Endothelial Cell Guidance in 3D Patterned Scaffolds

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A vascular network is required to provide engineered tissue constructs with the oxygen and nutrients needed for survival.[1,2] Endothelial cells (ECs), key cellular components of blood vessels, play important roles in the stem cell niche and thus their function in regenerative strategies extends beyond the vascular system.[3–5] To better understand the role of ECs in the stem cell niche, representative *in vitro* models of EC growth are required. Most studies of ECs have been performed in two-dimensional (2D) tissue culture;[6,7] however, given the morphological and functional differences exhibited by these cells in 2D and three-dimensional (3D) cultures,[8] the development of well-defined 3D culture models is necessary to better investigate vascular development.

During vascular development, the leading front of the sprout exposes a tip cell with numerous filopodia that express receptors to sense secreted and cell-bound guidance cues provided by surrounding cells.[9,10] One of the most studied factors that controls blood vessel formation and function is vascular endothelial growth factor A (VEGF-A).[9–12] VEGF-A exists in several isoforms which bind with different affinities to VEGF receptors (VEGFRs).[13,14] VEGF165, in particular, is a critical angiogenic factor. The binding of VEGF165 to its receptor, VEGFR2, causes EC proliferation and migration.[9,10,14,15] Tip cells have been shown to migrate up a concentration gradient of VEGF165.[9,10,16,17] Moreover, tip cells are trailed by stalk cells, which proliferate and self-organize to form lumens, when cultured in 3D, in response to VEGF-A.[9,10,16,17]

In this study, we designed a 3D culture system, comprised of concentration gradients of human VEGF165 in hydrogels, to guide endothelial cell growth with the ultimate goal of enhancing regenerative strategies and laying the foundation to investigate their role in the stem cell niche. By mimicking the cues that guide endothelial cells in vivo, we show, for the first time, that ECs follow an immobilized VEGF165 concentration gradient in a 3D hydrogel, with tip and stalk cells identified and tubular-like structures formed. Previously, only cell-adhesive cues have been studied for EC growth in 3D and this has limited the morphology of tip and stalk cells formed.[18]

In order to study the guidance of endothelial cells in 3D, agarose hydrogels were photochemically patterned with multiphoton lasers[19] to immobilize a series of concentration gradients of VEGF165. To promote cell adhesion, agarose was also modified with the ubiquitous cell adhesion peptide, glycine-arginine-glycine-aspartic acid-serine (GRGDS). Based on previous studies with neural stem cells, we hypothesized that the peptides and growth factors would be stably immobilized and remain bioactive after immobilization.[20,21] These comprised our first studies.

VEGF165 was covalently bound to agarose and compared to soluble VEGF165 and adsorbed VEGF165 in terms of endothelial cell proliferation. Agarose was chemically modified with cysteine-protected 6-bromo-7-hydroxycoumarin,[19] which, upon excitation with either UV light or a pulsed infrared laser, yielded agarose-sulphide. VEGF was modified with 4-(4-N-maleimidophenyl) butyric acid hydrazide (MI)[20] and Alexa Fluor 594 hydrazide (f), yielding MI-VEGF165-f which allowed Michael-type addition to agarose-thiol and facilitated quantification by fluorescence of immobilized VEGF165. By comparison to a standard curve of fluorescence intensity, 924 ± 25 ng mL^{-1} of MI-VEGF165-f was immobilized to agarose-sulphide (100 µL) whereas only 79 ± 15 ng mL^{-1} of MI-VEGF165-f was physically adsorbed. The bioactivity of the covalently-bound agarose-VEGF165 was characterized relative to adsorbed and soluble VEGF controls by the MTT cell proliferation assay using VEGFR2+ brain-derived endothelial (bEnd3) cells.[22] Soluble VEGF165 controls were prepared by mixing VEGF165 solution including 50 ng or 100 ng with 100 µL agarose. After 3 days of culture, ECs cultured on agarose-VEGF165 (924 ng mL^{-1}) showed a similar proliferation profile to those cultured in soluble 1000 ng mL^{-1} VEGF165 treatment ($n = 3$, **Figure 1**) yet a significantly different proliferation profile from those cultured in soluble 500 ng mL^{-1} VEGF165 ($p < 0.05$, $n = 3$) or in the absence of VEGF165 ($p < 0.05$, $n = 3$). In addition, physically adsorbed MI-VEGF-f did not stimulate bEnd3 cells and was not statistically different from the control treatment without VEGF165 ($n = 3$, Figure S1 in the Supporting Information). Thus, covalently-immobilized VEGF165 remained bioactive.

