

# Micron-Scale Spatially Patterned, Covalently Immobilized Vascular Endothelial Growth Factor on Hydrogels Accelerates Endothelial Tubulogenesis and Increases Cellular Angiogenic Responses

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Spontaneous formation of endothelial tubules was restricted to patterned micron-scale regions presenting cell adhesion ligands and angiogenic signaling protein on poly(ethylene glycol) hydrogels. Arginine-glycine-aspartic acid-serine (RGDS), an integrin ligand, and vascular endothelial growth factor (VEGF), a rate-limiting signaling protein involved in angiogenesis, were covalently bound through photopolymerization via laser scanning lithography to the surface of poly(ethylene glycol) hydrogels in patterned micron-scale regions. Endothelial cells cultured in this restricted environment underwent accelerated tubulogenesis, forming endothelial tubes within 2 days, whereas cells cultured on larger patterned areas remained spread and did not form tubules by day 2. Tubules formed in 2 days on RGDS and VEGF patterns were observed to possess lumens; however, tubule-like structures formed in 2 days on RGDS-only control patterns did not have observable lumens. Additionally, tubules that formed on restricted areas of RGDS and VEGF expressed more VEGF receptor 1, VEGF receptor 2, and ephA7 surface markers, in addition to higher expression of laminin, than cells remaining spread on wide patterned lines. This work reports spatial control and acceleration of endothelial tubule formation using biocompatible hydrogel materials to allow the formation of highly organized vascularized tissues.

## Introduction

**T**HE NATURAL PROCESS of angiogenesis relies on a complex system of biochemical signals and cell-cell/cell-matrix interactions. *In vivo*, capillaries form self-patterned networks to allow transport of nutrients, oxygen, and waste throughout tissue, connecting arterial and venous vasculature to ensure nutrient and oxygen transport via convection. Patterning occurs when endothelial cells respond to sequestered growth factors to form preliminary endothelial tubes, which are later reorganized via tubule regression and stabilization to meet the nutrient and oxygen requirements of the tissue. Initiation of angiogenesis occurs through an endothelial cell response to signaling factors such as vascular endothelial growth factor (VEGF). *In vivo*, endothelial cells respond to VEGF by forming preliminary endothelial tubules, which are later stabilized by supportive mural cells to form capillaries.<sup>1</sup> In the body, expression of VEGF is induced by low oxygen tension, as mediated through hypoxia-inducible factor-1.<sup>2</sup> Secreted VEGF diffuses from hypoxic cells to existing capillaries,<sup>3</sup> where it promotes endothelial permeability, mitosis, and angiogenesis.<sup>4</sup>

To promote patterned angiogenesis *in vitro* for tissue engineering applications, the combination of cells, scaffold material, adhesive peptides, angiogenic signals, and laser scanning lithography (LSL) patterning technique was used. The matrix, poly(ethylene glycol) diacrylate (PEGDA), is a biocompatible hydrophilic polymer that can be crosslinked to form hydrogels used for soft tissue scaffolds and implant encapsulation.<sup>5,6</sup> Crosslinking of polymer chains can be accomplished via photopolymerization.<sup>5,7,8</sup> PEGDA hydrophilicity acts to resist protein adsorption, preventing nonspecific cell adhesion, and thus acts as a blank slate that can be tailored to guide complex tissue organization.<sup>5</sup> A recent advancement, LSL, overcomes scale and reproducibility challenges often encountered in photolithography and soft lithography.<sup>8</sup> By using the precision of a confocal microscope, in which a laser is tightly focused onto a specimen and scanned with sub-micron precision, exposure to light and subsequent photopolymerization can be controlled spatially. The current studies explore the response of endothelial cells to patterned ligand and growth factor protein on the surface of biocompatible PEG hydrogels.

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Dike *et al.* originally elucidated the importance of spatially restricting presentation of adhesion molecules in promoting angiogenesis. In Dike's work, gold-coated glass coverslips were coated with a saturated density of fibronectin via microcontact printing of hexadecanethiol, followed by self-assembling monolayer formation of tri(ethylene glycol)-terminated alkanethiol and then fibronectin. Bovine capillary endothelial cells were attached to patterned fibronectin lines of either 10 or 30  $\mu\text{m}$  width and cultured in media saturated with basic fibroblast growth factor (FGF). Cells on 30  $\mu\text{m}$ -wide lines remained spread and flattened, whereas cells on 10  $\mu\text{m}$ -wide lines organized into linear cellular cords that appeared to possess a centralized hollow space, as observed by confocal microscopy.<sup>9</sup> Underlying tendrils of laminin and fibronectin were observed. After several days, a single, continuous, central lumen extended over several cell lengths, and single-cell bodies stretched around a negatively labeled lumen. Cells were observed to be partially retracted and elevated in height from the surface. PECAM staining showed strong cell-cell attachment and continuous linear presence, including at cell junctions.<sup>9</sup> Since geometric restriction of ligand presentation affects tubulogenesis, this phenomenon could be used to improve microvascularization of tissue-engineered constructs by tightly controlling ligand presentation in tissue engineering scaffold materials.

In this work, angiogenic cues were localized precisely on the surface of PEG hydrogels by using LSL. Patterning the presentation of biochemical signals to be highly localized allows control of the geometry of the capillary bed and also should discourage uncontrolled angiogenesis in undesired locations. The presented use of matrix-bound VEGF<sub>165</sub> in patterned locations within biocompatible polymer hydrogels is hypothesized to promote endothelial tubulogenesis, thereby leading to highly organized vessel formation. While restriction of cell-material interactions could be used to direct microvascular formation and anastomosis on the surface of biomaterials, two-dimensional patterning also acts as a foundation for future studies in three dimensions. This work introduces a system that promotes and spatially controls the formation of endothelial tubules by patterning the localization of integrin ligands and growth factors on the surface of PEG hydrogels.

