Physicochemical regulation of endothelial sprouting in a 3D microfluidic angiogenesis model

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Abstract: Both physiological and pathological tissue remodeling (e.g., during wound healing and cancer, respectively) require new blood vessel formation via angiogenesis, but the underlying microenvironmental mechanisms remain poorly defined due in part to the lack of biologically relevant in vitro models. Here, we present a biomaterials-based microfluidic 3D platform for analysis of endothelial sprouting in response to morphogen gradients. This system consists of three lithographically defined channels embedded in type I collagen hydrogels. A central channel is coated with endothelial cells, and two parallel side channels serve as a source and a sink for the steady-state generation of biochemical gradients. Gradients of vascular endothelial growth factor (VEGF) promoted sprouting, whereby endothelial cell responsiveness was markedly dependent on cell density and vessel geometry regardless of treatment conditions. These results point toward mechanical and/or autocrine mechanisms that may overwhelm pro-angiogenic paracrine signaling under certain conditions. To date, neither geometrical effects nor cell density have been considered critical determinants of angiogenesis in health and disease. This biomimetic vessel platform demonstrated utility for delineating hitherto underappreciated contributors of angiogenesis, and future studies may enable important new mechanistic insights that will inform anti-angiogenic cancer therapy. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2013.

Key Words: collagen, microfluidics, gradient, angiogenesis, VEGF


INTRODUCTION

During health, the growth of new blood vessels from preexisting ones (i.e., angiogenesis) is tightly regulated by a balance of pro and anti-angiogenic factors1,2 to maintain tissue homeostasis. However, in the context of diseases such as cancer, aberrant pro-angiogenic signaling leads to uncontrolled new vessel formation and subsequent tumor growth and metastasis.3 While drugs targeted against pro-angiogenic signaling, and particularly the humanized monoclonal vascular endothelial growth factor (VEGF) antibody Avastin,4 have demonstrated therapeutic effectiveness in certain cancer patients, their clinical performance has not yet achieved their anticipated potential.3 This is due in part to redundant, VEGF-independent biological and physical phenomena1,5 that are difficult to study with conventional in vitro and in vivo models. To increase our understanding of the diverse biological and physical parameters regulating tumor angiogenesis, there is a need for more sophisticated culture platforms that help reduce the disparity between petri dish and patient.

A variety of in vitro models exist to study endothelial cell behavior, but these are frequently limited in their ability to accurately mimic conditions regulating blood vessel formation in vivo. Chord or tube formation assays on Matrigel6,7 as well as wound or scratch assays on 2-D plastic surfaces8 are the most widely applied techniques for determining the pro-angiogenic action of isolated or cell-secreted soluble factors. However, batch to batch variations of Matrigel compromise the reproducibility of the first approach, while lack of capillary assembly and unphysiological substrate mechanics represent inherent limitations to the second. Better-defined biomaterials-based systems may overcome these shortcomings and promise to yield a more...
comprehensive understanding of cellular and molecular regulators of vessel formation. For example, encapsulation of single endothelial cells into collagen results in 3D morphogenesis and the formation of capillary networks for studies of vasculogenesis. In addition, maintenance of endothelial monolayers or endothelial cell-coated beads in physical contact with artificial extracellular matrices (ECM) can mimic sprouting events from an endothelial lining as typical during angiogenesis. All of the above biomimetic platforms have helped to isolate effects of global changes in soluble factors on endothelial cell behavior. Nevertheless, technological advancements are needed to recapitulate the physiological spatiotemporal heterogeneity of these factors in functional microvascular mimetics. 

To generate soluble factor gradients, microfluidic channels have been integrated into different in vitro angiogenesis models. For example, this approach has been used to measure endothelial cell migration responses to variations in pro-angiogenic factor gradient steepness and shape on 2-D surfaces and to assess the sprouting response of endothelial monolayers into hydrogels consisting of natural ECM polymers including collagen and fibronectin. Yet, models allowing in vivo-like invasion from endothelialized conduits are still limited, although viable and functional vessel mimics can be generated by seeding of endothelial cells onto the walls of ECM-embedded microchannels. Here, we utilize lithographically defined channels to generate fully-enclosed microvessels and stable VEGF gradients to study endothelial cell invasion dynamics in a remodelable collagen hydrogel scaffold. While providing in vivo-like vessel microenvironments, these models leverage an exquisite degree of experimental control and will afford an improved access to physiological and pathological mediators of angiogenesis.

