Geometrically Controlled Endothelial Tubulogenesis in Micropatterned Gels

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We present a novel approach to control endothelial tubulogenesis by spatially patterning cells within micromolded collagen gels. Endothelial cells cultured within microscale channels that were filled with collagen gel organized into tubes with lumens within 24–48 h of seeding. These tubes extended up to 1 cm in length, and exhibited cell–cell junction formation characteristic of early stage capillary vessels. Tube diameter could be controlled by varying collagen concentrations or channel width. The geometry of the microfabricated template also could be used to guide the development of branches during tube formation, allowing for the generation of more complex capillary architectures. Time-lapse imaging of tube formation revealed a highly dynamic process involving coalescence of endothelial cells, reorganization and alignment of collagen fibers into a central core, and arrangement of cells into cords. This platform may be of use to generate geometrically defined vascular networks for tissue engineering applications as well as a means to better understand the process of endothelial tubulogenesis.

Introduction

ENGINEERED TISSUES have the potential to address the shortage of replacement organs for patients who have suffered organ damage or failure. In particular, cell-based constructs are increasingly being examined as a means to recapitulate native organ structure and function. The inability to vascularize constructs whose dimensions exceed the diffusion limits of oxygen and nutrients has limited the practical application of large-scale engineered tissues.[1–4] Therefore, there is considerable interest in developing strategies to induce vascular development within tissue-engineered constructs. One such approach involves including factors within the engineered matrix to promote endothelial cell invasion into the construct after implantation.[5–7] An alternate approach involves seeding vascular precursors into these constructs before implantation in the hope that these cells will receive the appropriate cues to form vessels in vitro or in vivo.[8–11] A third approach involves preforming constructs containing endothelial tubules and promoting integration of construct vessels with in vivo vasculature after implantation.[12,13] Several recent studies suggest that incorporating cells or preexisting endothelial networks may accelerate the vascularization of an implant, thereby improving the likelihood for long-term implant survival.[10,13,14] However, the vascular networks that form when endothelial cells are implanted in vivo are typically randomly distributed in space, making it difficult to recapitulate the vasculature of organs such as the liver that are comprised of multiple cell types precisely distributed around a complex organized vascular network. Consequently, novel methods to spatially pattern endothelial cells and promote vascularization are needed for such applications.

While ultimately the functionality of vascular networks for tissue engineering applications must be tested in vivo, a variety of methods exist to develop partially organized in vitro networks of vascular cells. Factors that regulate in vivo vascular development have inspired methods for in vitro vascular network formation, and principally include stimulation by vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), an appropriate three-dimensional (3D) extracellular matrix (ECM), and sufficiently high cell densities to enable multicellular assembly.[15–20] Two processes associated with vascular development have been observed in culture—sprouting and tubulogenesis. In sprouting, cells from either an existing vessel network or a cluster invade and extend branches into the matrix.[21–23] In tubulogenesis, individual cells coalesce to initially form a loose network and subsequently organize into multicellular cords.[19,24,25] Although such in vitro methods have been optimized as basic research assays to gain insights into the roles of growth factors and different matrix materials in vasculogenesis, there is little control over initial cell position, and tubule formation is

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consequently randomly distributed throughout the matrix. This lack of control over the organization of the vasculature is not ideal for tissue engineering applications, which involve integration with other cell types to generate canonical tissue structures. Further, isolating individual tubules for manipulation or implantation *in vivo* is not possible from these macroscale hydrogels. Consequently, new approaches allowing spatial control over endothelial tubulogenesis would expand our ability to generate complex tissue-engineered substrates.

One approach to circumvent these limitations involves applying microfabrication technologies to spatially control the positions of cells within 3D ECMs. Several groups have demonstrated complex spatial patterning of cells within 3D hydrogel networks using photopolymerizable chemistries.[26–32] Others have used fabrication principles to organize natural polymers such as collagen into specific microscale structures, and have applied these substrates to examine epithelial morphogenesis.[33–36] In one such approach, Tien and coworkers developed a method to prefabricate hollow channels within collagen gels.[37] Endothelial cells were then seeded along the interior of the channel such that they formed a vessel-like structure that permitted flow of solution through the tube lumen. The authors demonstrated endothelial barrier function and appropriate barrier breakdown upon exposure to inflammatory cytokines. Although these and other studies demonstrate the potential for fabrication technologies to spatially engineer cellular constructs, how such geometric control actually impacts the process of endothelial cell self-assembly into tubules remains unknown.

Here, we present a novel method using microfabricated poly(dimethylsiloxane) (PDMS) templates to spatially arrange endothelial cells within collagen gels to promote *de novo* endothelial cord formation. We observed that individual endothelial cells organized into capillary tubes after stimulation with VEGF and bFGF over 24–48 h. We demonstrate that tube diameter can be modulated by both channel width and collagen concentration. Further, tubes with branched architecture could be generated with appropriate templates. This method provides a novel means to spatially organize endothelial cells for tissue engineering applications as well as to investigate the basic process of tubulogenesis.