## Materials and Methods

### Cell culture

Human umbilical vein endothelial cells (HUVEC; Cambrex/Lonza) were maintained in VEGF-free endothelial cell growth medium-2 (Cambrex/Lonza) containing human FGF-B, human epidermal growth factor, insulin-like growth factor (R<sup>3</sup>-IGF-1), heparin, hydrocortisone, ascorbic acid, GA-1000 (gentamicin, amphotericin-B), 2% fetal bovine serum (Bullectkit, Lonza), 1 U/mL penicillin, 1  $\mu\text{g}/\text{mL}$  streptomycin, and 2 mM L-glutamine (Sigma). Cells were maintained at 37°C in a 5% CO<sub>2</sub> environment and used between passages 3 and 6.

### Preparation and purification of PEGDA

PEGDA was prepared by reacting PEG (Fluka/Sigma; MW = 6000 Da) with acryloyl chloride (Sigma) in a 1:4 molar ratio in anhydrous dichloromethane (DCM; Sigma)

containing triethyl amine (Sigma) at a 1:2 (PEG:triethyl amine) molar ratio at 25°C under argon overnight. PEGDA underwent purification by phase separation using 2 M K<sub>2</sub>CO<sub>3</sub>. The organic phase, which contained PEGDA, was dried using anhydrous MgSO<sub>4</sub> and filtered. PEGDA was first precipitated in diethyl ether, then filtered, and dried overnight and under vacuum. <sup>1</sup>Hydrogen Nuclear Magnetic Resonance (<sup>1</sup>H-NMR) characterization of PEGDA was used to confirm acrylation (Avance 400 Hz; Bruker) with D<sub>2</sub>O as a solvent. PEGDA was stored under argon at -20°C.

### Preparation and purification of acryl-PEG-succinimidyl carbonate

Acryl-PEG-succinimidyl carbonate (PEG-SMC) was synthesized by reacting PEG (Fluka/Sigma; MW = 3400 Da) with 1.5 molar excess Ag<sub>2</sub>O (Sigma), 1.1 molar excess acryloyl chloride (Sigma), and 0.3 molar ratio KI (Sigma) in anhydrous dichloromethane (DCM; Sigma) overnight at 0°C-4°C to produce monoacrylated PEG. Silver oxide was removed from the resulting solution via filtration using Celite 521 (Spectrum Chemical Manufacturing Corp). The product was dried via Rotovap, then dissolved in deionized water (DI H<sub>2</sub>O), and the pH was titrated to 3 with HCl. This solution was heated at 35°C for 1 h, and activated charcoal (Fisher) was added for iodine removal, followed by subsequent filtration using Celite 521. Sodium chloride (NaCl) in DCM was added, followed by DCM extraction. Hydrochloric acid and chloride ions were removed through phase separation utilizing 2 M K<sub>2</sub>CO<sub>3</sub>. PEG-monoacrylate was dried with sodium sulfate (Fisher), concentrated via Rotovap, precipitated in ethyl ether, and vacuum filtered. Acryl-PEG-SMC was produced by reaction of monoacrylated PEG with four molar excess disuccinimidyl carbonate (Sigma) in pyridine (Sigma) and anhydrous acetonitrile (Sigma) under argon overnight. Acryl-PEG-SMC was dried via Rotovap, dissolved in anhydrous DCM, and filtered. The product was recovered via phase separation in acetate buffer (0.1 M, pH 4.5, 15% NaCl), dried using anhydrous MgSO<sub>4</sub>, filtered, precipitated in ethyl ether, filtered, and dried overnight and under vacuum. Acryl-PEG-SMC was characterized by MALDI-TOF (MS Autoflex) to determine molecular weight and <sup>1</sup>Hydrogen Nuclear Magnetic Resonance and stored at -80°C under argon.

### Preparation and purification of PEG-Arg-Gly-Asp-Ser

The cell-adhesive peptide Arg-Gly-Asp-Ser (RGDS) (American Peptide) was dissolved in dimethylsulfoxide (Sigma) at a concentration of 35 mM, and diisopropylethylamine (Sigma) was added in a 2:1 molar ratio (diisopropylethylamine: PEG-succinimidyl carboxymethyl [SCM, Laysan Bio, Inc.]). Acryloyl-PEG-SMC was added at a concentration of 30 mM in a 1:1.2 (PEG-SCM:RGDS) molar ratio and allowed to react for 24 h at 25°C under agitation. The product was dialyzed against DI H<sub>2</sub>O for 8 h using a dialysis membrane with a 3500 Da molecular weight cutoff (Spectrum Laboratories). PEG-RGDS was lyophilized and stored under argon at -80°C. Conjugation was determined by gel permeation chromatography using 0.1% ammonium acetate in dimethylformamide, a PLgel column (5  $\mu\text{m}$ , 500 Å; Polymer Laboratories), and evaporative light scattering detector (Polymer Laboratories), run against a PEG-SCM standard.

### *Synthesis of PEG-VEGF*

VEGF<sub>165</sub> (Sigma) was dissolved in sterile 50 mM sodium bicarbonate buffer (pH 8.5, on ice). Synthesized acryloyl-PEG-SMC was similarly dissolved and sterilized via filtration (0.2  $\mu$ m). PEG-SMC was added to VEGF in a 200:1 molar ratio under sterile conditions and allowed to react with agitation for 4 days at 4°C. PEG-VEGF was then lyophilized under sterile conditions, dissolved in HEPES buffered saline (100 mM NaCl, 10 mM HEPES in DI H<sub>2</sub>O; pH 7.4) with 0.1% bovine serum albumin (BSA), and stored at 4°C. PEG conjugation was confirmed via Western blot on a Tris-HCl precast polyacrylamide gel (Bio-rad), detected with rabbit polyclonal anti-VEGF primary antibody (Santa Cruz Biotechnology), HRP-conjugated goat anti-rabbit IgG secondary antibody (MP Biomedicals), and ECL<sup>TM</sup> chemiluminescent Western blot analysis system (GE Healthcare). The membrane was exposed to film (Kodak), which was developed using a Micromax Developer (Hope) with T<sub>2</sub> developer and T<sub>2</sub> fixer (White Mountain Imaging).

