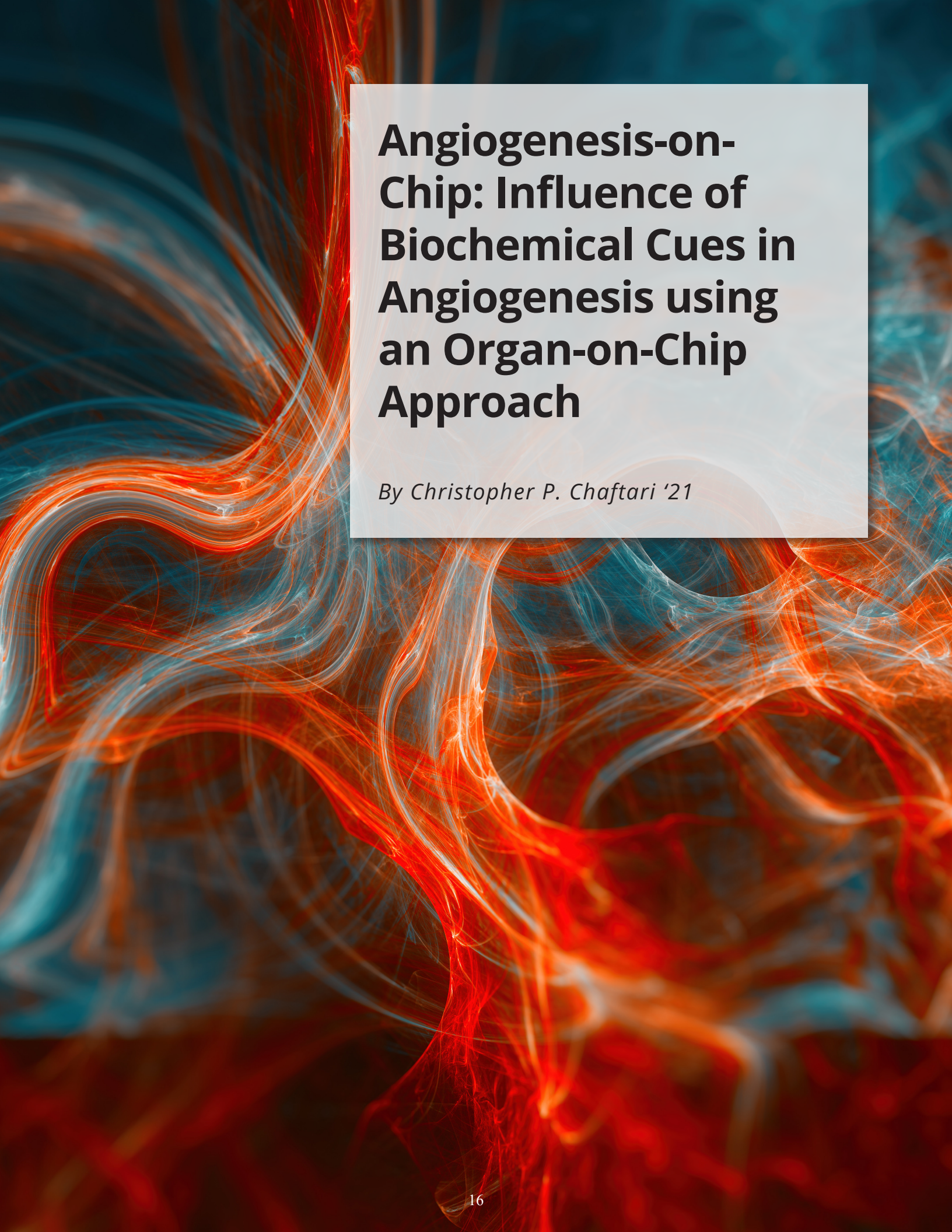