Since aggregates of ECs promote tubular formation due to cell-cell and cell-extracellular matrix interactions,[23,24] we tested the suitability of GRGDS-agarose for cell penetration and tubule-like structure formation. Primary endothelial cells that were isolated from the microvessels in the brains of adult mice[25] and cultured as EC spheroids,[26] were seeded on the GRGDS-agarose hydrogels. In the first 12 h of culture, sprouts were observed protruding from the spheroids into the hydrogels. After 24 h, ECs started forming tubule-like structures within the gel. Stalk and tip

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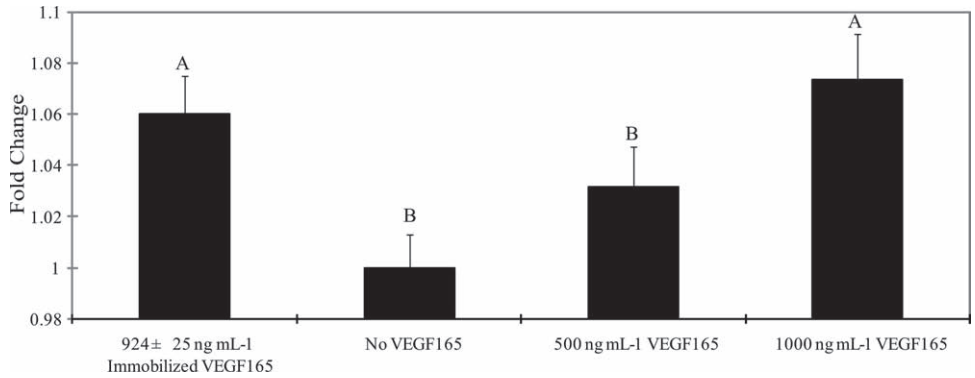

Figure 1. Analysis of bioactivity of immobilized VEGF165 relative to no VEGF165, or soluble VEGF165 in agarose hydrogels by MTT cell proliferation assay. Data are expressed as the fold changed in UV absorbance relative to the control (no VEGF165 treatment). ECs cultured on agarose-VEGF165 (924 ng mL^{-1}) showed a similar proliferation profile to those cultured in soluble 1000 ng mL^{-1} VEGF165 controls, yet significantly different profile from cultures with no VEGF165, or 500 ng mL^{-1} soluble VEGF165. (The different letters represent significant differences as measured by ANOVA, $p < 0.05$)

cells were first identified based on their morphology (**Figure 2a**) and then were further characterized by staining for junctional proteins with β-catenin, and for vessel wall extracellular matrix with laminin. We captured fluorescent confocal image stacks along the z-axis of the hydrogels to create 3D representations of the vessels (Figure 2b, c and d). We observed lumen-like structures in the organized EC cultures, suggesting that these 3-D cultured ECs in agarose were in the process of tubulogenesis (Figure S2 in the Supporting Information).

Having established the suitability of GRGDS-agarose hydrogels for EC migration and bioactive immobilization of VEGF165, we then created and tested the guidance capacity of a series of immobilized VEGF165 concentration gradients in agarose. Using a multiphoton Ti/sapphire confocal laser, a gradient of VEGF165 was immobilized within a defined volume in the agarose hydrogel, taking advantage of photolabile coumarin-protected agarose-sulphide groups and maleimide-modified VEGF165. We designed a series of vertical, linear concentration gradient volumes of fluorescently-tagged VEGF165 having dimensions of 300 μm × 300 μm × 600 μm (L × W × D, **Figure 3a**). The gradients were created by controlling the scanning number and scanning regions and quantified based on the fluorescence intensity: 2.48, 1.65, and 0.99 ng mL^{-1} μm^{-1} (Figure 3b).