### *Synthesis of PEG-RGDS-Fluor 350*

PEG-RGDS was dissolved in 50 mM sodium bicarbonate buffer (pH 8.5). Alexafluor 350 carboxylic acid, succinimidyl ester (Invitrogen) was dissolved in dimethylformamide (0.5 mg/mL). Alexafluor solution was added dropwise to PEG-RGDS in a 10:1 molar ratio with slow mixing and allowed to react overnight at 25°C. The product was dialyzed for 8 h using a dialysis membrane with a 3.5 kDa molecular weight cutoff (Spectra/Por). PEG-RGDS-Fluor350 was lyophilized and stored at -80°C under argon.

### *Formation of PEGDA hydrogels*

PEGDA (6 kDa) was dissolved at 10% w/v in HEPES buffered saline and sterilized via filtration (0.2  $\mu$ m). Ten microliters of 300 mg/mL 2,2-dimethoxy-2-phenylacetophenone in N-vinylpyrrolidone was added to each milliliter of polymer solution. Molds were constructed by placing 0.75 mm poly(tetra fluoroethylene) spacers between glass slides which were secured with binder clips, sealing the molds on three sides. Polymer solution was pipetted into the molds and crosslinked through exposure to long wavelength ultraviolet light (B-200SP UV lamp, UVP, 365 nm, 10 mW/cm<sup>2</sup>) for 30 s. After crosslinking, the PEGDA hydrogel slab was stored in sterile phosphate buffered saline (PBS) with 0.1% sodium azide for 24 h to allow swelling.

### *LSL patterning of ligand surface immobilization*

Five-millimeter-diameter disks were cut from PEGDA hydrogel slabs. Hydrogel disks were soaked for 1 h in sterile PBS to remove sodium azide. A polymer solution with 30  $\mu$ mol/mL PEG-RGDS, 420 pmol/mL PEG-VEGF, 1  $\mu$ mol/mL eosin Y, 1.5% triethanolamine, and 3.95  $\mu$ L/mL N-vinylpyrrolidone was prepared. Control solutions were made by omitting PEG-VEGF. Ten microliters of this solution was pipetted, as a drop, onto a #1 cover slip with attached chamber slide, and a hydrogel was placed on top of the drop, allowing the entire bottom surface of the gel to be in contact with the polymer solution. The gel and polymer solution were exposed to 514 or 532 nm laser excitation in specific locations using LSL. The surface-modified gel was again

soaked in sterile PBS for 1 day to allow nonreacted polymer, excess photoinitiator, and residual sodium azide to diffuse out of the hydrogel (Fig. 1).

This method allows immobilization of the PEG-monoacrylate derivatives in the polymer/photoinitiator solution at defined sites on the hydrogel surface. Specifically, a Zeiss LSM 510 or 5Live confocal microscope (Plan-Apochromat objective 10 $\times$ , 0.45 NA, area: 512 $\times$ 512 pixel, scan speed: 51.2  $\mu$ s/pixel, iterations: 10; zoom: 1, laser power: 0.26 mW/ $\mu$ m<sup>2</sup>) was focused onto the hydrogel interface and liquid polymer solution. The laser was then raster scanned across the sample in a predetermined geometry, as programmed using Zeiss software region-of-interest choices. Several patterns of varying dimensions were patterned onto each sample, providing both experimental and control groups on the same hydrogel. To compare the effects of restricted cell spreading, patterned lines of 10  $\mu$ m width by 1000  $\mu$ m length or 100  $\mu$ m width by 1000  $\mu$ m length were created side by side on the same hydrogel surface. This allows the experimental group (thin lines) and the control group (wide lines) to be on the same hydrogel sample. To determine feasibility of patterning capillary networks, patterns of branching 10  $\mu$ m lines were created on the surface of hydrogels.

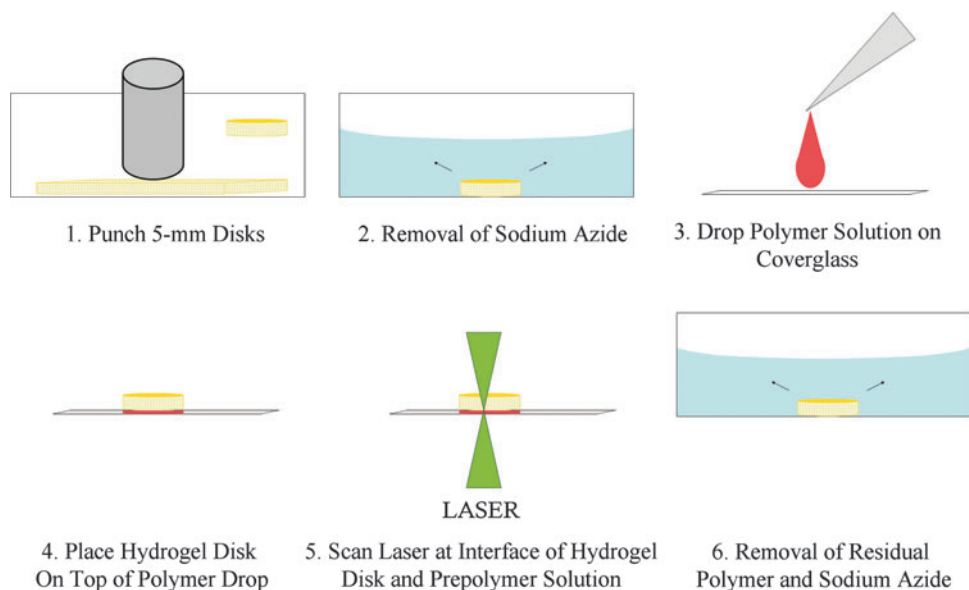
### *Quantification of surface-immobilized VEGF and RGDS*