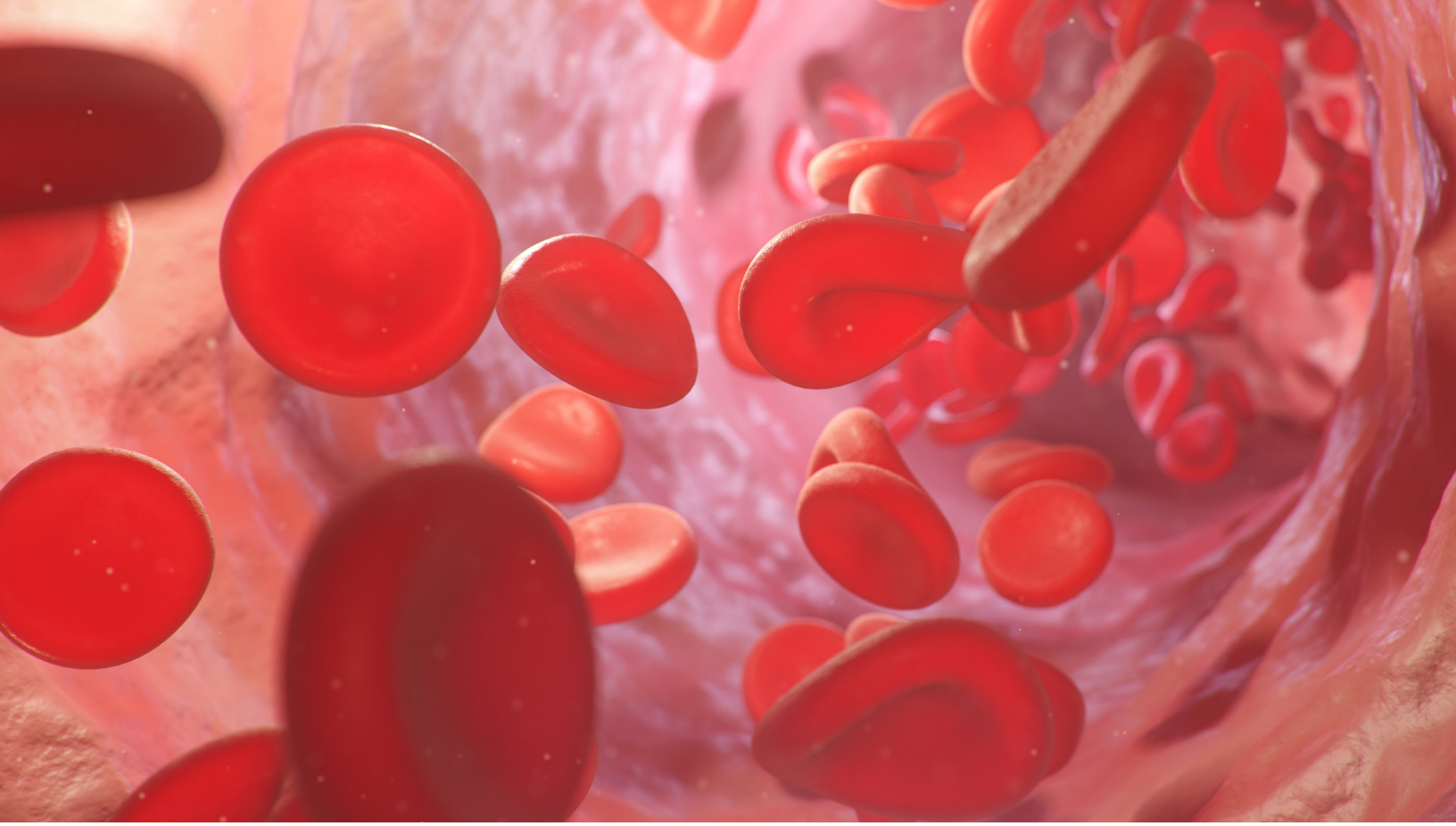