To study endothelial cell guidance in the immobilized VEGF165 concentration gradient hydrogels, GRGDS peptides were immobilized at a uniform concentration of 2.08 μM in the same volume using the confocal patterning technique. Dissociated primary ECs were seeded on top of the cell-adhesive, gradient agarose hydrogels, and formed small aggregates on top of the patterned volumes within the first 12–24 h of culture prior to penetrating the gels. Interestingly, after 3 days of culture, primary ECs had penetrated the agarose hydrogels to a depth of more than 200 μm and formed tubule-like structures in agarose hydrogels having VEGF165 gradients of both 1.65 and 0.99 ng mL^{-1} μm^{-1} (Figure 3c and d). In contrast, ECs cultured on the 2.48 ng mL^{-1} μm^{-1} VEGF gradient hydrogel developed a few sprouts from the EC aggregates, but showed no evidence of tubular formation in the gel (data not shown). Considering that the matrix-bound VEGF gradient in vivo is regulated by interstitial flow,[27] there is a physiological gradient range to which tip endothelial cells can respond. Our results suggest that the steeper 2.48 ng mL^{-1} μm^{-1} VEGF gradient may have saturated the VEGFR2 receptors on endothelial tip cells, thereby limiting their guidance response to this gradient.

To test whether the EC guidance observed resulted from the immobilized VEGF165 gradients, primary ECs were cultured on patterned GRGDS peptide hydrogels either

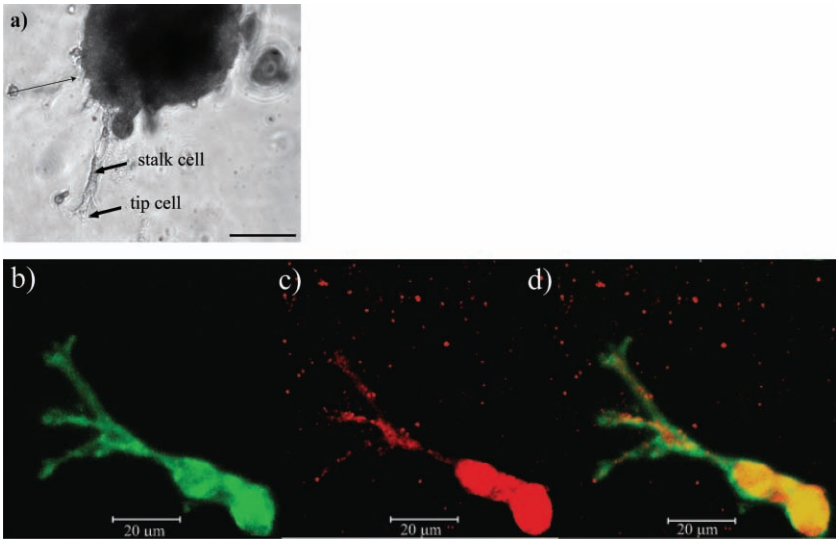

Figure 2. Representative images of primary endothelial cells from the brain microvaculature extend tip and stalk cells into GRGDS agarose hydrogels and form tubule-like structures: a) Phase-contrast microscopy image of an EC spheroid cultured on top of the GRGDS hydrogel after one day has stalk cells (arrow at top) and tip cells (arrow at bottom) growing into the agarose gel (arrows point the surface of the hydrogels Scale bar = 100 μm). Confocal images of these primary ECs immunostained with primary antibodies: b) β-catenin for junctional proteins, c) laminin for extracellular matrix, d) combined staining of β-catenin and laminin. (Scale bar = 20 μm).