**MATERIALS AND METHODS**

**Endothelial cell culture**

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and expanded in growth media consisting of BioWhittaker medium 199 (M199; Lonza, Walkersville, MD) with endothelial cell growth supplement

![Figure 1](https://example.com/figure1.png)
Fabrication of 3D microfluidic gradient device

A three-channel geometry was chosen for gradient generation\(^2\) [Fig. 1(a)]. Channel dimensions were 100 \(\times\) 100 \(\mu\)m in cross section, with 350 \(\mu\)m edge-to-edge spacing between parallel channels. The central microchannel was 3.7 mm in length. A silicon master was fabricated using standard lithographic processing and surface-treated with perfluorooctyl-trichlorosilane (Alfa Aesar, Ward Hill, MA), prior to casting a negative Sylgard\(^\circ\) 184 polydimethylsiloxane (PDMS; Dow Corning, Midland, MI) stamp for collagen micromolding. Milled plexiglass pieces (overall size 40 \(\times\) 40 mm) were fabricated to define the overall geometry of the collagen scaffold [Fig. 1(b)] and provide feeding reservoirs to micro-patterned channels. To promote collagen adhesion, surfaces to contact collagen were treated with 1\% polyethyleneimine (PEI, Aldrich Chemical, St. Louis, MO) and 0.1\% glutaraldehyde (GA, Fisher Scientific, Fair Lawn, NJ) followed by rinsing with de-ionized water. Rat tail-derived, acetic acid-extracted collagen (1 wt \%) was prepared as previously reported\(^2\) and injection-molded into the plexiglass piece against the PDMS stamp. Stainless steel pins were used to mold the inlet and outlet ports. Following collagen cross-linking at 37\(^\circ\)C for 30 min, the stamp was removed and channels were sealed with a collagen-coated (\(~\sim 100 \mu\m\) glass coverslip in the plexiglass frame [Fig. 1(c)].

Microfluidic endothelial cell culture

HUVECs (up to passage 4) were seeded in the central channel of the three-channel microfluidic device. To this end, culture media was aspirated from all six reservoirs, and HUVEC suspension was added to the center channel reservoirs (10 \(\pm\) 3 \(\mu\)L of a 2 \(\times\) 10\(^6\) cell/mL suspension to each reservoir). Complete media aspiration from side channel reservoirs and close balancing of cell seeding volumes resulted in a packed channel sidewall images, which were reconstructed from confocal \(z\)-stack images, to record the entire microchannel length. Images were viewed and analyzed with Image\(\text{J}\) (NIH), Volocity (PerkinElmer, Waltham, MA) and Matlab (Mathworks, Natick, MA).

Confocal microscopy and image analysis

Confocal microscopy (LSM 710; Carl Zeiss, Thornwood, NY) was used to capture \(z\)-stack images of endothelial cell-coated, immunostained channels. Images were taken with a 25\(\times\) water immersion objective, with 3.75-\(\mu\m\) \(z\)-step size. Eleven frames were captured as a tile image, to record the entire microchannel length. Images were viewed and analyzed with Image\(\text{J}\) (NIH), Velocity (PerkinElmer, Waltham, MA) and Matlab (Mathworks, Natick, MA).

Generation and characterization of VEGF gradients

Gradient experiments were carried out for 24 h on confluent endothelialized channels, after 48 h of culture under the above-described conditions. Gradient media consisted of growth media plus 1\% [v/v] L-ascorbic acid (50 \(\mu\)g/mL; Acros Organics, Morris Plains, NJ) and 0.16\% [v/v] tetradecanoil phorbol acetate (TPA; 50 ng/mL; Cell Signaling Technology, Danvers, MA). TPA, a reagent commonly used in angiogenesis assays, was included as it mimics endothelial cell activation typical of the tumor vasculature.\(^2,3\) Gradient media in the source channel was supplemented with 75 ng/mL VEGF-165 (R&D Systems, Minneapolis, MN) to generate a VEGF gradient of \(~\sim 90 \text{ng/mL/mm}\) across the cell-coated channel, which has been previously shown to achieve maximum sprout alignment.\(^1\) 250 \(\mu\text{M}\) 40 Kda FITC-dextran was also added to source media to verify the presence of a chemical gradient at the experimental endpoint. Growth media from input and output reservoirs of all three channels was analyzed for VEGF-165 content via enzyme linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN) according to manufacturer’s instructions.