PEG-VEGF and PEG-RGDS were immobilized via LSL to restricted 10  $\mu$ m $\times$ 1 mm or 100  $\mu$ m $\times$ 1 mm areas on 6 kDa hydrogels as described above. An ELISA was used to determine the amount of covalently attached VEGF. Briefly, gels were modified with PEG-VEGF and PEG-RGDS and allowed to soak in saline with 0.1% BSA overnight. The amount of unbound PEG-VEGF remaining in the saline was quantified using a VEGF ELISA kit (R&D) against dilutions of initial PEG-VEGF solution initially added to hydrogels. To quantify the amount of RGDS on the hydrogel surface, fluorescently labeled PEG-RGDS-Fluor350 was patterned to the hydrogels. Hydrogels were soaked for 2 days, and the amount of unbound fluorescently labeled PEG-RGDS was quantified using a fluorescent plate reader (Molecular Devices SpectraMax M2, excitation: 345 nm, emission: 446 nm) with fluorescently labeled PEG-RGDS standards created from the PEG-RGDS solution that was initially added to the hydrogels, so that fluorescence could be compared without bias due to photobleaching. The amounts of PEG-VEGF and PEG-RGDS conjugated to patterned areas were then calculated.

### *Endothelial tubule formation and quantification of angiogenic markers*

HUVECs were seeded (8.5 $\times$ 10<sup>4</sup> cells/cm<sup>2</sup>) onto hydrogels with PEG-RGDS and PEG-VEGF or PEG-RGDS only covalently attached to the surface in restricted areas of 10 $\times$ 1000  $\mu$ m or 100 $\times$ 1000  $\mu$ m. On day 2, images were taken of each patterned area and merged with Adobe Photoshop software. Each pattern was analyzed for tubule formation and measured for final dimensions of the area of cell attachment to the pattern. Due to inherent variability in focusing the confocal laser onto the interface of the hydrogel and polymer solution, some patterns were larger than designed, due to the

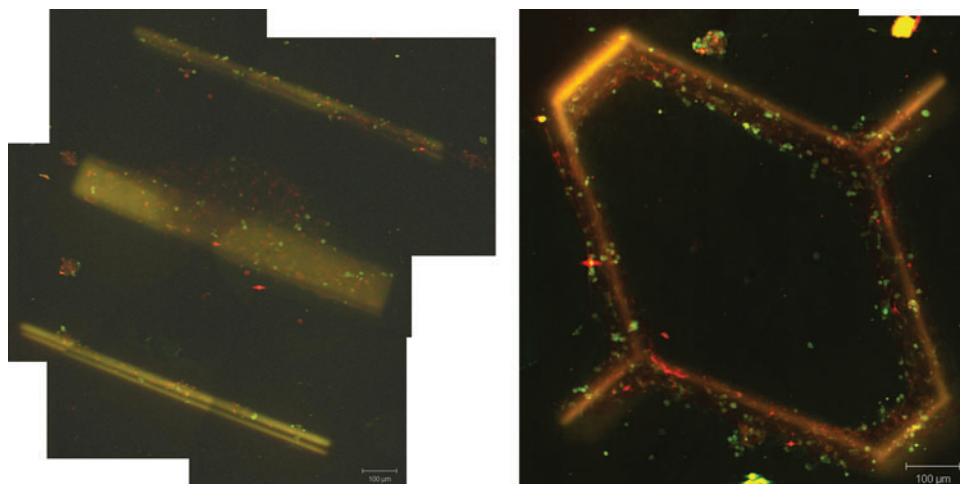
**FIG. 1.** Schematic of patterned surface modification process of PEG hydrogels. Hydrogel disks were placed on top of surface-modifying polymer solution. A laser was focused onto the interface of the hydrogel disk and polymer solution by utilizing confocal microscopy. The laser was scanned across user-defined regions of interest to control photopolymerization and subsequent surface modification to restricted areas. PEG, poly(ethylene glycol).



increased laser beam width on specimen areas that were not in exact focus. An analysis of variance was performed to determine significant differences between groups (RGDS, RGDS and VEGF, separated into bins depending on final dimension, with Tukey's Least Significant Difference *post-hoc* analysis,  $p < 0.05$  considered significant). After imaging on day 2, cells cultured on top of hydrogels were fixed with 4% formaldehyde, washed with PBS, blocked with 10% normal donkey serum (Sigma) for 1 h, incubated for 1 h with primary antibodies (two groups—group A: mouse anti-VEGF receptor 1 [VEGFR1] [Santa Cruz Biotechnology], goat anti-VEGFR2/Flk1 [Santa Cruz Biotechnology], and rabbit anti-epha7 receptor [Santa Cruz Biotechnology]; group B: mouse anti-fibronectin [Sigma], rabbit anti-laminin [Sigma], and goat anti-PECAM [Santa Cruz Biotechnology]; diluted 1:50 in PBS with 1% BSA). Samples were washed several times with PBS and incubated for 1 h with the following secondary antibodies: Alexafluor 555 donkey anti-rabbit, Alexafluor 647 donkey anti-mouse, and Alexafluor 488 donkey anti-goat (dilution 1:200; Invitrogen, Molecular Probes); then, they

were washed several times with PBS. Other samples were permeabilized with 0.1% Tween-20 for 30 min, blocked with BSA for 30 min, then treated with Alexafluor 568-conjugated phalloidin (10 U/mL; Molecular Probes) and 4'-6-diamidino-2-phenylindole (DAPI) (2  $\mu$ M; Invitrogen) for 45 min to label cell actin filaments and nuclei, and observed using confocal microscopy (Zeiss Live5, Plan-Apochromat 20 $\times$  objective with 0.8 numerical aperture, Plan-Neofluar oil-immersion 40 $\times$  objective with 1.3 numerical aperture, Plan-Apochromat oil-immersion 63 $\times$  with 1.4 numerical aperture, for Alexafluor 488: excitation = 489 nm, emission BP filter = 500–525 nm; for Alexafluor 555: excitation = 532 nm, emission BP filter = 560–675 nm, pinhole = 33  $\mu$ m; for Alexafluor 647, excitation = 635 nm, emission longpass filter = 650, pinhole = 100  $\mu$ m; for phalloidin: excitation = 532 nm, emission BP filter = 560–675 nm; for DAPI: excitation = 405 nm, emission BP filter = 415–480 nm, pinhole = 0.5  $\mu$ m). Images of tubules stained with phalloidin/DAPI were captured via z-stack images, which were then processed into three-dimensional projections using ImageJ Volume Viewer software. Fluorescent

