKit*. To do this, we used Suramin as our prototypical inhibitor; as it has been shown to significantly and potently inhibit angiogenesis *in vivo* and *in vitro*. The development of the *StemKit* assay allows flexibility in assay set-up because there is no requirement for media changes during the first 96 hours of the assay (neoangiogenic mode). In neoangiogenic

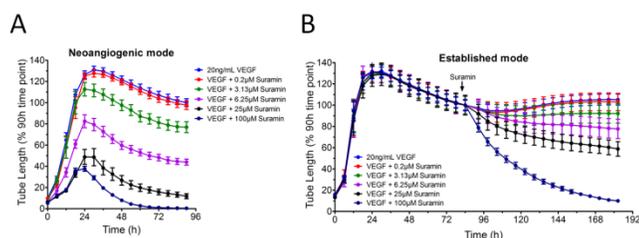


Figure 5. Different modes to study pharmacologic responses in CellPlayer StemKit. **A**, In neoangiogenic mode, addition of Suramin at the same time as VEGF ($t = 0$ h) inhibits tube formation in a concentration-dependent manner. **B**, In established mode, networks are stimulated with VEGF (or growth factor of choice) an allowed to develop for 96h. At 96h, Suramin is added with fresh VEGF and induces tube regression in a concentration-dependent effect. Treatment with $100\mu\text{M}$ Suramin induces complete regression of the established network.

mode, compounds are added at the same time as the growth factor, 3-4 hours after seeding, to determine the ability of the test agent to inhibit or stimulate tube FORMATION. As can be seen in Figure 5A, addition of suramin at the same time as VEGF results in a concentration-dependent inhibition of VEGF-mediated tube formation (Figure 5A). The *StemKit* assay has also been used to examine tube regression in established mode. In established mode, treatment with VEGF (or another growth factor) stimulates tube formation over the first 90-96 hours of the assay to develop stable networks. After 96h, there is a full media change including fresh growth factor and addition of test agent, in this case Suramin. This paradigm investigates the ability of the test agent to

DESTABILIZE established networks and induce tube REGRESSION. As shown in Figure 5B, Suramin induces a rapid and concentration-dependent regression of established networks. In fact, treatment with $100\mu\text{M}$ Suramin complete eliminates VEGF-driven tubes (Figure 5B).

Small molecule inhibitors disrupt established networks

Small molecule inhibitors of angiogenesis have been shown to be active in treating a wide variety of cancers, by targeting receptors or other targets in the multiple angiogenic signaling pathways (16). However, due to their non-specificity, these molecules tend to have adverse, off-target effects that have limited their clinical success (16). Here, we used two small molecules, sunitinib and combretastatin, to investigate their ability to inhibit angiogenesis. Sunitinib (Sutent) is a small molecule receptor tyrosine kinase inhibitor, targeting VEGFR and PDGF-R among others, which has been approved for treatment of renal-cell carcinoma and gastrointestinal tumors (16). Combretastatin, a tubulin disrupting agent, is effective at disrupting vasculature and is currently in clinical trials in combination with conventional chemotherapeutic agents (17). In the *StemKit*, addition of either sunitinib or combretastatin in neoangiogenic mode resulted in a concentration-dependent inhibition of VEGF-mediated angiogenesis (data not shown). Further, as seen in Figure 6, introduction of either sunitinib or combretastatin at 96 hours results in the regression of