Angiogenesis-on-Chip: Influence of Biochemical Cues in Angiogenesis using an Organ-on-Chip Approach

By Christopher P. Chaftari '21



INTRODUCTION

Microfluidic organs-on-a-chip, or microphysiological systems, are contemporary devices used to recreate healthy or pathological human-specific tissue microenvironments. One such process is angiogenesis, where a parent blood vessel made of individual endothelial cells (ECs) sprouts into new daughter blood vessels. Angiogenesis occurs in many biological processes, including during inflammation, wound repair, and growth of tumors.[1] Studying angiogenesis allows scientists to better understand how these processes occur and how to better treat them. At the cellular level, angiogenesis requires individual endothelial cells of a blood vessel to receive a pro-angiogenic stimulus, migrate toward that stimulus, proliferate, and form a cylindrical tube.[2] These three events are commonly referred to as tip cell selection and migration, stalk cell proliferation, and tube maturation, respectively.[3]

Angiogenesis can be induced on chip devices by adding biochemical cues that stimulate angiogenesis in endothelial cells. Additionally, these biochemical cues have varied roles and differing effects on the various stages of angiogenesis (Table 1). In the literature, angiogenesis chips are regularly reported, but culturing conditions widely vary because no standard protocol or quantification method exists.[4,5] Additionally, many reports misclassify endothelial cell sprouting as angiogenesis, whereas in physiology, angiogenesis also requires the final two stages of angiogenesis (endothelial cell proliferation and final maturation to cylindrical tubes). Due to the many outcomes that are possible with the plethora of biochemical cues and cell lines currently available, the standard culturing protocols for angiogenesis-chips are missing. By developing a standard for angiogenesis-on-a-chip, the findings of

STUDYING ANGIOGENESIS ALLOWS SCIENTISTS TO BETTER UNDERSTAND HOW THESE PROCESSES OCCUR AND HOW TO BETTER TREAT THEM.

future studies will be more accurate and reflective of angiogenesis due to the implementation of these standards.

To address these gaps, this study was aimed at identifying several cocktails of biochemical cues to set a standard for angiogenesis, which can readily be replicated and used for other scientific applications. The study first investigated the basic building blocks of angiogenesis: endothelial cells and biochemical cues. Using tri-channel microfluidic devices (Figure 1), parent vessels were seeded in a fluidic channel adjacent to a hydrogel that acts as a scaffold for angiogenic sprouts. In a third channel situated on the other side of the hydrogel, previously identified critical growth factors were supplied to one or all the events of angiogenesis: endothelial cell migration, proliferation, and tube maturation. The makeup and potency of the growth factor cocktails was analyzed to determine basal angiogenesis conditions, as well as cocktails that improve quantifiable sprout metrics such as maximum sprout length and average sprout diameter. With these results, standard culturing conditions were established and a quantification pipeline was introduced that can be adopted by other research groups.

Table 1. *Roles of Biochemical Cues in Angiogenesis.*

Category	*Growth Factor*	*Role in Angiogenesis*
Tip Cell Migration	VEGF	Motility, tip cell selection
	S1P	Motility, upregulate matrix metalloproteinase (MMP) secretion
	HGF	Motility
	MCP-1	Signaling, activation, MMP secretion
Stalk Cell Proliferation	VEGF	Mitogen, tube morphogenesis
	PMA	Mitogen, enhances other growth factor effects
	HGF	Mitogen, tube morphogenesis, branching
	bFGF	Mitogen, tube morphogenesis
Tube Maturation	VEGF	EC maintenance
	S1P	Tight junction formation, vascular maintenance

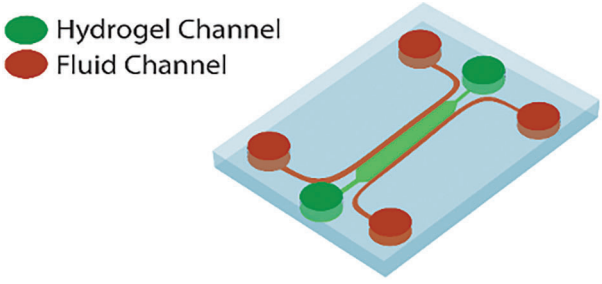


Figure 1. *Diagram of a tri-channel microfluidic device used in these experiments.*

