Published in final edited form as:

J Biomed Mater Res A. 2013 October ; 101(10): 2948–2956. doi:10.1002/jbm.a.34587.

Physicochemical regulation of endothelial sprouting in a 3-D 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 3-D 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.

Keywords

collagen; microfluidics; gradient; angiogenesis; VEGF

1. 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 factors^{1,2} 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 metastasis². While drugs targeted against pro-angiogenic signaling, and particularly the humanized monoclonal VEGF antibody Avastin®, have demonstrated therapeutic effectiveness in certain cancer patients, their clinical performance has not yet achieved their anticipated potential³. This is due in part to redundant, VEGF-independent biological and

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physical phenomena^{4,5} that are difficult to study with conventional *in vitro* and *in vivo* models. In order 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 Matrigel®^{6,7} as well as wound or scratch assays on 2-D plastic surfaces⁶ 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 3-D morphogenesis and the formation of capillary networks for studies of vasculogenesis⁸. Additionally, maintenance of endothelial monolayers^{9,10} or endothelial cell-coated beads^{11,12} 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 mimetics¹³.

In order 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 surfaces¹⁴ and to assess the sprouting response of endothelial monolayers into hydrogels consisting of natural ECM polymers including collagen and fibronectin¹⁵⁻¹⁷. 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 microchannels¹⁸⁻²⁰. 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.

2. Materials and Methods

2.1. 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 (ECGS; Millipore Co., 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] FBS, and 1% [v/v] PS.

2.2. Fabrication of 3-D microfluidic gradient device

A three-channel geometry was chosen for gradient generation²¹ (Fig. 1a). Channel dimensions were 100 × 100 μm in cross section, with 350 μm 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 perfluorooctyltrichlorosilane (Alfa Aesar, Ward Hill, MA), prior to casting a negative

Sylgard® 184 polydimethylsiloxane (PDMS; Dow Corning Co., Midland, MI) stamp for collagen micromolding. Milled plexiglass pieces (overall size 40×40 mm) were fabricated in order to define the overall geometry of the collagen scaffold (Fig. 1b) and provide feeding reservoirs to micropatterned 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 reported²², 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°C for 30 min, the stamp was removed and channels were sealed with a collagen-coated (~100 µm) glass coverslip in the plexiglass frame (Fig. 1c).

2.3. 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 added to the center channel reservoirs (10 ± 3 µL of a 2×10⁶ 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 and high cell seeding density (>1000 cell/mm² for confluent layers). After allowing cells to adhere, channels were perfused at 1 µL/min (~1 dyne/cm²) with growth media using gravity-driven flow or a syringe pump (Fig. 1c).

2.4. Generation and characterization of VEGF gradients

Gradient experiments were carried out for 24 hrs on confluent endothelialized channels, after 48 hrs of culture under the above-described conditions. Gradient media consisted of growth media plus 1% [v/v] L-ascorbic acid (50 µg/mL; Acros Organics, Morris Plains, NJ) and 0.16% [v/v] tetradecanoyl phorbol acetate (TPA; 50 ng/mL; Cell Signaling Technology Inc., Danvers, MA). TPA, a reagent commonly used in angiogenesis assays, was included as it mimics endothelial cell activation typical of the tumor vasculature^{22,23}. 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 ~90 ng/mL/mm across the cell-coated channel, which has been previously shown to achieve maximum sprout alignment¹¹. 250 µM 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.

2.5. Immunostaining

Staining reagents were delivered through all three channels by adding small volumes of solution to each inlet reservoir. A 1 hr 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 BSA diluted in PBS for 30 minutes at room temperature, and then incubated overnight at 4°C with mouse anti-human CD31 (1:200, Invitrogen Co., Carlsbad, CA). Alexa Fluor® 488 goat anti-mouse IgG (1:500, Invitrogen Co., Carlsbad, CA) secondary antibody was added in the presence of DAPI (1:5,000) and Alexa Fluor® 568 phalloidin (1:100, both from Invitrogen Co., Carlsbad, CA) and incubated for 1 hr at room temperature.

2.6. Confocal microscopy and image analysis

Confocal microscopy (LSM 710; Carl Zeiss, Inc., Thornwood, NY) was used to capture z-stack images of endothelial cell-coated, immunostained channels. Images were taken with a 25× water immersion objective, with 3.75 μm z-step size. 11 frames were captured as a tile image, to record the entire microchannel length. Images were viewed and analyzed with ImageJ (NIH), Volocity (PerkinElmer, Waltham, MA) and Matlab (Mathworks, Natick, MA).