Materials and Methods

Cell culture and reagents

Bovine adrenal microvascular endothelial cells (BAMECs; VEC Technologies) were cultured on collagen-coated dishes (collagen from BD Biosciences) in low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone), 10 ng/mL epidermal growth factor, 3 ng/mL bFGF, 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (all reagents from Invitrogen unless otherwise indicated). Before trypsinization, BAMECs were cultured in low glucose DMEM containing 10% calf serum (Hyclone), 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (all unspecified reagents from Invitrogen) for 4 h. BAMECs seeded into Microgel channels were cultured in low-glucose DMEM containing 2% (w/v) methyl cellulose (Sigma), 10% fetal bovine serum (Hyclone), 10 ng/mL epidermal growth factor, 3 ng/mL bFGF, 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (all unspecified reagents from Invitrogen).

Human umbilical vein endothelial cells (HUVECs, a gift from Dr. Guillermo Garcia-Cardena) were cultured in Medium 199 (Cambrex) containing 20% fetal bovine serum, 50 μg/mL endothelial cell growth supplement (Biomedical Technologies), 100 μg/mL heparin (Sigma), 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (all unspecified reagents were from Invitrogen). HUVECs cultured within PDMS channels were switched to EBM2 medium (Cambrex) lacking VEGF and bFGF. Samples were then supplemented with 3 ng/mL bFGF and 10 ng/mL VEGF.

Fabrication of PDMS templates

PDMS templates were fabricated as previously described.[38] Briefly, silicon masters were prepared by spinning SU-8 photoresist (Shipley) and photolithographically exposing and developing channel patterns at heights between 50 and 100 μm. To generate PDMS substrates, liquid PDMS prepolymer was poured onto silicon masters and cured. PDMS templates were sterilized with ethanol before use as culture substrates.

Preparation of micropatterned cells in gels

To prepare cells within micropatterned channels, channels were immersed in liquid (unpolymerized) neutralized collagen (BD Biosciences) and exposed to vacuum to remove air bubbles. Endothelial cells suspended in liquid collagen were distributed above the substrate before centrifugation to drive cells into the channels. Excess un-polymerized collagen and cells were removed by dewetting the surface of the substrate. After gelling at 37°C, an appropriate medium was added to each substrate. All samples were prepared at a concentration of 2.4 mg/mL collagen gel except where otherwise specified. Experiments were limited by the concentrations of available sources of collagen I to 3.3 mg/mL at the upper limit and by lack of gelation at the lower limit.

To facilitate the removal of endothelial tubes from the PDMS template by minimizing protein adsorption and cell adhesion to the surface, templates were precoated with a nonadhesive polymer, 0.1% Pluronic F127 (BASF), for 30 min before addition of cells and collagen. After rinsing, tubes were prepared as described above and allowed to form for 24 h. Liquid collagen I (2.4 mg/mL) was then added over the sample and gelled, thereby embedding the tubes in additional collagen. The embedded tubes were manually removed by gentle agitation of the culture dish and encased in additional collagen gel for stability before culturing in the absence of the template.

Microscopy

Immunofluorescence samples were fixed in 4% paraformaldehyde, blocked in 33% goat serum or donkey serum (Invitrogen), and stained. Antibodies used include anti-β catenin (Santa Cruz Biotechnology), anti-platelet endothelial cell adhesion molecule (PECAM; Santa Cruz Biotechnology), and anti-type I collagen (Biodesign International) followed by appropriate secondary antibodies labeled with Alexa 594 (Invitrogen), Alexa 488 (Invitrogen), or Cy2 (Jackson Labs). For actin and nuclear stains, TRITC phalloidin (Sigma) and Hoechst 33342 (Invitrogen) were used. Substrates were imaged using an inverted fluorescence microscope (Eclipse TE200, TE2000 [Nikon]; Axiovert 200M [Zeiss]). Confocal

images were acquired using a Zeiss LSM4. Spot software (Diagnostic Instruments) was used to measure tube diameters. For time-lapse imaging, samples were placed within a heated stage-top incubator (LiveCell™; Pathology Devices) mounted to an inverted microscope (TE2000; Nikon) and imaged for 24 h.

Statistical analysis

Linear regression fits to tube diameter measurements under different conditions were compared to determine inhomogeneity of slopes, followed by a multiple comparisons procedure (using Tukey's test) to assess pairwise significant differences between linear fits.