METHODS

Microfluidic Device Fabrication

Three-channeled microfluidic chips were used to analyze the effects of various growth factor cocktails (GFCs). These microfluidic chips were made of polydimethylsiloxane (PDMS). To fabricate the chips, a 10:1 PDMS base to curing agent mixture was made. This concentration allows for the optimization of mechanical properties for fluid flow and cell adhesion to form parent vessels in the device. The mixture was poured onto a silicon wafer master mold with channel features of the device. Next, the PDMS was degassed for 15 minutes and then baked at 70°C for 2 hours to cure. Cured PDMS slabs were peeled off the wafer and inlet and outlet ports were punched using 1 mm and 5 mm biopsy punches. Cut slabs and glass microscope slides were then plasma treated and bonded. Microchannels were then coated with 100 µg/mL poly-D-lysine (PDL) for 6 hours in a humidified chamber at 37°C.[6] PDL facilitates a stronger PDMS-hydrogel bond that prevents cell slippage during culturing. The devices were subsequently washed with 1X phosphate buffer solution (PBS) and put into an oven to dry overnight. The following day, a 2.5 mg/mL fibrin hydrogel was

Table 2. *Composition of growth factor cocktails (GFCs). All growth factors are supplied at a final concentration of 75 ng/mL, excluding S1P, which has a final concentration of 500 nM.*

Cocktail	*Growth Factors*
Control	N/A
GFC 1	VEGF, S1P, PMA
GFC 2	VEGF, S1P, PMA, bFGF
GFC 3	VEGF, S1P, PMA, HGF
GFC 4	VEGF, S1P, PMA, MCP-1
GFC 5	VEGF, S1P, PMA, HGF, MCP-1
GFC 6	VEGF, S1P, HGF, bFGF, MCP-1
GFC 7	VEGF, S1P, PMA, HGF, bFGF, MCP-1

injected into the middle channel.

Cell Culture

While the devices were being fabricated, human umbilical vein endothelial cells (HUVECs) were cultured from cryopreservation. Endothelial Basal Medium was supplemented with Growth Medium-2 (EGM-2) packs. The EGM-2 allows for cell proliferation and growth. Once cells reached 80% confluence, HUVECs were trypsinized and spun down into a pellet. Trypsin is an enzyme which breaks down the peptide anchors attaching the cells to the surface of the container in which they were being cultured. The pelleted cells were resuspended in fresh EGM-2 at a concentration of 10 million cells/mL and injected into the left-most-channel of the previously fabricated chips. Cells were left at a 90° angle for 30 minutes to allow cells to attach to the side of the hydrogel. After seeding, devices were flushed with fresh medium and left in a humidified chamber at 37°C overnight. The following day, growth factor cocktails (Table 2) were injected into the rightmost channel to induce angiogenesis. Daily media changes were performed for the following two days.

Immunofluorescence and Network Quantification

The staining process occurred in three main steps, each of which was followed by a wash with 1X PBS. Devices were first fixed with 4% paraformaldehyde for 30 min at room temperature and washed. Fixing halts cellular decomposition and keeps cellular components in place. Then, the devices were permeabilized with 0.1 % Triton X-100 in 1X PBS for 30 min at room temperature and washed again. Permeabilization is required to allow for the staining compounds to enter the cells. Next, the devices were stained with rhodamine phalloidin (1:100) and Hoechst (1:2000) in 3 % BSA in 1X PBS for one hour at room temperature. Rhodamine phalloidin stains F-actin in the cell, which is a common protein found in the cell membrane. Hoechst stains DNA found in the nucleus. Stained devices were then imaged under a microscope by collecting tiled Z-stacks, which were then orthogonally projected to render a 2D image from the 3D data. Images were analyzed using REAVER, a previously standardized MATLAB GUI, which quantifies the total vascularized area, total vascularized length, mean sprout length, mean sprout diameter, and maximum sprout length.[7] To compare the various groups to each other, Principal Component Analysis (PCA) was used to reduce the dimensionality of the data, resulting in two principal component scores (PC 1 and PC 2); these explain at least 80% of the variance of the data. PC 1 scores, which explain the most variance, were normalized and represented as the vas-

ADDITIONALLY, THIS PROJECT FOUND THAT INCREASING THE CONCENTRATION OF BIOCHEMICAL CUES DOES NOT HAVE A SIGNIFICANT EFFECT ON ANGIOGENESIS...