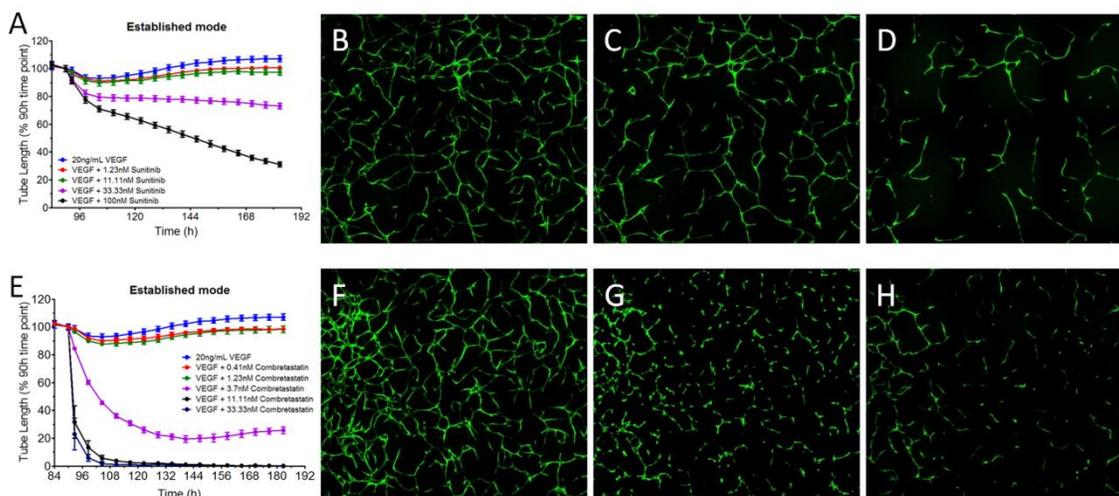


Figure 6: Small molecule inhibitors induce tube regression of established networks. **A**, Addition of Avastin after network development induces tube regression in a concentration-dependent manner. **B-D**, Representative images of Sutent treatment shown immediately before (B), 24h after (C), and 96h after (D) Sutent addition. Note the regression of the networks from the ends of the tubes. **E**, Combretastatin addition 96h after VEGF introduction rapidly and completely induces tube regression. **F-H**, Representative images of combretastatin treatment shown immediately before (F), 24h after (G), and 48h after (H) combretastatin treatment. Note the breaking apart of networks along the lengths of the tubes.

established networks (Figure 6A and E). The kinetic graphs show that combretastatin treatment results in a more potent and complete tube regression, exhibiting toxicity at the highest concentrations (Figure 6E). Interestingly, whereas sunitinib treatment causes regression of the angiogenic networks by retraction of the tubes from each end (Figure 6B-D), combretastatin seems to cause the tubes to break apart at multiple points along the length of the network (Figure 6F-H). Altogether, the kinetic graphs highlight the difference in pharmacology while the images reveal two separate phenotypic responses.

Pericyte biology stabilizes and protects established networks

VEGF is a major regulator of both physiological and pathological angiogenesis (3,18). Because of this, VEGF signaling has been a popular therapeutic target for inhibiting angiogenesis and treating tumors. However, not all cancer patients benefit from such anti-angiogenic therapies, and some responses are transitory due to the development of resistance to anti-VEGF therapy (3,19,20). Hence, there is an urgent need to elucidate the mechanisms that mediate resistance to anti-angiogenic agents. Here, we investigated the effects of Avastin on VEGF-mediated angiogenesis in both neoangiogenic and established modes (Figure 7). When added at the same time as VEGF, Avastin inhibits tube formation in a concentration-dependent manner (Figure 7A). Interestingly, unlike sunitinib or combretastatin, Avastin was unable to induce regression of established networks (Figure 7B). Smooth muscle actin staining suggests that this is due to the differentiation of ADSC into a pericyte-like phenotype and protection of the established networks from anti-angiogenic therapies. To test this, we used a γ -secretase inhibitor (GSI) that disrupts the pericyte / endothelial cell interaction without affecting global tube formation (data not shown). Adding the GSI or Avastin to established networks exhibited no effect on stabilized networks (Figure 7C). Treating with the GSI and Avastin in combination, however, induced a 60-70% regression of the established tubes. Thus, treatment with the GSI restores sensitivity of the tubes to anti-VEGF therapy.

The pericyte/endothelial cell interaction serves to strengthen and stabilize mature angiogenic networks and decrease vascular permeability (21). Signaling through the angiopoietin receptor, Tie2, is critical in promoting vessel assembly and maturation by regulating endothelial cell survival signals and recruiting mural cells and pericytes. The ligands for the Tie2 receptor, Angiopoietin 1 (Ang-1) and Angiopoietin 2 (Ang-2), exhibit agonistic (Ang-1) and antagonistic (Ang-2) effects, respectively (21, 22). Unlike the

