# INDUCTION OF ANGIOGENESIS IN MICROFLUIDIC DEVICES USING PROLYL HYDROXYLASE INHIBITORS AND SPHINGOSINE-1 PHOSPHATE

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## ABSTRACT

In this study, we stimulated the formation of capillary-like structure from human umbilical vein endothelial cells (HUVEC) by using prolyl hydroxylase inhibitor (PHi) and sphingosine 1-phosphate (S1P) in the presence of fibroblasts as mediating cells in microfluidic devices. Alone, either PHi or S1P is able to induce sprouting but together, they synergized and promoted a complex lumenized capillary-like network structure. With microfluidic devices, the subtle morphological differences are captured when conditioned medium from fibroblasts was used thus suggests that endothelial and fibroblasts cell-cell crosstalk was essential in PHi and S1P induced angiogenesis.

## KEYWORD

Angiogenesis, prolyl hydroxylase inhibitors (PHi), sphingosine 1-phosphate (S1P), fibroblasts

## INTRODUCTION

Angiogenesis is an inherently complicated biological event that requires careful orchestration of multiple cell types and growth factors in a temporal and spatial dependent manner [1]. With the application of microfluidic devices, the study of angiogenesis in a 3D environment with multiple cell type co-culture has been made possible. As such, microfluidic devices have been widely adopted for the creation of *in vitro* assays that are closer to *in vivo* conditions to study angiogenesis. Previous efforts have been dedicated to developing vessels in response to various known angiogenic factors such as vascular endothelial growth factors (VEGF) or certain cell types with angiogenic potential such as cancer cells and fibroblasts [2-4]. Our recent work develops microfluidic devices to study synergistic effects of two different angiogenic compounds, PHi and S1P in the presence of fibroblasts. PHis are compounds that prevent the degradation of hypoxia inducible factor-1α (HIF-1α) thus upregulating various angiogenic factors including VEGF as a response to the induced pseudo-hypoxic condition under normoxia [5]. S1P is a lysophospholipid that mediates multiple biological events including angiogenesis through specific cell surface G-protein coupled receptors. Previously, studies have shown mixed results for the interplay between HIF-1α and S1P where sphingosine kinase 1 was either the upstream regulator of HIF-1α or *vice versa* but it was confirmed that these two factors affect each other [6, 7]. On the other hand, fibroblasts have been reported to promote angiogenesis either through paracrine signaling via VEGF or through vascular stabilization [3]. Moreover, the pro-angiogenic effects of fibroblasts were even augmented by PHis as VEGF secretion by fibroblasts was increased when stimulated by PHis [5]. Together, we present the idea of combining PHi and S1P in the presence of fibroblasts for promoting angiogenesis by using *in vitro* microfluidic platform.

## METHODS

### Device Fabrication

The polydimethylsiloxane (PDMS) microfluidic devices are comprised of two gel regions and three cell seeding channels with two bead trap regions to contain alginate beads in the devices. The microfluidic devices were fabricated by using standard soft lithography as described earlier [3]. In short, PDMS was poured into SU-8 master mold and cured for 4 hours at 70 °C before peeling out and trimming and punching holes into features as shown in Figure 1. PDMS devices were autoclaved to ensure sterility and then they were bonded with cover glasses after plasma treatment for 90 s. Immediately, the devices were treated with 1 mg/ml poly-D-lysine (PDL) for 4 hours and then washed with sterile water and dried at 70 °C for 48 hours to render the surface back to hydrophobic. Collagen type I was then injected into the devices as 3D scaffold for endothelial cells to grow into capillary-like network. The devices are ready for cell culture after rehydration of cell culture channels with culture medium.

### Cell Culture

HUVEC were isolated from umbilical cord and kindly provided by Dr. Jerry Chan (National University of Singapore, Singapore). HUVEC were maintained in EGM-2mv culture medium until they were 80 % to 90 % confluent. IMR-90 human lung fibroblasts were obtained from ATCC (Manassas, VA) and maintained in DMEM basal medium that was supplemented with 10 % fetal bovine serum and 1 % penicillin. IMR-90 was encapsulated in 1% alginate solution by using microfluidic approach as described earlier [8]. Conditioned medium (CM) was prepared by conditioning EGM-2mv with 0.3 M IMR-90 in 6 well plate and then CM was concentrated through dialysing with a 3 kDa filter. The concentrated CM was then diluted with EGM-2mv to reconstitute into concentration comparable to concentration in microfluidic devices. All experiments were carried out with HUVEC at passages 5 to 6 and IMR-90 at passages 16 to 18. Cultures were kept at 37 °C in a humidified incubator with 5 % $CO_2$.