Results

Preparation and characterization of endothelial tubes

We present here a novel method to promote endothelial tubulogenesis using microfabricated templates that spatially organize cells within channels filled with collagen gel. After fabrication of PDMS templates with channel geometries using soft lithography techniques, we introduced endothelial cells suspended within collagen gel into the channels (Fig. 1). After stimulation with 3 ng/mL bFGF and 10 ng/mL VEGF, both BAMECs and HUVECs initiated tubulogenesis over 24 h (Fig. 2A). Patterning the cells and gel permitted the formation of a large array of spatially organized endothelial cords, in contrast to the nonuniform rate, length, diameter, and orientation of tubule formation characteristic of cells within unpatterned collagen gels. In addition, because all the cords are uniformly distributed and located within the same focal plane, simultaneous microscopic analyses of large arrays of tubules are facilitated. To examine whether these cords of cells were organized into open cylindrical conduits, we stained them for actin and nuclei, and imaged them using confocal microscopy. Cords formed by both HUVECs and BAMECs exhibited a tube-like morphology (Fig. 2B). Interestingly, tubes formed by HUVECs tended to have larger diameters than those formed with BAMECs, perhaps reflecting their distinct origins as large and small vessel cells, respectively. We also observed that the cells expressed markers for endothelial cell–cell junctions that were appropriately localized to cell–cell borders, including PECAM (Fig. 2C) and β-catenin, a marker of cadherin-mediated junctions (Fig. 2D). Three-dimensional rendering of such images indicated that cells formed a confluent and continuous barrier separating the lumenal and ablumenal spaces.

Dynamics of tubulogenesis

To better understand the dynamics underlying the tubulogenesis process, we cultured cells for 24 h in a heated microscope incubator and imaged them using time-lapse microscopy. Endothelial cells were initially individually distributed within the pattern, but after approximately 8 h, cells spread through the gels and reached out to their neighbors, formed contacts, and began to preferentially elongate along the direction of the channel (Fig. 3A and Supplemental Movie S1, available online at www.liebertonline.com). By 12 h after the start of imaging, cells had organized into an extended chain of cells, but still did not appear as a tubular structure. Subsequently, the cells retracted extensions and appeared to
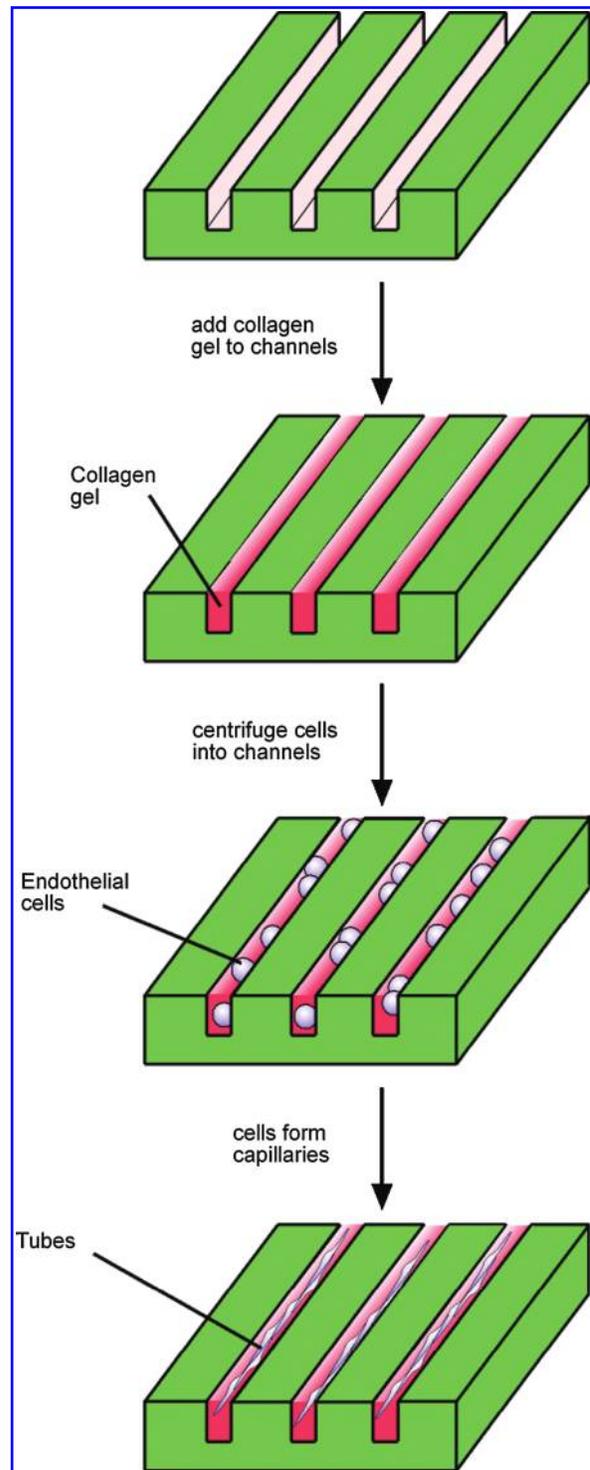
FIG. 1. Schematic of method to organize cells and collagen gel within microfabricated channels. Color images available online at www.liebertonline.com/ten.

