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Stable engineered vascular networks from human induced pluripotent stem cell-derived endothelial cells cultured in synthetic hydrogels

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Abstract

Here, we describe an *in vitro* strategy to model vascular morphogenesis where human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) are encapsulated in peptide-functionalized poly(ethylene glycol) (PEG) hydrogels, either on standard well plates or within a passive pumping polydimethylsiloxane (PDMS) tri-channel microfluidic device. PEG hydrogels permissive towards cellular remodeling were fabricated using thiol-ene photopolymerization to incorporate matrix metalloproteinase (MMP)-degradable crosslinks and CRGDS cell adhesion peptide. Time lapse microscopy, immunofluorescence imaging, and RNA sequencing (RNA-Seq) demonstrated that iPSC-ECs formed vascular networks through mechanisms that were consistent with *in vivo* vasculogenesis and angiogenesis when cultured in PEG hydrogels. Migrating iPSC-ECs condensed into clusters, elongated into tubules, and formed polygonal networks through sprouting. Genes upregulated for iPSC-ECs cultured in PEG hydrogels relative to control cells on

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Competing Financial Interests

J.A.T. was a founder and stockholder for Cellular Dynamics, Inc at the time of manuscript submission. D.J.B. holds equity in Bellbrook Labs, LLC, Tasso, Inc., Stacks for the Future, LLC and Salus Discovery, LLC. W.L.M. is a founder and stockholder for Stem Pharm, Inc., and Tissue Regeneration Systems, Inc.

tissue culture polystyrene (TCP) surfaces included adhesion, matrix remodeling, and Notch signaling pathway genes relevant to *in vivo* vascular development. Vascular networks with lumens were stable for at least 14 days when iPSC-ECs were encapsulated in PEG hydrogels that were polymerized within the central channel of the microfluidic device. Therefore, iPSC-ECs cultured in peptide-functionalized PEG hydrogels offer a defined platform for investigating vascular morphogenesis *in vitro* using both standard and microfluidic formats.

Keywords

Thiolene; Biomaterials; Blood Vessel; Tissue Engineering; Microfluidic; 3D culture

1. INTRODUCTION

The lack of a functional vasculature and pathological disruption of circulation are unresolved challenges that have limited the success for many tissue engineering and wound healing approaches [1–3]. Furthermore, the incorporation of a vascular component is expected to improve human cellular models by recapitulating cell-cell interactions during tissue formation [4, 5] and by supporting the function of model organ systems [6, 7]. Finally, while target organs such as the heart, liver or central nervous system have been the focus for many *in vitro* toxicity screening strategies [8], vascular models have also been identified as a promising tool for predictive toxicology [9, 10]. Therefore, several emerging applications would benefit from *in vitro* assays that enable systematic investigation of factors that promote blood vessel formation and stabilization [1–3].

Endothelial cells cultured *in vitro* will spontaneously “self-assemble” into organized networks [11–17], and several studies have demonstrated that capillary tubules can be perfused when subjected to flow [18–23]. While extracellular matrix (ECM) components such as collagen or Matrigel are often used as culture substrates when modeling vascular morphogenesis *in vitro* [12–14, 16, 17], these materials can be limiting for screening approaches due to batch variability, properties that are sensitive to reaction conditions, and poorly-defined compositions [24–26]. To address these limitations, synthetic strategies have increasingly been applied to investigate factors that instruct endothelial phenotypes [27–35]. Hydrogels formed via thiol-ene photopolymerization represent an emerging class of cell culture materials [36, 37] that are formed through a radical-initiated step-growth mechanism that couples thiols and alkenes with high specificity [38]. A growing body of literature has demonstrated the versatility of thiol-ene photochemistry for incorporating biomolecules such as peptides, growth factors, gelatin, and hyaluronic acid into synthetic hydrogels [4, 35–37, 39–47]. Hydrogels formed via thiol-ene photopolymerization enable spatial patterning of biochemical and mechanical properties [35, 39–41], sequestering and controlled release of growth factors [45], rapid photopolymerization for 3D bioprinting of encapsulated cells [44], and protein-free backgrounds for identifying ECM components deposited in the matrix during cellular remodeling [47]. Thus, thiol-ene chemistry offers a potentially powerful tool for modeling vascular morphogenesis by providing control over a wide range of matrix properties relevant to blood vessel formation [4, 35].