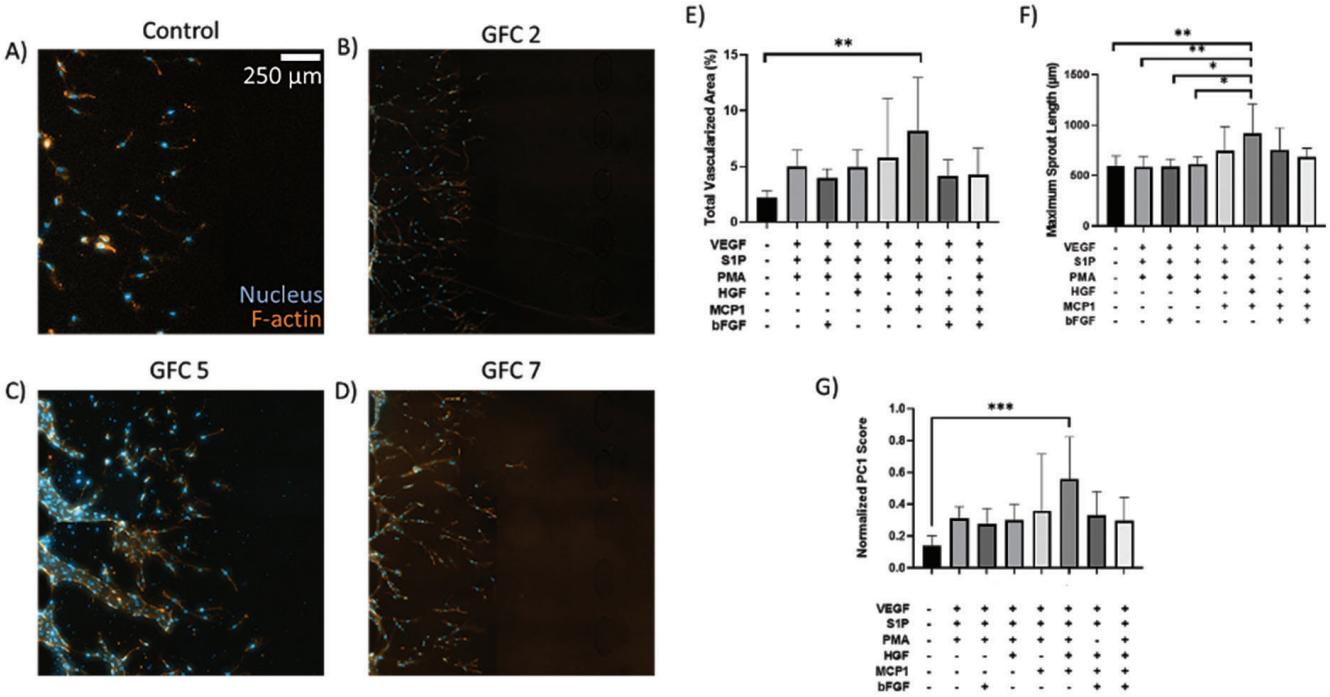


Figure 2. *Representative and quantitative results of angiogenesis-chips. A-D) Immunofluorescence images of angiogenesis-chips showing qualitative differences in sprout length, average sprout diameter, and total vascularized area. A) Control; B) GFC 2; C) GFC 5; D) GFC 7. E-G) Quantification of angiogenesis-chips. E) Total vascularized area; F) maximum sprout length, and; G) PC 1 score.*

cularization index that describes an optimized vascular network.[8] For all results, data is represented as mean ± the standard deviation, and one-way ANOVA was used to compare all the groups to each other.

RESULTS

In this project, the composition and concentration of biochemical cues were analyzed. Quantification of immunofluorescence images showed that the composition of biochemical cues can be a significant contributor in angiogenesis (**Figure 2**). First, controls, which had culturing medium without supplemental growth factors (containing 5 ng/mL VEGF and bFGF for EC maintenance and proliferation), show that some tip cells migrate into the hydrogel (**Figure 2A**). Because there are no additional cells following the tip cells, it appears that stalk cell proliferation does not occur, thus showing that angiogenesis is not faithfully recreated on-chip without growth factor supplementation. When we increased the concentration of VEGF and bFGF to 75 ng/mL and added 75 ng/mL PMA and 500 nM S1P, tip cells migrated followed by a thin trail of stalk cells, yet tube morphogenesis is not seen. When adding HGF instead of bFGF, which are two factors responsible for tube morphogenesis (**Table 1**), there is no significant differences in angiogenic sprouting. However, when adding monocyte chemotactic protein-1 (MCP-1) to this cocktail, wide sprouts yield a high vascularized area (**Figure 2C**). However, when adding all factors together (**Figure 2D**), the vascularization is decreased, possibly due to overstimulation.