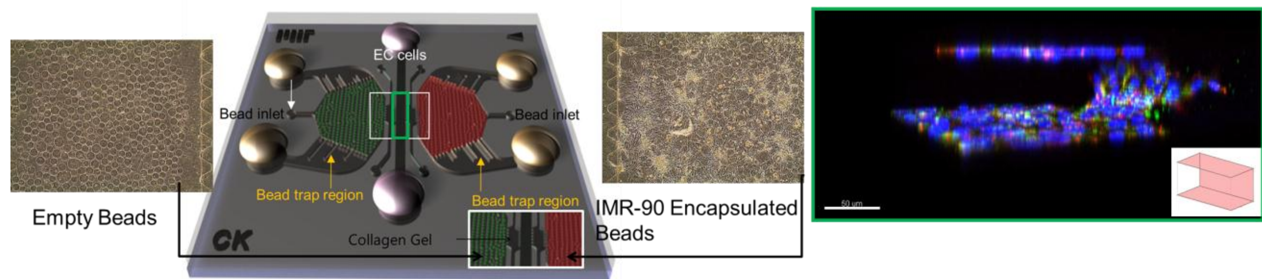


***Figure 1: Schematic drawing of microfluidic device and experimental setup.*** *Red region represents cells encapsulated beads and green represents empty alginate beads. The callout with white box represents the collagen gel that enables growing of sprouts. The callout with green box shows that HUVECs form a monolayer on collagen gel as well as top and bottom of the channel. Hoechst stained nuclei (blue), rhodamine phalloidin stained actin (red) and Alexa fluor 488 immunostained VE-cadherin (green). Scale bar denotes 50 µm*

**Immunohistochemistry**

HUVEC and IMR-90 cells in microfluidic devices were fixed with 4 % paraformaldehyd for 15 minutes at room temperature followed by PBS washing, twice. Cells were then permeabilized by 0.1 % Triton X-100 for 10 minutes in room temperature and washed with PBS once afterwards. Cells were blocked with 0.5 % BSA blocking solution for 2 hours at room temperature. VE-cadherin primary antibody was diluted into 1:100 and incubated with cells at 4 °C for overnight and then followed by incubation with Alexa Fluor-conjugated goat anti rabbit-secondary antibody in a dilution of 1:100 for 4 hours at room temperature. Cells were then counterstained with 10 µg/ml Hoechst and 3U/ml Phalloidin rhodamine for 30 minutes at room temperature. Images were acquired by using confocal microscope (OLYMPUS, FluoView FV1000). Images were then quantified by using Image J software (http://rsbweb.nih.gov/ij/) to count the number of cells, measure the skeletal length and maximum length of sprouts.

**RESULTS AND DISCUSSION**

We show that the interactions between endothelial cells and fibroblasts are essential in maintaining immature sprouts and converting them into functional vessels with lumens and these effects are most prominent when CPX works synergistically with S1P as shown in Figures 2.