Immunostaining

Staining reagents were delivered through all three channels by adding small volumes of solution to each inlet reservoir. A 1 h PBS rinse was carried out following formalin fixation and after both primary and secondary antibody incubations. HUVECs were initially permeabilized and blocked with a mixture of Triton-X and bovine serum albumin (BSA) diluted in PBS for 30 min at room temperature, and then incubated overnight at 4\(^\circ\)C with mouse anti-human CD31 (1:200, Invitrogen, Carlsbad, CA). Alexa Fluor\(^\circ\) 488 goat anti-mouse IgG (1:500, Invitrogen, Carlsbad, CA) secondary antibody was added in the presence of DAPI (4\(,\)6-diamidino-2-phenylindole; 1:5,000) and Alexa Fluor\(^\circ\) 568 phalloidin (1:100, both from Invitrogen, Carlsbad, CA) and incubated for 1 h at room temperature.

(CGS; Millipore, Billerica, MA), 0.05\% [v/v] heparin (10,000 U/mL stock, Sigma-Aldrich, St. Louis, MO), 1.3\% [v/v] L-glutamine (200 mM, Glutamax-1, Gibco), 20\% [v/v] fetal bovine serum, and 1\% [v/v] PS.

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the channel height) and projected pixel intensity was plotted.

Simulation of VEGF distribution
To simulate the concentration profile of VEGF throughout a cross-section of the entire construct, a 2-D mathematical model was defined. The construct included source, drain (endothelialized) and sink channels of dimensions 100 μm × 50 μm, similar to the microfluidic channel geometry following sealing with a collagen layer. The 2-D steady-state concentration profile was calculated from Fick’s Second Law using the finite difference method. Boundary conditions were defined by a constant concentration of VEGF (100 AU) at the source and a constant consumption rate at the walls of the drain (10 AU per unit time per channel, based on relative VEGF concentration measurements of culture media in the input and output reservoirs, data not shown). The concentrations were visualized across the construct using contour plots in MATLAB (The Mathworks, Natick, MA).

Simulation of endothelial cell-secreted IL-8 distribution
To simulate the concentration profile of cell-secreted interleukin-8 (IL-8) in the vessel vicinity, a 2-D model was developed similar to that used to calculate VEGF profile. A uniform cell density of 1400 cell/mm² was assumed to approximate the experimentally measured range of cell densities. An IL-8 secretion rate of 0.0035 pg/cell/24 h was experimentally determined with 2-D cultured HUVECs using ELISA, and incorporated into the model.

Statistical analysis
In analyzing directional bias in VEGF gradient experiments, only the highly invasive devices, referred to as type II devices in the figures (N = 3) were included; such devices displayed a large number of invasions, as compared with the type I devices which exhibited few or no invasions (N = 8). Invasions directed either toward the source or sink channel were quantified in Matlab, and differences were analyzed with Student’s t-test. For one of these type II devices, sprout intensity versus local nuclei density was plotted and statistically evaluated by linear regression. Statistical analyses were performed in Graphpad Prism.

RESULTS
Microfluidic endothelial cell culture
3D collagen-embedded microchannels were generated whose geometry deviated slightly from the lithographically defined dimensions (approximately 50 × 100 μm vs. 100 × 100 μm) due to sealing with the collagen-coated glass coverslip. Endothelial cells seeded into the central microchannel quickly adhered to collagen (10 min), and within 48 h of culture in endothelial cell growth media formed a confluent layer of cells coating all four walls [Fig. 1(d)]. Without any further stimulation, this monolayer was stable with no evident invasions. Cell viability was ensured via perfusion of media through the channel at 1 μL/min; cells cultured under static conditions within microchannels either did not reach confluence, or delaminated from channel walls after an initial growth period (data not shown). Applied average flow rates of ~1 μL/min corresponded to an average shear stress of ~1 dyne/cm². Although experimental shear stresses in these experiments were an order of magnitude or more lower than physiological values for similarly sized blood vessels, this flow rate ensured a media residence time within the cell channel that was significantly shorter than the calculated time for cellular oxygen consumption (~1 s vs. 60 s), providing an explanation for reduced cell viability at significantly slower flow rates.