**FIG. 2.** Confocal images of LSL patterns with attached and fluorescently labeled cells. LSL patterns were used to evaluate the effects of restricted spreading as well as to control branching capillary patterns. LSL patterns are fluorescent due to incorporation of fluorescent eosin Y, used as the photoinitiator for the polymerization cross-linking reaction between the PEG diacrylate hydrogel base and the PEG-Arg-Gly-Asp-Ser (PEG-RGDS) and PEG-VEGF modifying the surface. Scale bar = 100  $\mu$ m. LSL, laser scanning lithography; VEGF, vascular endothelial growth factor.



immunocytochemistry images were processed with ImageJ to quantify expression of markers by measuring pixel intensity per cell. ImageJ returned intensity data in histogram format. Threshold levels were determined for each image by measuring the intensity of any background fluorescence. Data points were processed by weighting the number of pixels at each intensity level by the value of the intensity (range: 0–256). Data from each experiment were normalized to the average intensity measured in the control group (wide lines), thereby allowing comparison without bias due to photobleaching. Normalized data from several experiments were pooled into one data set per marker analyzed. Significance was determined using Student's *t*-test within each marker group, with  $p < 0.05$  considered significant.

## Results

### Polymer characterization

Polymer products were determined to be successfully synthesized. Gel permeation chromatography results confirmed that the PEG-RGDS product increased in molecular weight relative to the initial PEG-SCM reactant, showing successful conjugation. Western blot results showed that PEG-VEGF had a higher molecular weight than native VEGF, thereby confirming successful pegylation of VEGF.<sup>10</sup>

### Photolithographic patterning of ligand surface immobilization

PEG-RGDS and PEG-VEGF were patterned successfully on the surface of 6 kDa PEGDA hydrogels. Eosin Y, present in patterned areas, was observed via confocal microscopy, which suggested that different patterns from 10 to 100  $\mu\text{m}$  in width and 1000  $\mu\text{m}$  in length were produced (Fig. 2). PEG-RGDS was covalently attached to patterned regions of all PEGDA hydrogels at a concentration of  $74 \pm 21 \mu\text{mol}/\text{cm}^2$ . PEG-VEGF was covalently attached to patterned regions at a surface concentration of  $1.18 \pm 0.3 \text{ nmol}/\text{cm}^2$ . Previous work showed that higher levels of RGDS, as compared to full-surface modification levels, are required for cells to successfully attach to thin lines.<sup>11,12</sup> Additionally, the initial amounts of PEG-RGDS and PEG-VEGF in the polymer solution were equal to those used in previous work,<sup>10</sup> and higher final concentrations are due to the different laser used for photopolymerization.

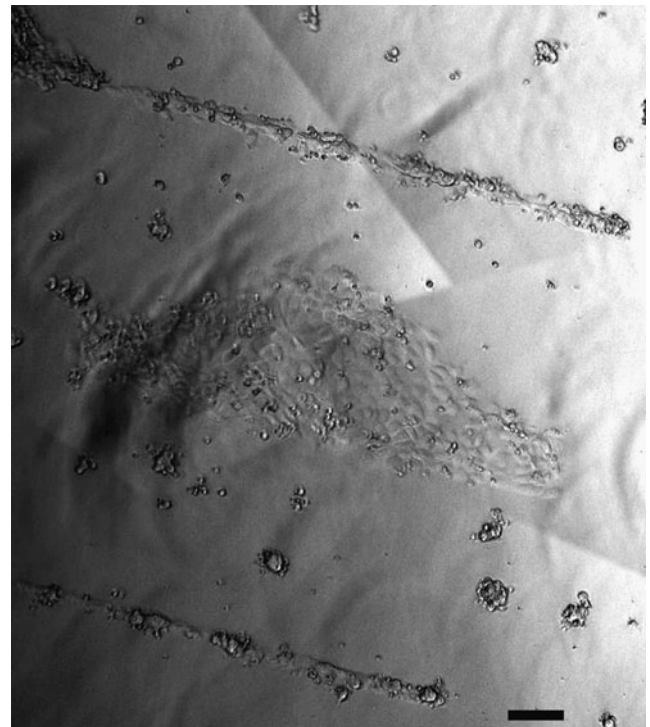
### Endothelial response to patterned PEG-RGDS and PEG-VEGF

Presented results of patterning PEG-VEGF and PEG-RGDS on the surface of PEGDA hydrogels, suitable for tissue engineering matrices, are in agreement with work performed on glass cover slips by Dike *et al.*<sup>9</sup> Using LSL, lines of  $\sim 10$  and  $\sim 100 \mu\text{m}$  of covalently bound PEG-RGDS and PEG-VEGF were patterned on the same hydrogel surface. Two days after seeding, HUVECs formed tube-like structures on thin lines of RGDS and VEGF but remained spread on wide lines (Fig. 3). Additionally, tubule formation occurred on thin lines at a significantly higher proportion than on wide lines, where it was virtually nonexistent. Further, tubule formation occurred on RGDS and VEGF thin lines at a significantly higher proportion than on RGDS-only thin lines (width

of cell attachment  $< 70 \mu\text{m}$ ) (Fig. 4). Cross-sectional images of phalloidin-labeled tubules on 10- $\mu\text{m}$ -width patterned lines of RGDS and VEGF show negatively labeled lumens along these tubules, suggesting patent lumens. Patent lumens were not observed on the phalloidin and DAPI-labeled RGDS-only control patterns of 10  $\mu\text{m}$  lines (Fig. 5). These results suggest that lumen formation may be accelerated by the presence of PEG-VEGF on the surface of the hydrogel; however, even thinner patterns of RGDS-only modification should be studied to confirm this hypothesis.