contract such that the long axis of the tube stretched, condensing into a tubular structure by 20 h after initiation. The cells appear to actively remodel the ECM, as collagen fibers initially visible by phase-contrast imaging were re-positioned so that they were no longer visible by the end of the experiment. When we stained the samples for collagen I, we found
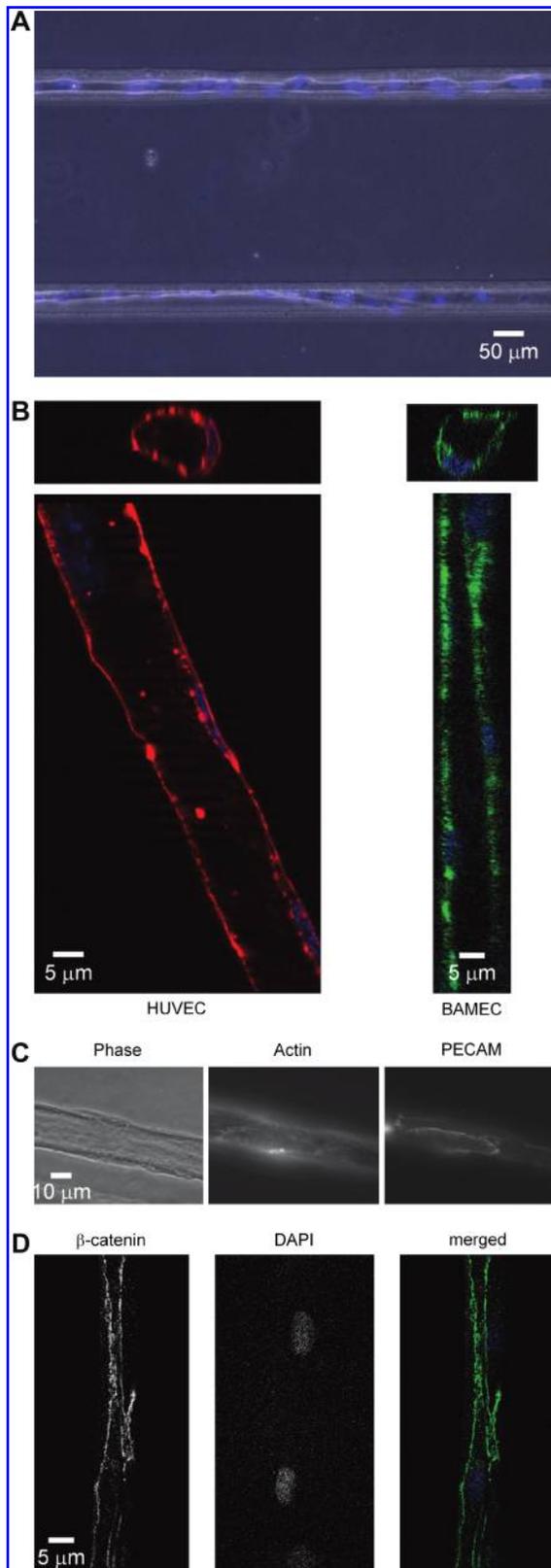
that cells had bundled the collagen into the core of the tubule and formed a cylinder around it (Fig. 3B). These data reveal a highly dynamic, multistep process, and further studies into the mechanisms underlying endothelial cord formation are needed.
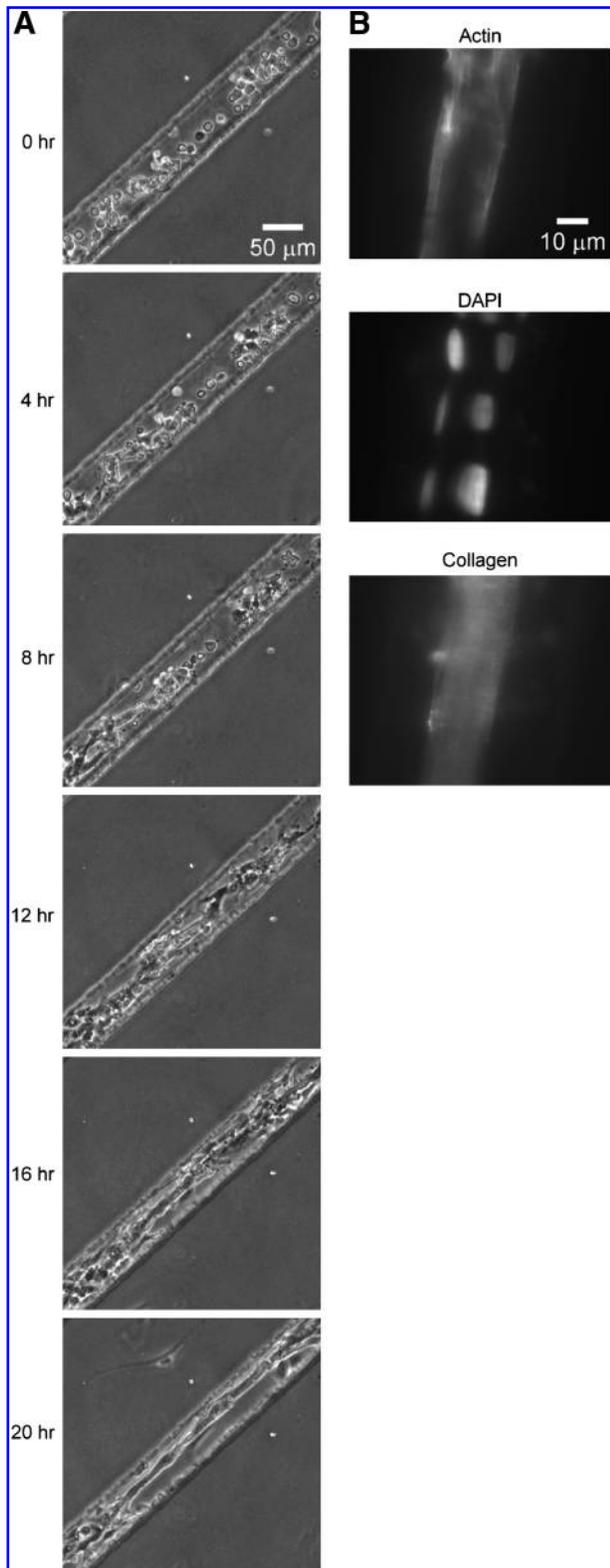
Controlling tube diameter with collagen concentration and pattern dimensions