When quantifying the images of each angiogenesis-chip after a 3-day culture with the GFCs, we saw that only GFC 5 had a significant difference compared to the culture in total vascularized area (**Figure 2E**) and PC 1 score (**Figure 2G**). We used these metrics as a vascularization index that linearly combines all quantified metrics into a dimensionless unit. GFC 5 has a significant difference between the control and three of the other six GFCs when quantifying maximum sprout length (**Figure 2F**). Because the key difference between GFC 5 and the other cocktails is the use of MCP-

1 instead of bFGF for tube morphogenesis without overloading the system, we theorize that MCP-1 plays a significant role in angiogenesis on-chip. This is also noted in literature, which suggests that MCP-1 induced angiogenesis is mediated by MCP-1 induced protein (MCPIP) and plays a key role in tube formation.[9]

Based on the results in **Figure 2,** the three-best performing GFCs (4, 5, and 6) were selected as possible standard cocktails for on-chip angiogenesis and doubled the concentrations of the biochemical cues in them to investigate if increasing the concentration affects angiogenesis. As shown in **Figure 3**, increasing the concentrations of biochemical cues does not seem to affect the scoring of angiogenesis within the same GFC. Figures 3G-J do not indicate any significant differences in angiogenesis between different concentrations of the same GFCs. This suggests that GFC composition is more important for angiogenesis than concentration of biochemical cues within the GFC. In addition, as seen in the 2x images (**Figure 2**), increasing the concentration may overstimulate the migrating tip cell, causing the migration rate to exceed tube formation and maturation rates. This inequality in rate might result in suboptimal angiogenesis as the networks are not fully perfusable because the ECs are not connected to each other.[10]

CONCLUSION

The purpose of this project was to determine basal angiogenesis conditions, establish standard culturing procedures, and introduce a scoring method to quantify angiogenesis in chip devices. This is important because there is no current standard for modelling and quantifying angiogenesis, thus limiting the use of angiogenesis-chips in drug toxicity and efficacy evaluation as well as healthy and pathological tissue modeling. In this project, we identified several key biochemical cues which are essential to inducing angiogenesis in chip devices. Furthermore, we identified three potential