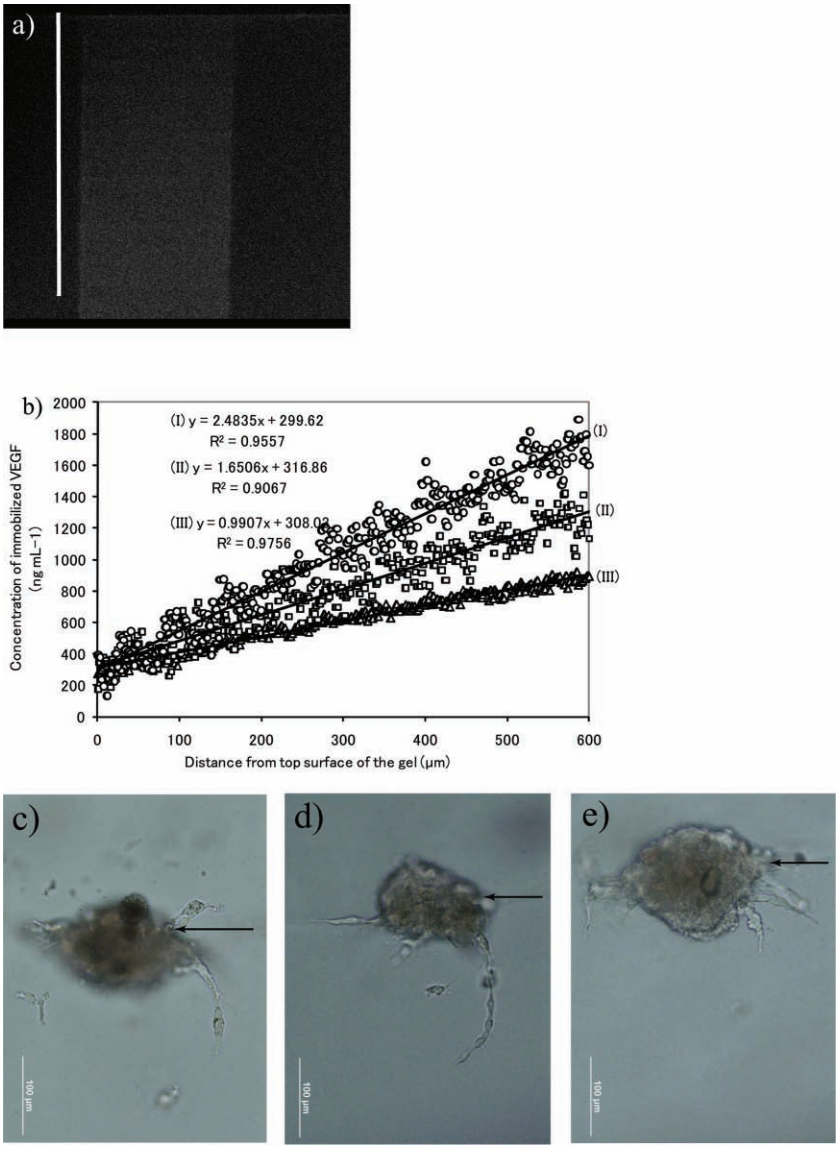

Figure 3. Creation of an immobilized gradient of VEGF165 in the agarose hydrogel to guide primary EC growth. a) Confocal image of the gradient of fluorescently tagged immobilized VEGF165. As the number of scans by the confocal laser increases from top to bottom, the fluorescent intensity increases correspondingly, reflecting the greater number of deprotected thiol groups that react with maleimide modified VEGF165 (Scale = 600 μm). b) Quantification of a gradient of immobilized VEGF165 in the agarose hydrogel based on the standard curve of fluorescently tagged VEGF. Each gradient was calculated at (I) 2.48, (II) 1.65 and (III) 1.00 ng mL^{-1} μm^{-1}. c–e) Phase-contrast microscopy of cross-sectional images of primary ECs growing into the hydrogels after culturing for 3 d. ECs formed aggregates on top of the patterning areas (arrows point the surface of the hydrogels) and then were guided into the agarose hydrogels, in tubular-like formations, following the VEGF165 gradients: c) 1.65 ng mL^{-1} μm^{-1}, d) 1.00 ng mL^{-1} μm^{-1}; EC growth was minimal into the agarose hydrogel control with no VEGF165 immobilized. Scale bars (= 100 μm) are parallel to the Z-direction in the gels.

in the absence of VEGF165 (Figure 3e) or in the presence of homogeneously immobilized VEGF165 (ie., no gradient): 195 ± 98 ng mL^{-1}, 521 ± 106 ng mL^{-1}, or 722 ± 172 ng mL^{-1} (Figure S3 in the Supporting Information). In all cases, tubular-like sprouts grew from EC aggregates; however, these penetrated the agarose to only a limited depth (<30–50 μm), just below the surface. In contrast, the mean ± standard deviation tubule extension was 195 ± 21 μm (n = 6) for an immobilized VEGF165 concentration gradient of 1.5–1.8 ng mL^{-1} μm^{-1} and a starting concentration of 250–300 ng mL^{-1} (Figure 3c). The mean ± standard deviation tubule extension was 210 ± 41 μm (n = 5) for an immobilized VEGF165 concentration gradient of 0.8–1.0 mL^{-1} μm^{-1} and a starting concentration of 230–300 ng mL^{-1} (Figure 3d).