Gradient generation and vessel barrier function
Generation of a chemical gradient across the endothelialized microchannel was accomplished using two parallel side channels that served as a source and a sink for the factor of interest. Using this setup, gradient formation and steady-state stability were first tested using fluorescently-labeled 40 kDa dextran, approximating the molecular weight of VEGF. Image analysis of fluorescence intensity across the three-channel device suggested that a steady-state gradient of FITC-dextran was established within 3 h of initial delivery (data not shown) and was maintained for up to 3 days of cell culture [Fig. 2(a)]. Consistent with these results, analysis of VEGF transport using ELISA of input and output media after 12 h of media flow confirmed that excess VEGF delivered via the source channel diffused across the microvessel to reach the sink channel at low, but detectable concentrations. Importantly, no detectable levels of VEGF were measured in the output reservoir of the endothelialized center channel [Fig. 2(b)], consistent with a diffusion barrier provided by the endothelial vessel as reported in our previous paper. We note that flow rate in the side channels was ~20% slower than in the central channel due to the increased resistance inherent to these slightly longer channels. This lower flow rate did not compromise the ability of the sink channel to evacuate solute; the output VEGF concentration was measured to be ~0.8% the source concentration. Together, these results suggest that gradient formation was driven by diffusion of VEGF through the collagen hydrogel to the sink channel rather than leakage into the center channel. Furthermore, mathematical modeling [Fig. 2(c)] confirmed the generation of relevant gradients as cells on the source side of the cell channel were exposed to approximately twice the concentration of VEGF as compared to cells on the sink side, with a similar enhancement of VEGF concentration in the vicinity of cell corners as compared to central side positions [Fig. 2(c), inset].

Invasion response to VEGF gradient
To test invasion response to VEGF gradients, the endothelial lining of the center channel, as established by culture in growth media for 48 h, was exposed to VEGF gradient conditions for 24 h. Unexpectedly, this treatment resulted in two types of responses. In the first response type, vessels remained largely stable, exhibiting few sprouts into the surrounding collagen [Fig. 3(a–i)]. In the second response type, application of a VEGF gradient resulted in the formation of abundant endothelial sprouts [Fig. 3(a–ii)] that were up to...
Invasion response to variations in cell density
Enhancement of invasions at vessel corners led us to hypothesize that cell density may play a role in determining invasion behavior. Cell density analysis indicated an increase in nuclear density in the vicinity of vessel corners as revealed by projecting a 45 degree cross-section rotation and plotting average pixel intensity across this projection for the DAPI fluorescence [Fig. 5(a)]. Lower corners, that is, corners embedded deeper within the collagen scaffolds, similarly exhibited increased average pixel intensity relative to sides, albeit at reduced levels due to depth-dependent reduction of optical signal in confocal imaging [Fig. 5(a)].

As changes in cell number and geometry can affect cell behavior due to varied autocrine signaling,26 we next assessed the chemical environment generated by endothelial cell-secreted factors. While ELISA analysis of HUVEC secretion revealed no detectable levels of VEGF (data not shown), IL-8—an endothelial cell-secreted signaling molecule that may modulate sprouting in an autocrine manner27—was released at 0.0035 pg/cell/24 h. Using finite element modeling of IL-8, we demonstrated steeper gradients of this factor in the vicinity of vessel corners [Fig. 5(b)]. This enhancement was due to direct geometrical effects as well as increased cell numbers in the corners, and it was consistent with augmented invasion observed at these locations. These changes may be compounded by the presence of exogenous VEGF (mathematically predicted to be 5% of the maximum concentration near endothelial cells facing the sink channel [inset Fig. 2(c)]) that is known to up-regulate IL-8.28 These results motivated a re-quantification of invasions for the type II response VEGF...
gradient experiments \((N = 3)\) to assess if removal of corner effects would reveal a directional bias toward the VEGF source in sprouts originating on the flat channel walls. While this approach revealed a nominal 2.2-fold increase in source-to-sink invasion ratio, this trend was not statistically significant \((p = 0.16)\). However, quantification of endothelial cell density for all experiments indicated that inter- as well as intra-vessel variations in cell density may regulate endothelial cell sprouting. Specifically, plotting total invasion frequency per bin along the channel length (source + sink, for each of 10 bins) versus average wall nuclear density for a highly invasive VEGF gradient vessel [Fig. 5(c-i)] revealed a statistically significant non-zero slope \((p < 0.01)\). This suggested that cell density and invasion frequency are locally correlated within a single vessel. Furthermore, analysis of multiple devices confirmed that invasion frequency represents a function of cell density [Fig. 5(c-ii)]. Together, these data suggest both intra- and inter-vessel correlation of cell density and sprouting.