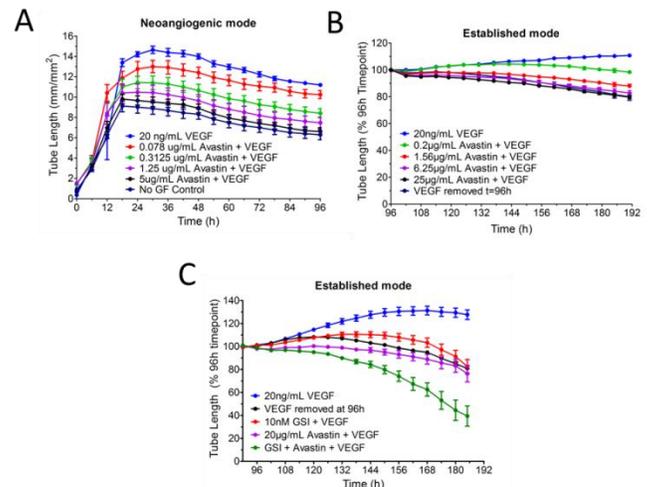


Figure 7: Avastin inhibition of VEGF-mediated tube formation is context-dependent. **A**, Addition of Avastin at the same time as VEGF inhibits the development of angiogenic networks. **B**, ECFC were treated with 20ng/mL VEGF to allow network development. At 96h, Avastin was introduced at the indicated concentrations in the continued presence of VEGF. Avastin treatment did not successfully induce network regression. **C**, Similar to (B), tubes were formed upon VEGF treatment for initial 96 hours of assay. After 96h, Avastin was added in the presence or absence of a γ -secretase inhibitor (disrupting endothelial cell/pericyte interactions). Inclusion of the GSI restores sensitivity to Avastin, inducing tube regression. Each data point represents mean \pm SEM (N=3).

endothelial expression of Ang-2, Ang-1 is expressed primarily by the mesenchymal cells and is involved in pericyte recruitment and vessel stabilization (21,22). On the contrary, Ang-2 expression and signaling leads to the disruption of the pericyte/endothelial cell interaction, pericyte dropout, and destabilization of the established vasculature (21,22). During our characterization of the *StemKit*, we have detected Tie2 expression in mature ECFC networks (data not shown). As such, we investigated if signaling through the Tie2 receptor altered VEGF-mediated angiogenesis. To do this, co-cultures were treated with increasing concentrations of Ang-2 in both neoangiogenic and established modes. When added at the same time as VEGF, Ang-2 inhibited tubule development in a concentration dependent manner (Figure 8A). Interestingly, addition of Ang-2 to established networks in the continued presence of VEGF resulted in tube regression (Figure 8B). Further, images of the networks immediately before and several days after Ang-2 addition support the pharmacologic findings and demonstrate the tube regression (Figure 8C-D). Together these data show that Ang-2 signaling alters angiogenesis in the *StemKit*, presumably by disrupting the pericyte/endothelial cell interaction, destabilizing the established networks, ultimately resulting in tube regression.

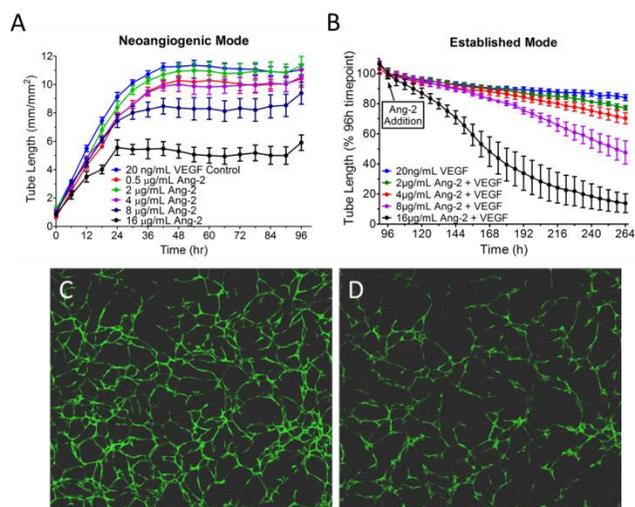


Figure 8: Effect of Ang-2 on vessel formation and disruption of stable networks. A-B, Kinetic curves of co-cultures treated with Ang-2 in neoangiogenic (A) and established (B) modes show dose-dependent responses. C-D, Representative images illustrate the effect of Ang-2 on established networks. (C) Image of VEGF-mediated network development immediately prior to Ang-2 addition. (D) 48h after Ang-2 addition, networks become destabilized, showing disruption of the vascular network.

Intra- and Inter-plate Analysis Demonstrates the Reproducibility of the CellPlayer StemKit

A key feature of any *in vitro* approach is examining the well-to-well and experiment-to-experiment variability to assess assay validity. The goal is to develop an assay format that combines low variability with reliable, reproducible results. To test this in the *StemKit*, we performed a series of experiments using VEGF and Suramin treatments to examine whole plate reproducibility. ADSC and ECFC co-cultures were treated with 20 ng/mL VEGF and imaged in an IncuCyte™ FLR every 6 hours for the 4-5 day assay period. Representative data from three separate experiments show that there is no significant difference in tube lengths measurements based on plate position (Figure 9A).