These data demonstrate that VEGF165 stimulated the migration of ECs in a concentration gradient manner. To gain greater insight into the 3D tubule-like structures, the 1.65 ng mL^{-1} μm^{-1} VEGF165 concentration gradient agarose hydrogels were stained for: ECs with CD31, junctional proteins with β-catenin and vessel wall extracellular matrix with laminin. Fluorescent confocal image stacks along the z-axis of the hydrogels show 3D representations of the vessels (**Figure 4**).

To explore the impact of the VEGF165 concentration on the elongation of the tubular-like structures, a series of agarose hydrogels were synthesized with the same immobilized VEGF165 gradient (of 1.8 ng mL^{-1} μm^{-1}) and different initial VEGF165 concentrations: 566 ng mL^{-1}, and 256 ng mL^{-1} (Figure S4 in the Supporting Information). We observed that ECs did not migrate beyond depths where the VEGF165 concentration was 600–800 ng mL^{-1}, suggesting that the VEGFR2 receptors on endothelial stalk cells may have been saturated and thus they were neither proliferating and nor promoting EC migration. While the Kd of soluble VEGF165 for VEGFR2 is 75-125 pM,[28] our data suggest a significantly higher saturation of 15.7–20.9 μM, indicating that VEGF165 immobilization may affect its binding to VEGFR2.

In summary, we demonstrated that ECs can be guided to form tubule-like structures by a gradient of immobilized VEGF165 in 3D hydrogels. By using coumarin chemistry and a multiphoton patterning technique, maleimide-modified VEGF165 and GRGDS were immobilized within agarose-sulphide hydrogels. The ECs showed stereotypical tip and stalk cell morphology in the agarose gels and exhibited tubule-like formation as they migrated in response to the VEGF165 gradient. To our knowledge, this is the first demonstration of guided EC tubule-like formation in 3D hydrogels. The effect of VEGF165 concentration and concentration gradients on tubule-like structure in 3D environments has not been previously reported. This 3D model of EC growth may be useful for engineered tissues and to study the stem cell niche. Ultimately, the ability to fabricate 3D-patterned scaffolds opens up a broad array of applications in tissue engineering.