We next explored whether tube diameter could be controlled by pattern dimensions and collagen concentration, or if it was intrinsically determined by the type of cell used in the system. HUVECs were seeded into channels of differing widths but with the same height at three different collagen concentrations. After 24–36 h in culture, samples were fixed and stained for actin and nuclei, and tube diameters were measured. Cells formed tubules under all conditions. We observed that at the same channel width, cells cultured in higher concentrations of collagen formed larger-diameter tubes (Fig. 4A). In addition, for a given collagen concentration, cells formed larger tubes as channel width was increased (Fig. 4B, C). It appeared that cells cultured at lower collagen densities formed tubes more rapidly than cells at higher collagen densities. The effects of collagen concentration on tube diameter were more distinct at larger channel widths, suggesting that the combination of more cells due to larger channels and higher collagen concentration promoted formation of larger tubes.

Guiding branched tube formation with template geometry

Using PDMS templates to guide tubulogenesis provides the potential to control the fabrication of more complex patterns of capillary network growth *in vitro*. To explore this possibility, we seeded HUVECs in PDMS templates containing branched patterns. We observed tubulogenesis within the branched pattern, with the new tubes conforming to the prespecified geometry such that bifurcations in the channels resulted in bifurcations in the ensuing endothelial tubes (Fig. 5A). Cells formed tubes at several branch angles, including 90° bends. When we stained and imaged the branched samples for actin and β-catenin, we observed lumens for each branch (Fig. 5B). In addition, the septation point was clearly visible in cross sections progressing along the trunk of the pattern to the branch point and beyond. A 3D reconstruction of the branched tube demonstrated that cells lined

FIG. 3. Dynamics of tube formation. (**A**) Phase-contrast images taken from a time-lapse video of endothelial tubulogenesis. BAMECs were cultured in 2.4 mg/mL collagen gel within channels 50 μm wide and 50 μm tall and imaged in a heated stage-top microscope incubator for 24 h. (**B**) Immunofluorescence staining reveals collagen I within the tube lumen. BAMECs were cultured in 2.4 mg/mL collagen gel within channels 50 μm wide and 50 μm tall for 24 h before fixation.

the exterior of the tube but were not present in the tube lumen, with cell–cell junctions demarcated by β-catenin labeling (Fig. 5C). These data demonstrate a means to guide endothelial tubulogenesis along branching geometries that may be useful for tissue engineering applications.

Removal of tubes from the PDMS template

To facilitate integration with other tissue-engineered constructs, we developed a means to remove endothelial tubes from the PDMS template while preserving spatial organization. After allowing tubes to form within the templates for 24 h, we added unpolymerized collagen I matrix over the sample and gelled, thereby embedding the tubes in additional collagen gel. The embedded tubes were released from the PDMS molds by gentle agitation of the culture dish. The endothelial tubes were then encased in additional collagen gel before culturing in the absence of the template. This approach retained the spatial orientation of the patterned endothelial tubes while transferring them completely to a physiologic matrix (Fig. 6A, B), ultimately allowing for easier integration with other tissue-engineered constructs.

Discussion

Tissue-engineered constructs hold great promise for alleviating the shortage of available organs for transplantation, but proper organ function and long-term viability are predicated upon vascularization of these constructs. Therefore, developing the means to form stable vascular networks that can be integrated with other cell types in specific patterns to create an engineered tissue is of significant interest. Endothelial cells cultured in the absence of a template typically form randomly distributed networks, making it difficult to define the vascular architecture of engineered tissues. We have presented here a novel method to organize cells within channels filled with collagen to promote *de novo* endothelial tubulogenesis. The endothelial cords that self-assembled over 24 h with bFGF and VEGF stimulation exhibited appropriate markers of *in vivo* vasculature. Although others have formed patterned tubules by seeding cells that attach to the walls of an open channel,[37] here we demonstrate the self-organization of cells into patterned multicellular tubules of smaller diameter than those obtained by seeding cells through channels. It has previously been shown that endothelial cells seeded onto flat substrates micropatterned with thin (10 μm) lines of matrix can form cords.[39] While such an approach also leads to geometrically patterned cords, here we demonstrate the ability to adjust tube dimensions and geometry by manipulating collagen concentration, channel width, and template pattern. The novel ability of this system to also form complex branched patterns of tubes by controlling template geometry alone may be of use in designing engineered tissues with prespecified vascular networks that more closely recapitulate native tissue structure. Removal of these tubes from the PDMS template by embedding within collagen gel while retaining spatial organization allows for integration with other tissue-engineered substrates. Further, because the engineered vessels are generated in a separate step from their release into a second material, one can imagine including cells or other gel types in that material, thereby generalizing the approach for inclusion in an engineered implant. Alternatively, a layered approach could be