While engineering platforms provide control over the 3D microenvironment when modeling vascular morphogenesis [1–3], the heterogeneity and donor-to-donor variability of primary human endothelial cells may be limiting for applications that require standardization or scale-up [1, 48]. Human umbilical vein endothelial cells (HUVECs) can be used for standardized screening of angiogenesis inhibitors *in vitro*, but require processing and pre-validation to identify donor sources with similar function [10]. Human pluripotent stem cells [49–51] offer promise for predictive toxicology [8], and have been used to derive endothelial cells that form vascular networks *in vitro* and functional blood vessels *in vivo* [23, 30, 52, 53]. Importantly, human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) can be produced with high batch uniformity [23], which may be beneficial for vascular disease models or screening approaches that require standardization or scale-up [9, 54].

The strategy reported here combines a uniform endothelial cell source [23], a tunable synthetic ECM [36], and a tri-channel microfluidic device [55] to model vascular morphogenesis *in vitro*. Thiol-ene photopolymerization was used to fabricate peptide functionalized PEG hydrogels permissive towards cellular remodeling [36] and the iPSC-ECs were previously characterized by high lot-to-lot purity to at least 6 passages [23]. The passive flow concept that is the essence of the microfluidic device described here uses standard culture techniques for loading cells and exchanging media [55], and therefore provides an accessible format that takes advantage of microscale features such as decreased requirements for reagents and cells [56]. Our combined results provide evidence that iPSC-ECs self-assemble into vascular networks through physiologically-relevant mechanisms when cultured in PEG hydrogels, and capillary tubules with lumens were stable for at least two weeks when the hydrogels were polymerized within the microfluidic device.

2. MATERIALS AND METHODS

2.1 Cell culture

Human induced pluripotent stem cell-derived endothelial cells (“iPSC-ECs”, Cellular Dynamics, iCell® Endothelial Cells) were cultured according to the manufacturer’s protocol. Briefly, iPSC-ECs were expanded to passage 3 and cryopreserved for additional use. Passage 3 iPSC-ECs were thawed and plated at 10,000–15,000 iPSC-ECs/cm² onto tissue culture plates treated with 3 µg/cm² fibronectin (Invitrogen) and passaged every 3 to 4 days with TrypLE (Invitrogen). The manufacturer’s recommended growth medium was used for culturing iPSC-ECs, which consists of Vasculife VEGF Medium (Lifeline Cell Technologies) that was modified as follows: 10 mL glutamine supplement was added to 500 mL medium (rather than the 25 mL provided) and the FBS supplement was replaced with iCell® Endothelial Cells Medium Supplement (Cellular Dynamics). Cells were incubated at 37°C and 5% CO₂ for all experiments. **Microfluidic channels.** Microfluidic experiments were conducted using iPSC-ECs cultured in the growth medium described above (“Control”), or in growth medium supplemented within 100–1000 ng/mL VEGF-165 (Catalog # 293-VE, Lot 114714051, 97% purity from R&D), as described in Results and Discussion. The iPSC-ECs were encapsulated in PEG hydrogels that were polymerized within the central channel of the tri-channel device (see details below). All fluid was removed from both outer channels daily and replaced with a total of 10 µL of fresh medium.

2.2 Microfluidic Device Fabrication

The tri-channel microfluidic device was fabricated as previously described without modification [55]. Briefly, polydimethylsiloxane (PDMS, Sylgard 184 Silicone Elastomer Kit, Dow Corning) elastomer base and curing agent were mixed at a 10:1 ratio and degassed under vacuum for 45 min (room temperature). The degassed PDMS was poured over SU-8 master molds, which were generated using standard soft lithography methods [57]. The PDMS was cured for 4 hr (80°C), allowed to cool to room temperature, and removed from the master mold. The PDMS device was autoclaved for 20 min at 120° C. Six hr before loading the cell/monomer solution, devices were oxygen-plasma-treated to bond the PDMS channels to the inside of a glass-bottom Petri dish (MatTek).

2.3 Poly(ethylene glycol) (PEG) hydrogel preparation

Poly(ethylene glycol) (PEG) hydrogels were formed using thiol-ene photopolymerization chemistry (Fig. 1A) [36]. For most experiments, 8-arm PEG-norbornene was purchased from a commercial source (JenKem USA: 20,000 MW, 8ARM (TP)-NB-20K). For some experiments, 8-arm PEG-NB monomer was synthesized as previously described [35]. Stock PEG solutions were prepared by adding 0.8 mL 1× PBS to 300 mg lyophilized 8-arm PEG-NB powder (final volume = 1 mL) and filtered through a 0.2 µm nylon syringe filter (Fisher) for a final concentration of 300 mg/mL sterile monomer. Monomer solutions for cell encapsulation were prepared in 1× PBS with 40 mg/mL 8-arm PEG-NB in which 40–60% of the available norbornene arms were cross-linked with a matrix metalloproteinase (MMP)-degradable peptide with cysteines flanking the active sequence (KCGGPQG*IWGQGCK, GenScript; active sequence in bold; * = cleavage site) [58, 59]. To promote cell adhesion [60], 2 mM CRGDS (GenScript, active sequence in bold) was incorporated as a pendant group through the terminal cysteine.