**DISCUSSION**

We have developed a microfluidic gradient device to investigate sprouting from an endothelial cell-coated vessel in response to spatial variations of VEGF. Our results suggest that responsiveness to pro-angiogenic factor gradients may be affected by other microenvironmental parameters including cellular density and vessel geometry. The device was configured within a remodelable hydrogel scaffold and consisted of a three-channel arrangement. The two side channels allowed gradient generation, while culture of endothelial cells in the center channel created a responsive, 3D, biomimetic endothelium. Our experiments were conducted in a pseudo-static regime in which flow is sufficient to provide nutrition and eliminate cellular waste without inducing mechanical stimulation that would typically result from physiological flow rates. The resulting low shear values appropriately recapitulate blood flow conditions within dysfunctional tumor vasculature, which can be slow or even stagnant.\(^{29,30}\) We note that physiological shear stress can

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**FIGURE 3.** Endothelial cell invasion in response to VEGF gradient. (a) Confocal analysis of HUVEC invasion following 24 h of exposure to exogenous VEGF gradient revealed non-responsive (type #1) and responsive vessels (type #2). (b) Cross-sectional projection of confocal images of devices shown in (a). Triangle indicates direction of VEGF gradient. (c) Quantification of sprouting frequency towards the source and sink channel via fluorescence intensity analysis indicated no statistical bias in sprouting direction for the type #2 VEGF gradient devices \((N = 3)\). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
inhibit angiogenesis,\textsuperscript{16} and therefore abnormally low shear may promote endothelial sprouting in tumor vasculature. However, the lack of shear-induced mechanical stimulation was not the primary regulator in our studies as the generated endothelium underwent quiescence under basic culture conditions, and sprout initiation only occurred when stimulated with biochemical factors. Furthermore, geometry-dependent differences in local shear stress are unlikely to contribute to our results as quiescence is typically induced at significantly higher shear stresses (3 dyne/cm\textsuperscript{2}) than explored in this work.\textsuperscript{16} The complete lack of invasions in our low-density, VEGF-activated, low shear experiments further supports this conclusion. Nevertheless, integrating controlled variation in shear and studying synergistic effects will be an important component of future work with this system.

The developed biomaterials-based platform permits studies of endothelial cell invasion dynamics in response to pro-angiogenic factor gradients from a fully-embedded channel, rather than individual monolayer. The appropriate dimensionality is of particular relevance as it may partially explain the surprising lack of directional bias in our sprouting experiments. Specifically, an endothelial cell layer presents a barrier to diffusion of large molecules, while also consuming and secreting potent signaling molecules. Accordingly, our computational model suggested that in the vicinity of a fully-enclosed vessel the exogeneous VEGF profile is perturbed locally such that it may drive invasions from all points on the vessel surface. Similarly, finite element modeling of IL-8 secretion and diffusion suggested the formation of an autocrine gradient that may affect endothelial invasion in our microfluidic device. While a variety of other factors may be similarly important, we selected IL-8 as a specific example because this factor directly enhances angiogenic processes.\textsuperscript{10,27} is secreted at greater levels in the presence of VEGF\textsuperscript{28} and elevates response to VEGF via up-regulation of VEGF-R2.\textsuperscript{31,32} Endothelial monolayers do not allow comprehensive recapitulation of similarly complex soluble factor distributions and therefore may obscure bona fide sprouting characteristics of \textit{in vivo} blood vessels.