Confocal z-stacks were quantified for cell density and sprouting frequency using ImageJ and Matlab. Stacks were re-sliced with 2.5 μm steps along the axial channel direction using ImageJ. The fluorescence intensity of the actin channel from the cross-sectional image was used for analysis of invasion frequency and direction. Top and bottom channel walls were analyzed for local nuclear density because of the higher image resolution for these planes, as compared to sidewall images, which were reconstructed from confocal z-stacks. Channels were binned into ten sections along a 2.5 μm portion of channel length. Nuclear density (number of nuclei per area, for 50 μm wide region of top and bottom channel walls) was quantified in Matlab for each bin, as was total invasion pixel intensity from either the full channel height, or a 25 μm tall central portion of sidewalls, and either towards the VEGF source or sink. To quantify sprouting intensity, channel sidewalls were identified in cross-sectional images via intensity thresholding, and cellular actin extending beyond the channel boundary was quantified by summing the projected pixel intensity with a local bin, normalized to the bin intensity threshold used for wall identification. To quantify corner effects on cellular density, channel cross-sections were projected (average intensity) along a direction 45 degrees rotated from the y-axis (defined along the channel height) and projected pixel intensity was plotted.

2.7. 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 $\mu\text{m} \times 50 \mu\text{m}$, 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).

2.8. 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 1,400 cell/ mm^2 was assumed to approximate the experimentally measured range of cell densities. An IL-8 secretion rate of 0.0035 pg/cell/24 hrs was experimentally determined with 2-D cultured HUVECs using ELISA, and incorporated into the model.

2.9. 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 to the type i devices which exhibited few or no invasions ($N = 8$). Invasions directed either towards 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.

3. Results

3.1. Microfluidic endothelial cell culture

3-D collagen-embedded microchannels were generated whose geometry deviated slightly from the lithographically defined dimensions (approx. $50 \times 100 \mu\text{m}$ vs. $100 \times 100 \mu\text{m}$) 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 hrs of culture in endothelial cell growth media formed a confluent layer of cells coating all four walls (Fig. 1d). Without any further stimulation, this monolayer was stable with no evident invasions. Cell viability was ensured via perfusion of media through the channel at $1 \mu\text{L}/\text{min}$; 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 $\sim 1 \mu\text{L}/\text{min}$ corresponded to an average shear stress²⁵ of $\sim 1 \text{ dyne}/\text{cm}^2$. Although experimental shear stresses in these experiments were an order of magnitude or more lower than physiological values for similarly sized blood vessels^{24,25}, this flow rate ensured a media residence time within the cell channel that was significantly shorter than the calculated time for cellular oxygen consumption ($\sim 1 \text{ s}$ vs. 60 s), providing an explanation for reduced cell viability at significantly slower flow rates.

3.2. 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 3-channel device suggested that a steady-state gradient of FITC-dextran was established within three hours of initial delivery (data not shown) and was maintained for up to three days of cell culture (Fig. 2a). Consistent with these results, analysis of VEGF transport using ELISA of input and output media after 12 hrs 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. 2b), consistent with a diffusion barrier provided by the endothelial vessel as reported in our previous paper²⁰. We note that flow rate in the side channels was $\sim 20\%$ 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 $\sim 0.8\%$ 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. 2c) confirmed the generation of relevant gradients¹¹ 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. 2c, inset).

3.3. 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 hours, was exposed to VEGF gradient conditions for 24 hrs. 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. 3a-i). In the second response type, application of a VEGF gradient resulted in the formation of abundant endothelial sprouts (Fig. 3a-ii) that were up to ~ 100 μm in length, typically remained connected to the parent cell layer, and consistently contained multiple nuclei. Sprouts emanated from all vessel walls and corners (Fig. 3b-ii). These differences in invasion behavior were not related to geometrical variations between type i and type ii devices as similar channel cross sectional areas and thus flow and shear rates exhibited very different invasion behaviors (Fig. 3b). Quantification of invasion frequency by normalizing the sum of invasion pixel intensity over a 2.5 mm center region of the vessel revealed no statistically significant directionality ($N=3$) of invasions towards the VEGF source channel for the second response type (Fig. 3c); this lack of bias was unexpected based on previous reports of directional sprouting¹⁷ in 3-D gradient devices and was reproducible for multiple experiments.

Because multiple invasion response patterns were observed to result from seemingly identical treatment conditions, control experiments were conducted in which vessels were exposed to gradient media lacking VEGF. Under these nominally uniform culture conditions, vessels similarly exhibited two types of behavior. In the first class, vessels remained stable with no sprouts (Fig. 4a-i), whereas a large number of sprouts were observed in the second class (Fig. 4a-ii). However unlike in the VEGF gradient experiments, sprouts were observed to originate preferentially from channel corners (Fig. 4b). Culture of endothelial cells in open channels (i.e., channels not sealed by a second collagen layer) that were fully immersed in culture media also demonstrated 3-fold invasion enhancement at channel corners relative to sides confirming the relevance of this trend independent of flow conditions (data not shown).

3.4. 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. 5a). Lower corners, i.e., 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. 5a).