FIG. 4. Mechanisms to control endothelial tube diameter. (**A**) Representative phase-contrast images of altered tube diameters due to changing collagen concentration. HUVECs were cultured in collagen gels of specified concentration within channels 100 μm tall and 200 μm wide for 24–36 h before fixation. (**B**) Representative phase-contrast images of altered tube diameters due to changing channel width. HUVECs were cultured in 2.4 mg/mL collagen gels within 100 μm tall channels of varying widths for 24–36 h before fixation. (**C**) Graph of average tube diameter as a function of channel width and collagen concentration. $*p < 0.001$ as determined by a multiple comparisons procedure (using Tukey's test) for pairwise comparison of slopes determined by linear regression fits to tube diameter measurements.
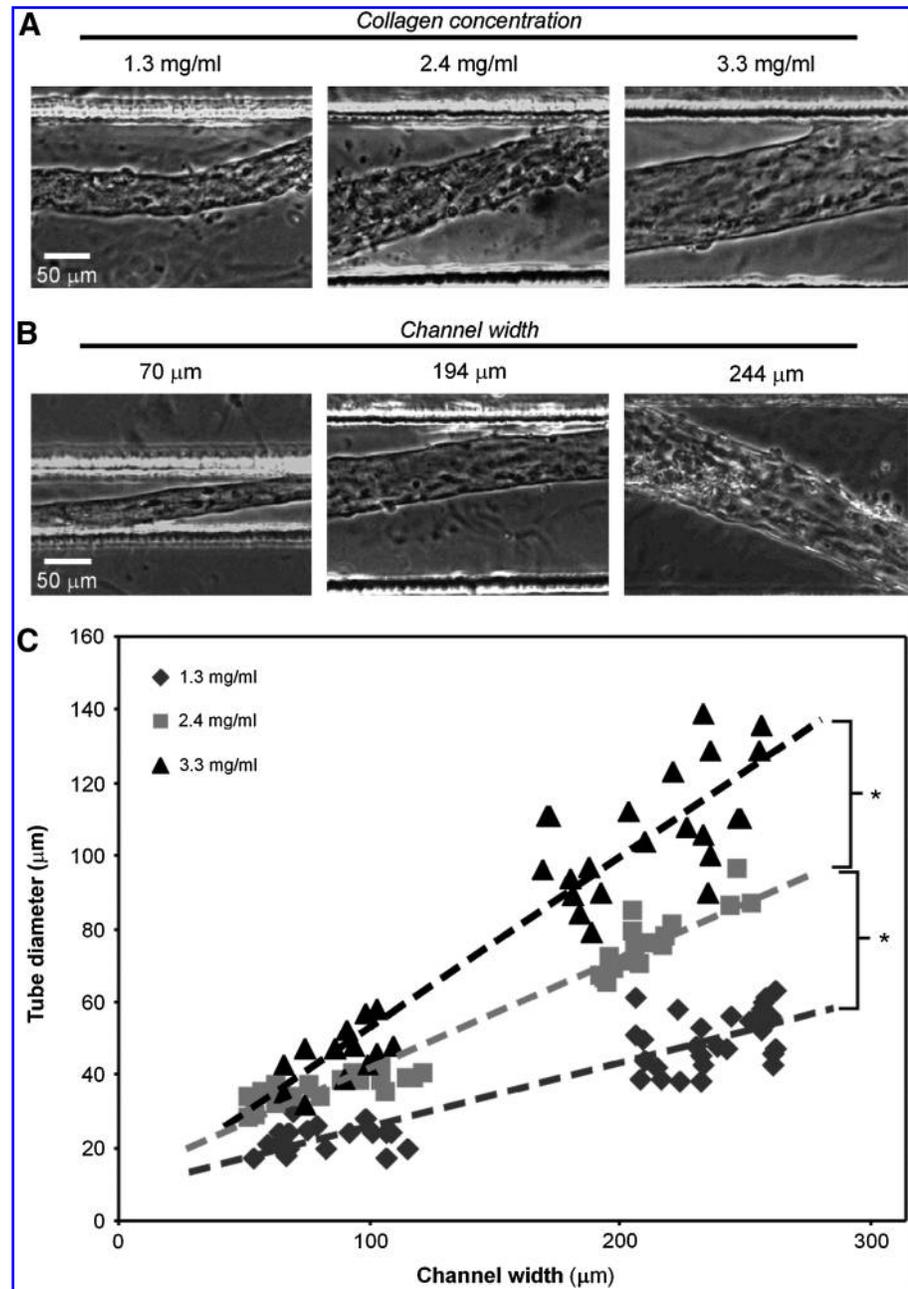
used to interdigitate these vascular networks with other cell types to form a multicellular 3D patterned construct for *in vivo* implantation.