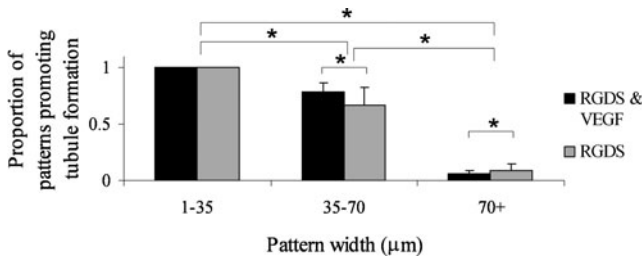
### Cell-surface markers and protein expression

After 2 days, cells cultured on restricted patterns of PEG-RGDS and PEG-VEGF were observed for expression of VEGFR1, VEGFR2, ephA7, PECAM, fibronectin, and laminin. Expression of angiogenic markers at 2 days after seeding was observed via confocal microscopy, which suggested higher expression in cells forming tubules on thin lines (Fig. 6), and quantification confirmed a statistically significant increase in angiogenic marker expression on tubules formed on thin lines. Quantification of pixel intensity per cell shows that restricted spreading and subsequent tubule formation of HUVECs on thin lines corresponds to increased levels of expression of ephA7 by 1.4-fold, VEGFR1 by 12.3-fold, VEGFR2 by 1.5-fold, and laminin by 2.4-fold as compared to cells spread on wide lines (Fig. 7,  $p < 0.05$ ). Recent research has elucidated the role of cell-surface Eph receptors and their



**FIG. 3.** Phase-contrast image of patterned endothelial cells. Cells patterned on  $\sim 10 \mu\text{m}$  lines of RGDS and VEGF formed capillary-like structures by day 2, whereas those allowed to spread to  $\sim 100 \mu\text{m}$  remained in a cobblestone morphology. Scale bar = 100  $\mu\text{m}$ .





**FIG. 4.** Quantification of the effect of pattern size on tubule formation. All patterned lines  $<35\mu\text{m}$  promoted tubule formation. As the pattern size increases, the percentage of patterns that promote tubule formation decreases. For patterns between 35 and  $70\mu\text{m}$ , patterns with RGDS and VEGF promote more tubule formation than RGDS only. Error bars show standard deviation ( $\text{SD} = \text{square root}(pq/n)$ , where  $p$  = proportion of success,  $q$  = proportion of failure, and  $n$  = sample size). Statistical differences ( $*p < 0.01$ ) between all groups except groups showing 100% tubule formation (RGDS, RGDS and VEGF  $1\text{--}35\mu\text{m}$ ).

cell-surface ephrin ligands in patterning the localization of arterial and venous vasculature.<sup>13</sup> Because the Eph/ephrin system is instrumental in capillary remodeling, expression of these cell-surface receptors is also of interest when working toward a functional microvasculature. The accelerated formation of endothelial tubes on thin lines may be related to these higher levels of angiogenic marker expression, allowing cells to react to angiogenic signaling sequestered in the matrix. The ability to pattern PEG hydrogels allows control

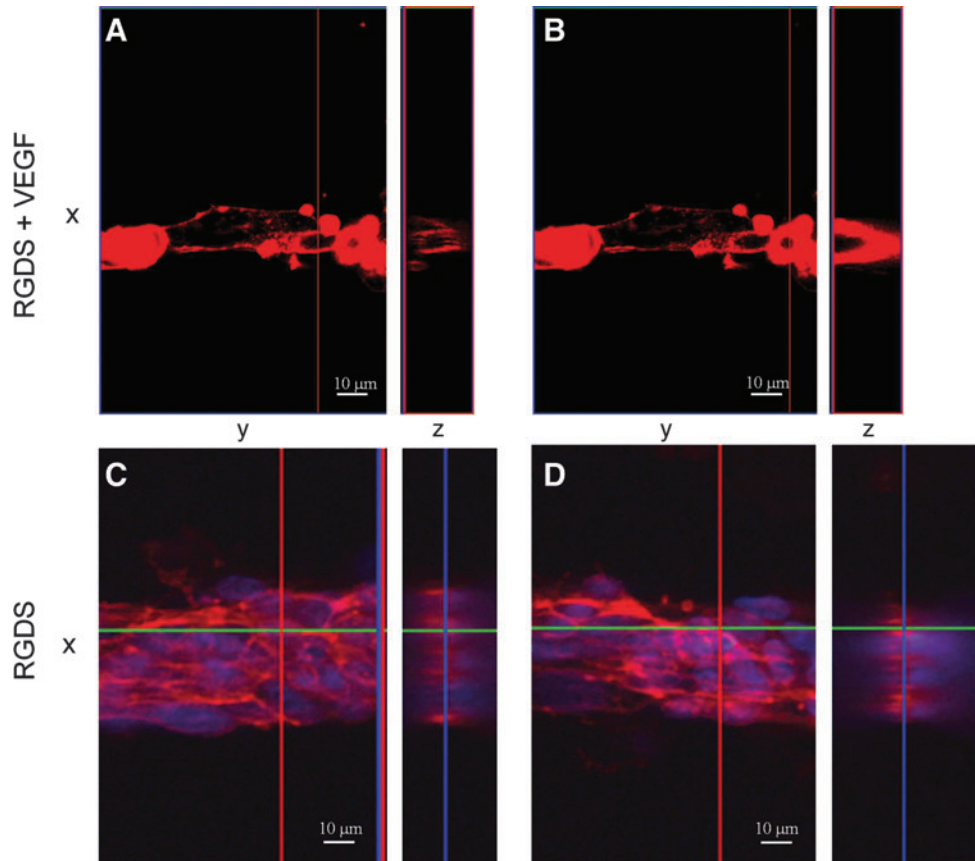
of tissue organization, whereas patterned presentation of ligands contributes to activation of angiogenic pathways. Spatial restriction of angiogenic factors and integrin ligands on PEG hydrogels accelerates and tightly controls the angiogenic response in these scaffolds.