As changes in cell number and geometry can affect cell behavior due to varied autocrine signaling²⁶, 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 hrs. Using finite element modeling of IL-8, we demonstrated steeper gradients of this factor in the vicinity of vessel corners (Fig. 5b). 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. 2c]) that is known to up-regulate IL-8²⁸. These results motivated a re-quantification of invasions for the type 2 response VEGF gradient experiments ($N=3$) to assess if removal of corner effects would reveal a directional bias towards 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 ten bins) versus average wall nuclear density for a highly invasive VEGF gradient vessel (Fig. 5c-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. 5c-ii). Together, these data suggest both intra- and inter-vessel correlation of cell density and sprouting.

4. 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, 3-D, 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 inhibit angiogenesis¹⁶, 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/cm²) than explored in this work¹⁶. 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 exogenous VEGF profile is perturbed locally such that it may drive invasions from all points on the vessel surface. Likewise, 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^{10,27}, is secreted at greater levels in the presence of VEGF²⁸, and elevates response to VEGF via up-regulation of VEGF-R2^{31,32}. Endothelial monolayers do not allow comprehensive recapitulation of similarly complex soluble factor distributions and therefore may obscure bona fide sprouting characteristics of *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^{33,34}. Microfluidic gradient systems using biomaterials in which growth factor release is controlled in a cell-specific manner³⁵ would be highly valuable to this end. Furthermore, such systems would enable studies of endothelial cell responses to newly developing vs. established gradients that may differentially modulate cell behavior due to variability in receptor responsiveness following chronic exposure to signaling molecules³⁶.

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. 3b-ii and 4b). 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 density²⁶. Our observation of increased cell density in corners vs. sides may further enhance this effect in part by limiting cell spreading, which can stimulate angiogenic gene expression and tubulogenesis³⁷. 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 properties³⁸. 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 proliferation³⁹, migration⁴⁰, secretion of proteolytic enzymes such as matrix metalloproteinases (MMPs)⁴¹, and VEGF receptor-mediated signal transduction⁴² are all positively correlated with greater stiffness and together may contribute to the observed increase in cell 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^{3,43}. 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 invasion⁹; 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 therapy³. 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.

5. 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.

Acknowledgments

We thank Glenn Swan for hardware fabrication, and Ying Zheng and Michael Craven for helpful discussions. This work was supported by NIH/NCI (RC1 CA146065), the Cornell Center on the Microenvironment & Metastasis through Award Number NCI U54 CA143876, and NSF (Graduate Research Fellowship for P. DelNero). The content is solely the responsibility of the authors and does not necessarily represent the official views of NCI or NIH.

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Fig. 1. Model development

(a) Schematic of the three-channel microfluidic gradient generator, with center channel used for 3-D culture of HUVECs, and outer channels used as source (C_1) and sink (C_2) for gradient establishment. (b) Photograph of plexiglass jig, comprising media reservoirs, collagen injection ports, and location of hydrogel scaffold. (c) Step-by-step assembly process of microfluidic devices; cross-sectional view of center channel is shown. (d) Top-down (i), and cross-sectional (ii) views of a confocal micrograph showing HUVEC-lined microchannel, immunostained for CD31 (green), actin cytoskeleton (red), and nuclei (blue) after 48 hours of culture in growth media.

Fig. 2. Experimental and computational characterization of VEGF gradient

(a) Characterization of VEGF gradient by perfusion with a 40 kDa FITC-dextran tracer molecule and fluorescent image analysis. (b) VEGF concentration in the inlet and outlet reservoirs of the three channels as quantified by ELISA 12 hours after initiating VEGF delivery through the source channel. (c) Finite difference model contour plot of VEGF concentration across the device, as a result of the experimental source-sink concentrations, vessel barrier function, and VEGF consumption by cells. Inset depicts a magnified view of the local VEGF profile in the vicinity of the center channel.

Fig. 3. Endothelial cell invasion in response to VEGF gradient

(a) Confocal analysis of HUVEC invasion following 24 hrs 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).

Fig. 4. Endothelial cell invasion in absence of VEGF gradient; effect of vessel geometry
 (a) Confocal analysis of HUVEC invasion following 24 hrs of exposure to invasion media lacking exogenous VEGF gradient revealed non-responsive (type #1) and responsive vessels (type #2). (b) Cross-sectional projection of confocal image of type #2 device shown in (a-ii) demonstrates a distinct preponderance of sprouts initiating at the channel corners.

Fig. 5. Effect of cell density on endothelial cell invasion

(a) Rotated cross-sectional and top-down projection of confocal images visualizing nuclear density and corresponding pixel intensity profile. This method revealed increased nuclear density associated with channel corners. Lower corners, i.e., corners embedded deeper within the collagen scaffold, also exhibit increased average pixel intensity relative to sides, albeit at reduced levels due to depth-dependent reduction of confocal signal. (b) Finite difference model of autocrine IL-8 profile, based on experimentally determined HUVEC IL-8 secretion (0.0035 pg/cell/24 hrs). (c) Quantification of sprouting as a function of intrachannel variations of cell density by image analysis of 10 sections (bins) of a 2.5 mm channel portion (i), and total sprouting intensity per microchannel as a function of total nuclei density for distinct vessels exposed to a VEGF gradient (ii).