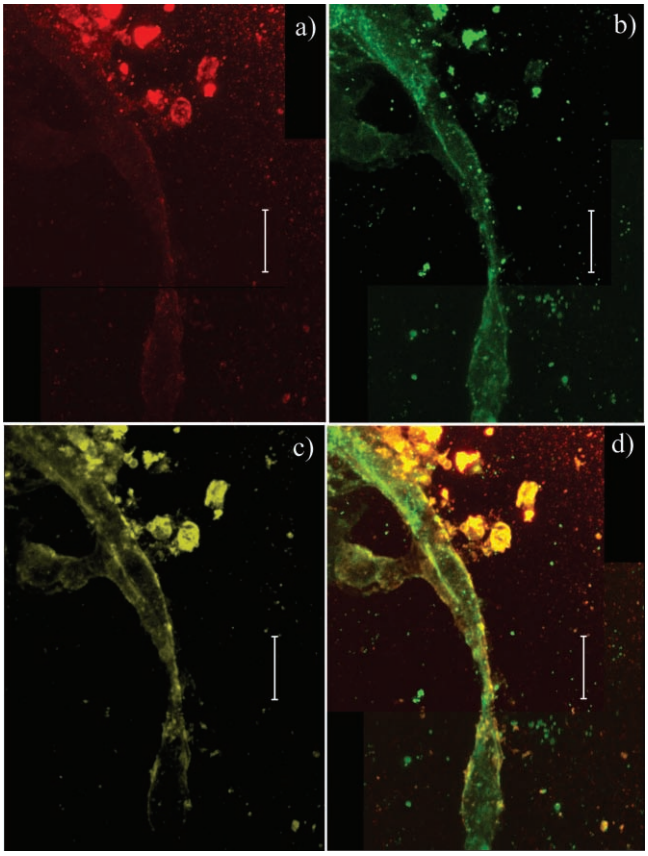

Figure 4. Tubular formations of ECs guided into the 3D GRGDS-agarose hydrogels following a VEGF165 gradient of 1.65 ng mL^{-1} μm^{-1} , having a starting concentration of 316 ng mL^{-1} (as shown in Figure 3c). Confocal images were taken at cross-sectional views of ECs immunostained with primary antibodies: a) CD31 for ECs; b) β–catenin for junctional proteins; c) laminin for extracellular matrix proteins; and d) combined image of (a), (b), and (c). Scale bars (= 20 µm) are parallel to the Z-direction in the gels.

Experimental Section

Immobilization of VEGF165 and GRGDS Peptides on Photolabile Agarose: Agarose was modified with a photolabile thiol protected-6-bromo-7-hydroxycoumarin as described previously.[19] Modification and immobilization of VEGF165 was performed based on the previously described method.[20] Briefly, VEGF165 (100 µL, 500 µg mL^{-1}) was dissolved in 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (50 mM, pH 6; Sigma-Aldrich, Oakville, ON, Canada) including NaCl (400 mM), 1,2-propanediol (10 wt%). 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 50 µL, 44 mM; Sigma-Aldrich) and N-hydroxysulfosuccinimide (sulfo-NHS, 50 µL, 48 mM; Pierce Biotechnology, Rockford, IL, USA). After mixing and reacting the above reagents at room temperature for 15 min, Alexa Fluor 594 hydrazide sodium salt (100 µg) dissolved in DMSO (10 µL, Invitrogen, Carlsbad, CA) and 4-(4-N-maleimidophenyl) butyric acid hydrazide (MPBH, 7 mM, Pierce Biotechnology, Rockford, IL) in sodium acetate buffer (200 µL, 100 mM, pH 5.5) were added to the VEGF165 for 75 min at room temperature. The maleimide- and Alexa Fluor-modified VEGF165 (MI-VEGF165-f) was purified with AKTA-FPLC (Amersham Pharmacia, Piscataway, NJ) using Sephadex G-25 (Sigma-Aldrich) column (10 × 200 mm, Amersham Pharmacia) equilibrated in sodium phosphate buffer (100 mM, pH 7.0) including NaCl (400 mM), propanediol (5 wt%). Conjugation of MI-VEGF165-f to photolabile agarose hydrogel was investigated using coumarin-agarose (100 µL, 0.75 wt%) gelled in a 96-well plate. For covalent immobilization of the MI-VEGF165-f, photolabile agarose solution and MI-VEGF165-f solution in PBS were mixed and irradiated for 2 min with a UV lamp (EFO X-Cite XC60000) after forming the hydrogel. Controls for MI-VEGF165-f adsorption were treated identically without irradiation of photolabile agarose. Non-covalently bound MI-VEGF165-f was removed by washing the hydrogels in PBS for 2 days. Cell-adhesive peptides, maleimide-glycine-arginine-glycine-aspartic acid-serine dissolved in PBS (mi-GRGDS, 100 µL, 0.5 mg mL^{-1}, Ana Spec, San Jose, CA) were added to each well and the entire plate was irradiated for 3 min with a UV lamp to form GRGDS-agarose hydrogels. Unreacted mi-GRGDS was rinsed by successive washing in PBS for 12 h.

Creation of VEGF165 Immobilized Gradient within the Hydrogels: VEGF165 was modified with MPBH and Alexa Fluor 488 hydrazide sodium salt (Invitrogen) as described above. A solution of photolabile agarose (0.3 wt%) containing MI-VEGF165-f solution (8 µg mL^{-1}) was pipetted into a 1 mm glass cuvette (Starna Cells, Inc, Atascadero, CA). After cooling at 4 °C for 40 min to ensure complete gelation, the cuvette was mounted on the stage of a Leica TPS SP2 confocal microscope equipped with a Spectra-Physics Mai Tai broadband Ti-Sapphire laser, tuned to 740 nm.[19] After focusing the laser to a plane in the interior of a gel at low power, the Leica software was used to define a region of interest (300 µm × 300 µm) and the laser power was increased through software controls to the maximum available. A macro program was previously written using Leica software to create concentration gradient patterns of the desired shape and dimension.[19] After patterning, gel samples were immersed in PBS buffer to remove unreacted MI-VEGF165-f for 2 days. mi-GRGDS including tris(2-carboxyethyl) phosphine hydrochloride (Sigma-Aldrich) in PBS (100 µL, 0.5 mg mL^{-1}) was then added to each cuvette and immobilized as described above using confocal laser patterning. Unreacted mi-GRGDS was rinsed by successive washing in PBS for 12 h.