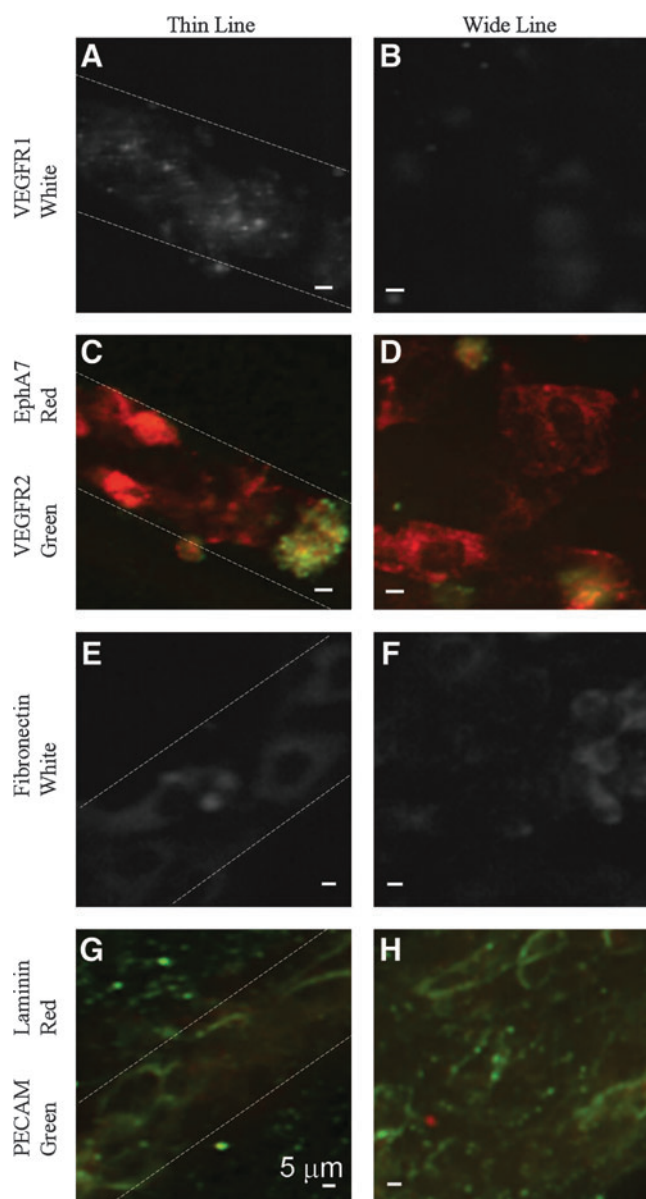
## Discussion

The need for organ transplants has risen steadily in the past decade.<sup>14</sup> Tissue engineering of functional tissues and organs could meet demands of quantity and increase quality of tissue transplantation by incorporating techniques to reduce tissue rejection. A major limitation in tissue engineering is providing microvascular networks throughout engineered tissue for transport of nutrients, oxygen, and waste. One proposed way of promoting angiogenesis is through the presentation of appropriate ligands in matrix materials.

VEGF was used in this system as signaling by VEGF is considered a rate-limiting step in the initiation of angiogenesis.<sup>2</sup> VEGF binds to two receptors, fms-like tyrosine kinase (Flt-1 or VEGFR-1) and kinase domain receptor or VEGFR-2.<sup>15</sup> VEGFR-2 is the main receptor for VEGF, and signal initiation for survival, mitogenesis, chemotaxis, capillary morphogenesis, and vessel permeability occurs via this receptor. VEGFR-1 has been suggested to act as a decoy receptor, which would remove VEGF as a ligand for VEGFR-2.<sup>2</sup> However, this interaction is thought to modulate more subtly the levels and spatial localization of VEGF required for proper vessel sprouting and branching, and indeed loss of VEGFR-1 results in defective angiogenic sprouting.<sup>16,17</sup>

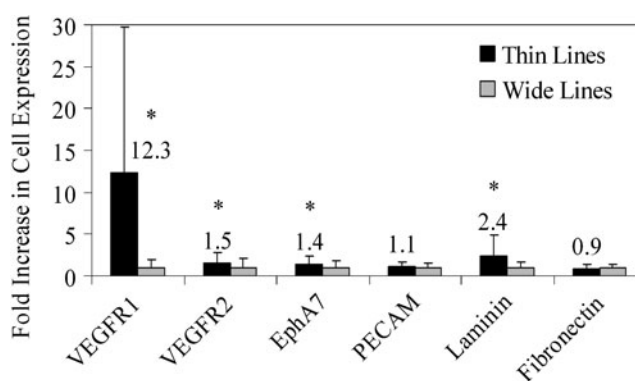
**FIG. 5.** Top: Observation of actin filaments (red) in tubule formed on patterned RGDS and VEGF. (A) Top view and cross section of tubule shows lumen. (B) Top view and cross section of cell-forming vacuole. Bottom: Observation of actin filaments (red) and 4'-6-diamidino-2-phenylindole (DAPI) (blue) in tubules formed on patterned RGDS only. (C, D) Cells cultured on only RGDS were not observed to form tubules with continuous lumens. Scale bar =  $10\mu\text{m}$ . Red lines shows region of cross section.





**FIG. 6.** Confocal images of fluorescent immunocytochemistry. Cells on both thin and wide patterned lines of PEG-RGDS and PEG-VEGF on the same sample were fixed at day 2 and stained for (A, B) VEGFR1 (white), (C, D) VEGFR2 (green) and ephA7 (red), or (E, F) fibronectin (white), (G, H) laminin (red), and PECAM (green). Dotted lines indicate area of thin line. Images of wide lines show cells within the patterned region, with unpatterned regions not shown. Background signals arise from incorporated eosin Y, which was used as the photoinitiator; the background signal was subtracted before quantification. VEGFR, VEGF receptor.