A strong threshold effect in invasion frequency as a function of both local as well as global vessel cell density seemingly supported our hypothesis that invasions may be modulated via an autocrine mechanism. Nevertheless, future experiments and computational simulations evaluating the individual and combined contributions of VEGF with autocrine factors are needed to confirm this connection. Such experiments should also evaluate the importance of growth factor sequestration and proteolytic release on gradient formation and endothelial cell response. This is of particular importance in the case of tumor angiogenesis, in which heparin-binding affects both the spatial distribution and signaling potency of VEGF and IL-8.\textsuperscript{33,34} Microfluidic gradient systems using biomaterials in which growth factor release is controlled in a cell-specific manner\textsuperscript{35} would be highly valuable to this end. Furthermore, such systems would enable studies of endothelial cell responses to newly developing versus established gradients that may differentially modulate cell behavior due to variability in receptor responsiveness following chronic exposure to signaling molecules.\textsuperscript{26}

The specific morphology of endothelialized microchannels in this study suggests the importance of geometrical effect in the formation of new blood vessels. Sprouting was reliably increased at corners relative to sides of microchannels. The particular device construction leads to the existence of an interface between bulk and sealing collagen layers, which did not influence invasion frequency as opposite corners (i.e., corners not associated with the sealing collagen layer) similarly exhibited enhancement of invasion relative to sides [Figs. 3(b–ii) and 4(b)]. The observed geometrical effects were consistent with previous studies describing the role of tissue geometry in guiding local cell invasion as a function of autocrine signaling mechanisms independent of cell density.\textsuperscript{26} Our observation of increased cell density in corners versus sides may further enhance this effect in part by limiting cell spreading, which can stimulate angiogenic gene expression and tubulogenesis.\textsuperscript{27} Nevertheless, other corner-associated phenomena may play a similar role. For example, asymmetric tissue geometry can lead to cell-induced changes in ECM density that modulate local collagen mechanical properties.\textsuperscript{38} The consequent increase in stiffness can enhance cell contractility, which, in turn, may affect the spatial distribution of sprouting in our model due to varied mechanotransduction. In particular, endothelial cell proliferation,\textsuperscript{39} migration,\textsuperscript{40} secretion of proteolytic enzymes such as matrix metalloproteinases (MMPs),\textsuperscript{41} and VEGF receptor-mediated signal transduction\textsuperscript{42} are all positively correlated with greater stiffness and together may contribute to the observed increase in cell density.
number in corners relative to sides. While we appreciate the relatively unphysiological nature of square vessels, geometrical irregularities are common to vascular branch points and tortuous tumor vessels, both sites of augmented angiogenesis. Based on our results, we propose that altered vessel geometry may increase neovascularization at these sites.

It was previously reported that endothelial cell confluence is a necessary precursor to invasion; however, a strong dependence on cellular density within a confluent layer has not to our knowledge been reported. This study defines cell density as a potentially important design parameter for engineered angiogenesis models, in which small variations in cell density might confound the effects of other variables such as exogenous VEGF or fluid mechanical properties. Finally, our results may inform the widely observed therapeutic resistance of cancer patients to VEGF-based anti-angiogenic therapy. Specifically, the reported data suggest that other physicochemical factors currently not targeted in anti-angiogenic therapies may significantly modulate blood vessel response to VEGF or even potentiate sprouting independent of VEGF. Future use of this model system may help to identify the underlying cellular, molecular, and tissue-level phenomena regulating angiogenesis in health, disease, and therapy.

CONCLUSIONS
We have demonstrated the use of lithographically defined microchannels to fabricate biomimetic in vitro blood vessels with stable biochemical gradients for studies of microenvironmental regulation of angiogenesis. Our results suggest vessel geometry, cell density, and the subsequent changes in autocrine signaling loops as novel mediators of blood vessel sprouting. Future studies with the developed model system will increase our understanding of angiogenesis in health and disease and lead to important new insights with impact on a variety of basic sciences, tissue engineering, and clinical questions. Further adaptation of this platform, for example by integration of additional cell types (e.g., pericytes, circulating bone marrow progenitors), modification of the biomaterial used to generate the microfluidic hydrogel scaffold, or application as a screening platform for anti-angiogenic drugs, has the potential to elucidate novel therapeutic targets and inform more efficacious anti-angiogenic therapies for cancer patients.

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