Further, VEGF responses across eleven independent whole plate assays using the *StemKit* measured an average tube length of $13.36 \text{ mm/mm}^2 \pm 0.67$ (CV = 5.0%; Table I). To determine if the *StemKit* is amenable to a higher throughput screening approach we treated five independent plates with 20 ng/mL VEGF (N=48 wells per plate) alone or with 100 μM Suramin (N=48 wells per plate). As can be seen in Figure 9B, treatment with 100 μM Suramin completely suppresses tube formation, giving a large signal window for measuring angiogenesis. As the networks have matured and established by the 96 hour time point (data not shown), and the signal

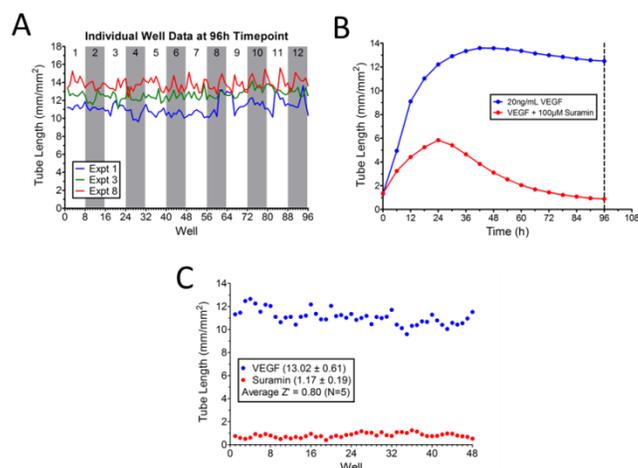


Figure 9. Longitudinal data demonstrates reproducibility of the *StemKit* Assay. A, Representative whole plate data from three separate experiments show reproducibility of the co-culture in response to 20ng/mL VEGF stimulation. There is no significant difference in the tube lengths of the outer, inner, and corner wells of a 96-well plate. B, Results from a representative 96-well assay illustrate the assay signal dynamic range as well as the low variation in sample measurements. The *StemKit* has been shown to form stable tubes beginning at 48 hours through 96 hours (data not shown), without the need to re-feed the co-culture. C, A representative individual well analysis of VEGF and VEGF + Suramin treated wells at 96 hours in a single *StemKit* assay. Due to stability of networks at 96h, this time point was chosen for Z' analysis. Across 5 separate experiments, the Z' was calculated at 0.80, indicating a very strong assay.

window is nearly maximal, this time point was used to determine Z' values. The calculated Z' for the combined experiments was 0.80, indicative of a high quality assay (Figure 9C). Together these data show that the *StemKit* is a low variable, highly reliable, and highly reproducible approach to studying both pro- and anti-angiogenic responses *in vitro*.

Conclusions

We have previously developed the NHDF/HUVEC co-culture approach for studying angiogenesis *in vitro*. Here, we introduce our newest approach to studying angiogenesis, the CellPlayer *StemKit*. This assay format combines two stem cell types; an endothelial stem cell, ECFC, with a mesenchymal stem cell, ADSC. Using the IncuCyte™ Live-Cell Imaging System in conjunction with the *StemKit* for the measurement of angiogenesis has demonstrated a highly quantitative and reproducible measurement of endothelial tube formation. The ability of the ADSC/ECFC co-culture to form tubes in 3-5 days eliminates the need to feed cells during the length of the assay in neoangiogenic mode. An initial feeding and treatment of the co-culture is all that is required for



stimulating or inhibiting network formation. Further, kinetic monitoring of cells allows for the detection of both stimulators and inhibitors of angiogenesis in physiologically relevant conditions eliminating the need for determining a universally suitable end point a priori. This feature allows for profiling time-dependent biological activity. In addition, networks are stable 48-72 hours post assay initiation, allowing the user to investigate the ability to induce tube regression of established networks (established mode). The ADSC in the **StemKit** differentiate into perivascular cells, providing the opportunity to kinetically study the complex process of endothelial cell and pericyte interactions. The presence of pericyte biology stabilizes the established networks and enables measurement of potential resistance mechanisms. As shown in this application note, disrupting the pericyte/endothelial cell interaction with a γ -secretase inhibitor re-sensitizes the system to anti-VEGF therapy and induces tube regression. As well as being able to measure complex biological responses, the **StemKit** is a robust and reproducible assay, enabling a higher throughput approach to investigating angiogenic responses. Finally, the IncuCyte™ allows for automated data acquisition of phase contrast and fluorescent images that can be used in combination with the Angiogenesis Analysis Module to more completely describe the angiogenic response. Together, the family of angiogenesis assays provides quantitative and powerful approaches for studying angiogenesis *in vitro*.





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