Our time-lapse studies revealed that the endothelial cells coalesce and appear to bundle the surrounding ECM into a core around which they arrange themselves, even though at the time of seeding the cells were embedded within the matrix. These events resemble early processes in vasculogenesis, which involves the *de novo* formation of the heart tube and early vasculature by coalescence of isolated endothelial precursor cells and subsequent lumen formation, in contrast to angiogenesis, which involves the extension of new vessels from preexisting ones.[40,41] The observations of increased tube diameter at higher collagen densities and larger channel widths are consistent with the mechanism

observed by time-lapse imaging. At higher collagen densities (with equivalent channel volume), more collagen was available for cells to organize into larger bundles. At larger channel widths, both the greater number of cells and larger amount of collagen likely contributed to tubes of larger diameter. Although these data suggest that further increasing channel width or collagen density will result in increased tube diameter, it is likely that there are limits to the collagen densities conducive to tube formation. We speculate that at high collagen densities, cells would no longer be able to easily remodel the collagen to organize into tubes. Several reports have demonstrated decreased length of endothelial sprouts with increased matrix density,[42,43] and we suspect that there would also be an upper limit to effective collagen concentration in our assay.
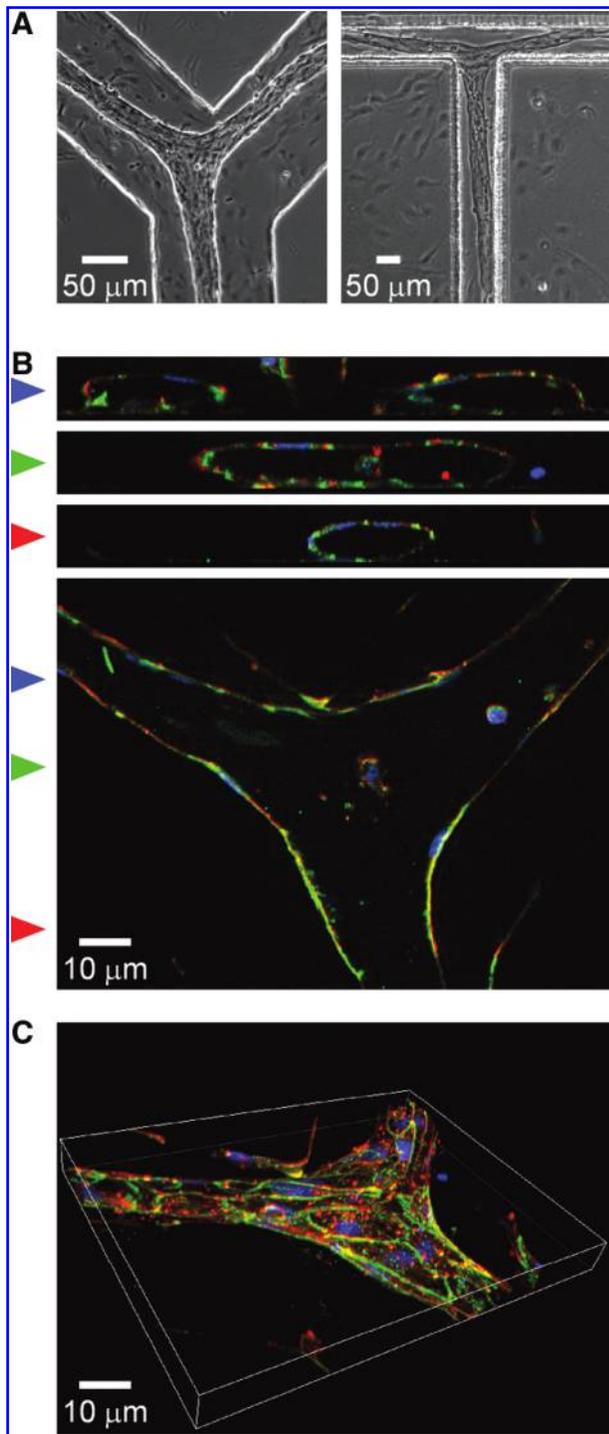
FIG. 5. Formation of branched tubes. (**A**) Representative phase-contrast images of branched tubulogenesis. HUVECs were cultured in 2.4 mg/mL collagen gel within 45° branched channels 125 μm wide and 100 μm tall (left) or 90° branched channels 75 μm wide and 100 μm tall (right) for 48 h before fixation. (**B**) Immunofluorescence images of a branched tube labeled for β-catenin (green), actin (red), and nuclei (blue). Arrowheads indicate cross sections at different points. HU-VECs were cultured in 2.4 mg/mL collagen gel within 45° branched channels 50 μm wide and 50 μm tall for 48 h before fixation. (**C**) Three-dimensional reconstruction of branched tube labeled as above. Color images available online at www.liebertonline.com/ten.
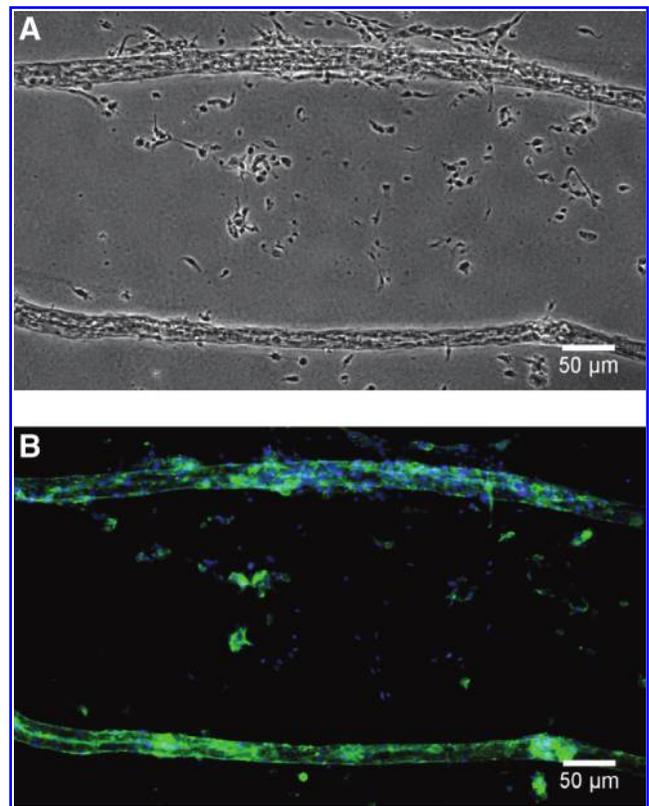
FIG. 6. Removal of tubes from the PDMS template. Phase-contrast (**A**) and immunofluorescence (**B**) images of HUVEC tubes cultured for 24 h in 2.4 mg/mL collagen gel within channels 160 μm wide and 100 μm tall, and subsequently embedded in collagen gel (2.4 mg/mL) and removed from the template. Cells are labeled for actin (green) and nuclei (blue). Color images available online at www.liebertonline.com/ten.