As in most physiological processes, angiogenesis must be tightly regulated to avoid pathophysiology. In the current study, angiogenic signaling is controlled and limited to distinct regions by covalently binding a growth factor to the matrix. Zisch *et al.* showed an angiogenic response due to signaling from pegylated VEGF, which was cleaved by cellular enzymes from a PEG matrix.<sup>18</sup> Further work has shown that covalently bound VEGF elicits an angiogenic response



**FIG. 7.** Analysis of protein expression of six angiogenic proteins and cell surface receptors expressed by endothelial cells on patterned lines of PEG-RGDS and PEG-VEGF at day 2. Fluorescent immunocytochemistry images were analyzed for intensity of pixels in images to quantify expression of angiogenic cell surface receptors and proteins. Increased expression was determined for VEGFR1, VEGFR2, ephA7, and Laminin. \* $p < 0.05$  using Student's *t*-test comparison between thin and wide lines within each marker category.

from endothelial cells cultured on PEGDA hydrogels and that PEG-matrix dissociation is not necessary for this response.<sup>10</sup>

The work presented in this article demonstrates the ability to pattern PEG hydrogels that allows control of tissue organization, whereas patterned presentation of ligands contributes to activation of angiogenic pathways. Spatial restriction of angiogenic factors and integrin ligands, in this case VEGF and RGDS, on PEG hydrogels accelerates and tightly controls the angiogenic response in these scaffolds. The reported work illustrates that patterning localization of integrin ligands and signaling factors not only allows a more engineered, highly organized design for a vascularized engineered tissue, but also actively accelerates tubulogenesis and upregulates angiogenic genes within endothelial cells.

Certainly, many factors contribute to endothelial tubule formation on patterned substrates. Dike *et al.* suggested that because binding of integrins stimulates chemical signaling in angiogenesis, cytoskeletal tension-dependent changes in cell shape may have a role in determining cell fate. Substrate-immobilized fibronectin promotes integrin clustering and activates integrin signaling pathways in capillary cells. They suggested that by restricting spreading, spatial restriction of extracellular matrix ligands promoted cell-to-cell contact and thus promoted tube formation. Additionally, they hypothesized that cell-generated traction forces causing partial retraction of the tendril away from the surface and matrix remodeling into thread-like configuration could be required in tube development.<sup>9</sup> The current work is in agreement, showing that restricted spreading of integrin ligands and growth factors promotes endothelial tube formation. Additionally, partial retraction of endothelial tubules was observed in the PEG hydrogel model as well.

Further, localization of growth factors in the extracellular matrix has been suggested to guide vessel formation.<sup>1,19</sup> For example, *in vivo*, FGF-2 accumulates at areas of capillary branching<sup>20</sup> and may play a role in patterning capillary geometry. In addition, localization of VEGF has been shown

to affect cell function, as cleaved, soluble VEGF<sub>165</sub> induces endothelial cell proliferation, whereas matrix-bound VEGF<sub>165</sub> promotes vascular sprouting and branching.<sup>21,22</sup> *In vivo*, proteoglycans containing heparin sulfate act to bind and concentrate certain growth factors, such as basic FGF, in the extracellular matrix as well as near cell surfaces.<sup>19</sup> Localization of growth factors and cell-adhesive ligands can similarly be achieved in PEG-based hydrogels by covalently crosslinking these substrates to the hydrogel via photopolymerization.<sup>6,23</sup> Because PEG hydrogels can be cross-linked and modified via chemical reactions initiated by light, localization of hydrogel components can be precisely patterned. Moon *et al.* showed that spatial restriction of PEG-RGDS in 50- $\mu$ m-width lines on PEGDA guided endothelial tubule formation by 18 days as compared to 200- $\mu$ m-width lines, which supported cell spreading but not tubulogenesis.<sup>11</sup> In previous work, branching networks of endothelial tubes formed within 30 days on hydrogels modified with PEG-VEGF and PEG-RGDS when no spatial restriction of attachment and signaling was applied. Significantly less tubulogenesis occurred on hydrogels modified with PEG-RGDS only.<sup>10</sup> Patterning the presentation of cell adhesive ligands and signaling factors allows geometric restriction of cell location and function in a tissue engineered construct.

In the LSL patterning process, because the scanning laser is under the control of computer software, it is facile to restrict the laser excitation to particular areas of the specimen, as is frequently used in fluorescence-bleaching experiments. The user-defined region of interest is drawn with exact coordinates using the software, allowing precisely calculated areas of excitation. The spatial area of laser scanning, power of the laser, laser scan speed, and number of iterations of scanning can be controlled via software which runs the microscope assembly. Additionally, because this method does not require the production of photomasks, patterns can be easily varied.

Combining tools of growth factor and cell-adhesive ligand localization via photopolymerization in PEG hydrogels and novel LSL as a photolithographic alternative, this work presents the design of tightly regulated tissue engineering matrices that support endothelial tubulogenesis along specified boundaries, allowing design of highly organized tissues. Micron-level spatial restriction of endothelial cell attachment to extracellular matrix and availability of localized biochemical signaling accelerates endothelial tubule formation from 30 days to 2 days and upregulates expression of VEGFR1, VEGFR2, ephA7, and laminin within 2 days.

Recent developments of several technologies have made possible the promise of design and creation of tissue engineering matrices with high levels of tissue organization preprogrammed to ensure extracellular matrix-mimetic niches for metabolically active cells. Covalently attaching RGDS and VEGF to PEGDA hydrogel matrices promoted endothelial cell fate determination toward tubulogenesis. Spatially restricting cell integrin ligands and angiogenic signals on physiologically relevant PEGDA matrices through patterning pushed endothelial cells into a tubulogenic response. Two- and three-dimensional capillary networks patterned throughout PEGDA hydrogels are hypothesized to allow the culture of functional engineered tissues larger than 200  $\mu$ m, the current oxygen diffusion

limit for implanted tissues. Additionally, by restricting immobilization of VEGF, physiological responses to this growth factor will remain localized, leading to an expected, engineered tissue response.

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### Disclosure Statement

No competing financial interests exist.

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