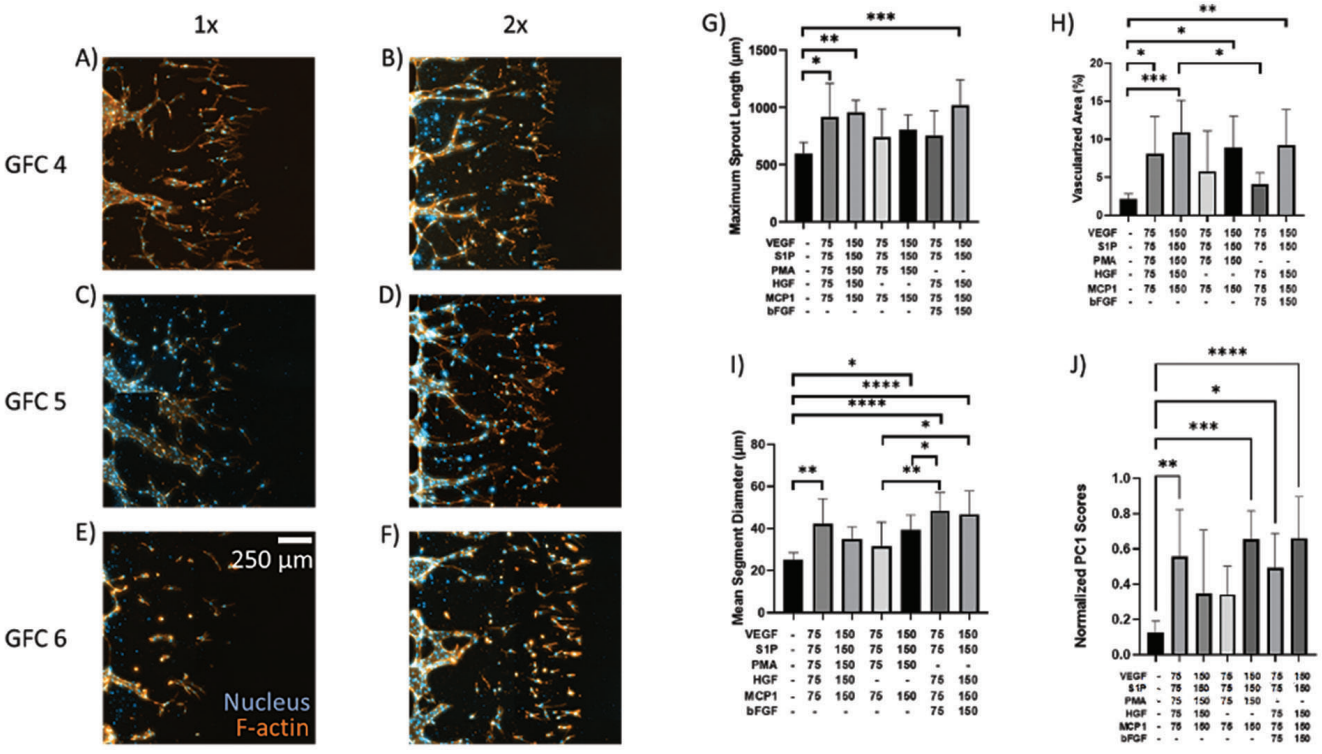


Figure 3. *Immunofluorescence images and quantification of angiogenesis-chips with 1X and 2X concentration of GFCs. A-F) immunofluorescence images A) GFC 4 at 1X concentration; B) GFC 4 at 2X concentration; C) GFC 5 at 1X concentration; D) GFC 5 at 2X concentration; E) GFC 6 at 1X concentration, and; F) GFC 6 at 2X concentration. G-J) Quantified results comparing each of the six immunofluorescence results. G) maximum sprout length; H) total vascularized area; I) mean segment diameter. Results from G-J were used to develop a normalized PC 1 score to compare overall vascular network quality between all groups and a control.*

WE IDENTIFIED THREE POTENTIAL COMBINATIONS OF BIOCHEMICAL CUES WHICH COULD BE USED AS STANDARDS FOR ANGIOGENESIS.

combinations of biochemical cues which could be used as standards for angiogenesis. Also, we presented how PCA can be used as a possible quantitative method to score angiogenesis and standardize angiogenesis across research studies. By using PCA, we can combine multiple variables to reduce dimensionality as opposed to presenting single network metrics and better analyze the overall quality of the angiogenic sprouts.

Additionally, this project found that increasing the concentration of biochemical cues does not have a significant effect on angiogenesis when compared to a 1X concentration. This can be an important consideration for projects limited by material availability. In fact, higher concentrations may overload the biological system and lead to suboptimal angiogenesis due to unequal rates of tube formation, cell migration and tube cell maturation. Increasing the concentrations of biochemical cues caused the tip cell migration to occur much faster than the stalk cells proliferated, resulting in the separation of tip and stalk cells and the elimination of stalk cells' guide to angiogenic factors. Therefore, it is important to use a concentration which precisely induces angiogenesis without overwhelming the biological system.

In conclusion, the findings of this project can be implemented into future studies mimicking biological environments, which include microphysiological systems and microfluidic technology. Since organs and tissues are usually highly vascularized in vivo, incorporating a perfusable endothelium into a model will yield better biomimetic results in future experiments and will lead to significant advances in types of studies which can implement organ-on-a-chip technology.[11] Additionally, pathological tissue models such as cancer-on-a-chip require angiogenesis to mimic how a tumor supports itself and subsequent metastasis. Besides the GFC makeup and potency, the scoring standard for microvascular self-assembly developed can be implemented in further studies, which require microvascular networks to have a complete model.

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