While the presence of collagen within the lumens of endothelial cords is a potential limitation of this approach, additional cues from the local environment after implantation *in vivo* may promote lumen opening. Other cell types such as pericytes and macrophages appear to provide critical cues to endothelial cells for vessel maturation *in vivo*.[44–46] In addition, recent evidence has suggested that the activation of matrix metalloproteases is necessary for the development of vessels, and may be particularly important for the matrix remodeling required for lumen formation.[46–48] As such, further studies are needed to examine whether coculture with the appropriate support cells and exposure to additional *in vivo* cues after implantation will provide these endothelial tubules with the necessary signals to promote matrix remodeling, more complete lumen opening, and the formation of a functional patterned vascular network within a tissue-engineered construct.

In addition to tissue engineering applications, these substrates may be of use in high-throughput assays of endothelial function. In contrast to traditional tubulogenesis assays where cells are randomly distributed, the planar nature of the PDMS template and the formation of precisely arrayed tubes enable easy analysis by microscopy. These substrates are amenable to rapid automated microscopic quantification of the number

and length of tubules in high-throughput screening assays of endothelial tube formation. Further, smaller volumes of antibodies are needed for immunolabeling, as these samples can be stained in a manner similar to cells cultured on coverslips rather than cells in traditional macroscale collagen gels, resulting in lower costs for sample processing and analysis. The spatial templating of cord formation in conjunction with control over matrix composition, channel geometry, and culture environment allows for rapid comparison of endothelial tubules formed under different conditions. For example, if integrated with combinatorial protein spotting techniques, this platform would be applicable to high-throughput screening of inhibitors of endothelial tube formation. In addition, culturing endothelial cells from different vascular lineages (e.g., large vs. small vessels, and arterial vs. venous vessels) in the microfabricated channels under a variety of conditions might provide additional insight into the functional and developmental variation between different vascular types. That is, by allowing endothelial self-assembly, this approach enables examination of how intrinsic differences between endothelial cell subtypes might contribute to variations in the process of vascular morphogenesis, in contrast to methods that seed endothelial cells onto preformed ECM templates. As we observed in our studies, endothelial cells isolated from large vessels (HUVEC) tended to form larger cords than cells isolated from the microvasculature (BAMEC). By culturing different endothelial lineages under a variety of inhibitor scenarios, this platform could be used to identify and dissect signaling pathways that lead to functional differences in endothelial tubulogenesis and *in vivo* behaviors.

Conclusions

We have presented here a new method to geometrically control endothelial cord formation by spatially organizing endothelial cells within 3D collagen hydrogels before inducing tubulogenesis. Endothelial tubes formed spontaneously within these constructs after stimulation with appropriate growth factors over 24–48 h. Additionally, template architecture can guide the formation of branched tubes. These studies highlight the importance of tissue architecture in tissue function, and provide an approach to engineer multicellular organization to control morphogenetic events.

Acknowledgments

Disclosure Statement

The authors declare that they have no competing financial interests.

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