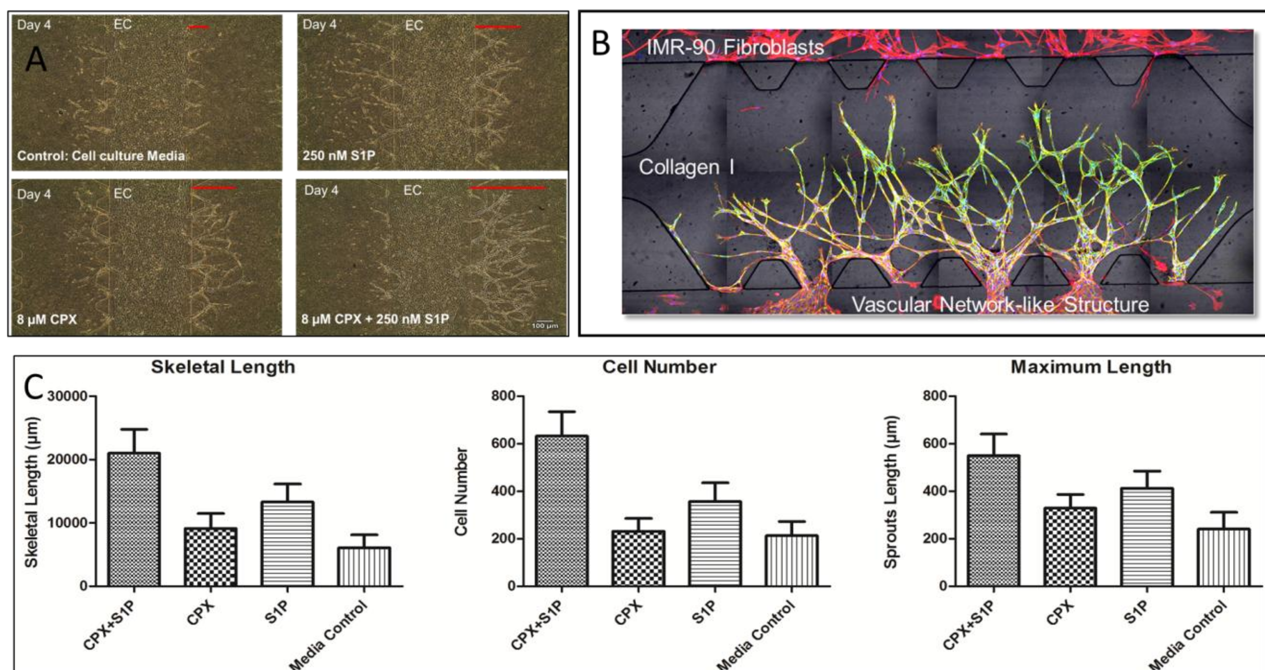


***Figure 2: CPX and S1P promote angiogenesis synergistically.*** *(A) 4x phase contrast images of devices treated with only cell culture media (control), S1P alone, CPX alone and the combination of both CPX and S1P. Sprouts on the right side grow toward IMR-90 encapsulated beads. Red lines denote the approximate length of sprouts showing that CPX works synergistically with S1P in promoting angiogenesis as compared to the other three conditions. (B) Stitched 20x confocal images of Hoechst stained nuclei (blue), rhodamine phalloidin stained actin (red) and Alexa fluor 488 immunostained VE-cadherin (green) shows sprouts that anastomose under 8 µM CPX and 250 nM S1P induction. (C)Quantification of skeletal length and cell number shows that CPX is synergistic with S1P in inducing formation of longer and more complex network-like-structures. Scale bar denotes 100 µm. Error bars denote standard error.*

The premature sprouts that invade into collagen gel would regress within 48 hours when they are growing towards empty alginate beads but the structure could be maintained and slowly turned into lumenized capillary-like structure when they are growing towards IMR-90 encapsulated alginate beads on the right side of the devices. The presence of CPX and S1P acts as stimulus for the formation of more complex and developed network within 4 days. We further quantified the capillary-like network structure through measuring the skeletal and maximum sprouts lengths and counting number of cells in the sprouts. From the quantification data, we are positive that CPX and S1P could induce more complex capillary network due to longer skeletal length and higher number of cells in the sprouts.

As fibroblasts can secrete multiple angiogenic factors, we carry out proteomic analysis of the fibroblast-conditioned medium to determine if there is any difference between CPX/S1P treated and untreated fibroblasts. We observe an increase in the secretion of VEGF by fibroblasts when stimulated by CPX (data not shown). Previously it has been reported that VEGF could upregulate the expression of $S1P_1$ receptor on endothelial cells hence promote the interplays between VEGF and S1P [9]. This could be the underlying mechanism for the synergy observed between CPX and S1P where CPX increase VEGF expression from fibroblasts and then VEGF sensitizes endothelial cells towards S1P stimulation which leads to increase angiogenesis.

We hypothesize that paracrine effects of fibroblasts are the major contributors for the observed increase in angiogenic stimulus. To confirm this, we collect conditioned medium from CPX and S1P treated fibroblasts and then apply it to EC monoculture in microfluidic devices. Strikingly, we observe increased angiogenic events as expected, but the morphologies of the sprouts are distinctly different from the experiment in which ECs are co-cultured with fibroblasts. With conditioned medium, we observe highly irregular sprouts that are less directional and invade into the collagen gel collectively (Figure 3). This interesting observation demonstrates the usefulness of microfluidic devices as functional assays in studying angiogenesis, since these more subtle morphological differences could be easily missed in conventional assays.

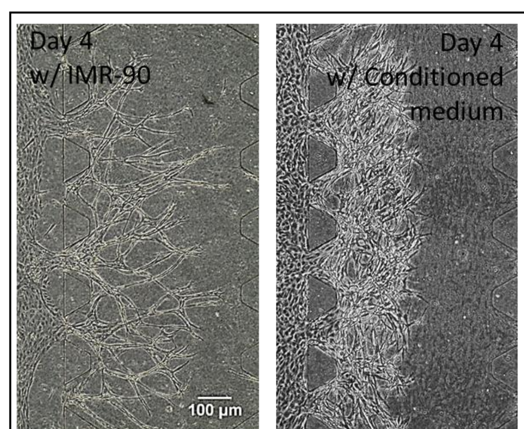


***Figure 3: Morphological difference between IMR-90 encapsulated alginate beads and CM mediated angiogenesis.*** *4x phase contrast images show different sprouting behaviors under stimulation of CPX and S1P: when EC co-cultured with IMR-90 (left) and with conditioned medium from IMR-90 (right). Conditioned medium induced sprouts are irregular and less directional as compared to the sprouts induced by IMR-90 encapsulated alginate beads. Scale bar denotes 100 µm.*

## CONCLUSION

The synergistic effects of PHis and S1P are confirmed in our 3D endothelial-mesenchymal co-culture model while the morphological differences in fibroblast-conditioned media-induced angiogenesis also suggest that crosstalk between the two cell types is critical in fibroblast-mediated-angiogenesis. The combination of PHi and S1P could be further exploited for the application in regenerative medicine.

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