Cell Culture: Brain derived endothelial cell lines (bEnd3, ATCC, Manassas, VA, USA) were routinely grown in DMEM supplemented with fetal bovine serum (FBS, 10% Gibco-Invitrogen, Carlsbad, CA, USA) and penicillin-streptomycin (P/S, 100 µg mL^{-1}, Sigma-Aldrich) in a 25 cm^2 tissue culture flask (VWR, Mississauga, ON) at 37 °C and 5% CO_2. Cell numbers and viability were determined with a haemocytometer and the trypan blue exclusion assay (Sigma-Aldrich) before seeding. Primary mouse brain derived endothelial cells were isolated and cultured in DMEM/F12 medium (Gibco-Invitrogen) containing FBS (10%), horse serum (10%, Gibco-Invitrogen), heparin (100 µg mL^{-1}; Sigma-Aldrich), endothelial cell growth supplement (100 µg mL^{-1}; Sigma-Aldrich) and P/S (100 µg mL^{-1}) in a 24-well plate coated with murine collagen. Experiments were performed on cells at passage 0.

MTT Assay: The MTT Proliferation Assay (Promega, Madison, WI, USA) was performed following the manufacturer's protocol on bEnd3 cells cultured on the hydrogels containing either immobilized VEGF165, physically adsorbed VEGF165, or soluble VEGF165 (n = 3). After 3 days of culture, the labeling mixture (40 µL) was added to each well containing the hydrogel immersed in the media (200 µL). The plate was incubated at 37 °C and 5% CO_2 for 1 h. Then supernatant (100 µL) was added by pipette from each well and put into a new plate. The absorbance was measured at 490 nm using a UV plate reader (Molecular Device, Sunnyvale, CA).

Immunocytochemistry and Imaging: The following primary antibodies were purchased from Abcam (Cambridege, MA) for the immunocytochemical studies: monoclonal rat anti-CD31 (1:10); polyclonal chicken anti-laminin (1:500); and monoclonal rabbit anti-*beta* catenin (1:250). For all immunocytochemical procedures, appropriate controls were obtained by omission of the relevant primary antibody. Cells on the hydrogel were fixed with PBS solution containing PFA (4%) for 1 h and then washed with PBS. After cell membrane permeation and blocking by treating with BSA (1.5 wt%) and Triton X-100 solution (0.2%) at room temperature for 1 h, each specific primary antibody solution described above, was added for 5 h at room temperature. After washing with PBS, samples were exposed to goat Alexa 633 anti-chicken, goat Alexa 488 anti-rabbit IgG (1:200, Invitrogen) and goat Cy3 anti-rat IgG (1:100 Jackson Immunoresearch, West Grove, PA) for 5 h at room

temperature and then washed with PBS. For imaging, fluorescent Z-series image stacks were captured on Zeiss MicroImaging LSM510 microscope using a water-immersion 40× numerical aperture plan Apochromat objective and compiled into three-dimensional rendering with LSM software (University of Toronto, Faculty of Medicine, ON, Canada). Excitation wavelengths were 488 nm (Argon laser), 543 nm and 633 nm (He-Ne laser) for Alexa 488, Cy3, and Alexa 633 respectively. For all EC guidance studies, images of tubule-like structures were photographed using a BX 61-microscope (Olympus) using a 20× objective.

Statistical Analysis: All statistical analyses were performed using JMP IN 5.1 (SAS Institution Inc., Cary, NC). Differences among groups were assessed by one-way ANOVA with the Tukey's *post hoc* analysis to identify statistical differences among three or more treatments. All data are presented as mean ± SD.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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