2.4 Quantification of Shear Modulus

To measure the shear modulus of PEG hydrogels, 72 µL precursor solutions were pipetted into 8.0 mm diameter, 1.2 mm depth Teflon wells and cured for 8 sec using 365 nm UV light at a dose rate of 90 mW/cm². The resulting hydrogels were swollen to equilibrium in 1× PBS for 24 hr. The samples were tested using an Ares-LS2 rheometer (TA Instruments). A 20 g force was applied to the samples via parallel plate crossheads and a strain sweep test at 1 Hz fixed frequency was performed from 0.1 to 20% strain. If the sample was not robust enough to withstand a 20 g force the gap between the parallel plates of the rheometer was set to 1.0 mm distance. Measurements for complex shear modulus for each hydrogel formulation were taken at 1 Hz, 2–20% strain.

2.5 Cell encapsulation and polymerization of PEG hydrogels

Cells pellets were re-suspended in a 2× photoinitiator solution (0.1 % wt./wt. Irgacure 2959) and mixed 1:1 with a 2× PEG monomer solution. For experiments using standard plates, 5 µL of the cell/monomer solution was pipetted onto tissue culture treated surfaces and polymerized for 2 min using ~5–10 mW/cm² UV light centered at ~365 nm (multiwell plates were placed on the top shelf of the exposure stand for a UVP XX-15L series UV

lamp, Fisher). **Microfluidic device.** For microfluidic experiments, 2.5 μL of the cell/monomer solution was polymerized in the middle channel of the tri-channel device.

2.6 Microscopy

Immunofluorescence images were collected using a Nikon A1R laser scanning confocal microscope with Plan Apo 10 $\times$, Plan Fluor 20 $\times$ Ph1 DLL, or Plan Apo 20 $\times$ DIC M objectives (0.95–3.35 μm z-steps) unless otherwise noted. Some confocal z-stacks were drift corrected before creating maximum projection images using the “Align Current ND Document” command in NIS Elements. Time-lapse and viability images were collected using a Nikon TI Eclipse fluorescence microscope (20 μm z-steps). For time-lapse imaging, cells were housed in a Nikon environmental chamber with external heater (in vivo Scientific) and CO₂ regulator (in vivo Scientific) to control temperature and CO₂ levels. Some time-lapse images were drift corrected using the StackReg plugin [61] for ImageJ [62, 63].

2.7 Quantification of cell viability

Cell viability was quantified for iPSC-ECs encapsulated in PEG hydrogels using the LIVE/DEAD[®] Viability/Cytotoxicity kit (Life Technologies). Cells were rinsed with 1 $\times$ PBS and stained with calcein AM and ethidium homodimer-1 for 30 min using the manufacturer’s recommended dilutions. Samples were then washed with 1 $\times$ PBS and fixed in 10% buffered formalin (4% formaldehyde, Fischer) for 30 min. Following fixation, cell nuclei were stained with 1:500 DAPI (Sigma-Aldrich) for 30 min. The fraction of dead cells was quantified by determining the ratio of ethidium homodimer⁺ nuclei to total nuclei.

2.8 Immunofluorescence (IF) imaging

Standard well plates. Antibody dilutions were 1:200 mouse anti-CD31 (MAB2148, R&D Systems or M082301–2, DAKO), 1:100 goat anti-VE cadherin (AF938, R&D Systems), and 1:200 secondary antibodies (Alexa fluor 488 donkey anti-mouse, A-21202 or Alexa fluor 568 donkey anti-goat, A-11057, Life Technologies). Cells encapsulated in PEG hydrogels were fixed in 10% buffered formalin (4% formaldehyde, Fischer) for 30–60 min. Following fixing, samples were rinsed 2 $\times$ 15 minutes with wash buffer (0.05% Triton X-100 in 1 $\times$ PBS). Cells were then permeabilized and blocked for at least 60 min using 0.25% Triton X-100 and 1% (wt/wt) bovine serum albumin (BSA, Fisher Scientific) in 1 $\times$ PBS, followed by an additional two rinses with wash buffer. Samples were then incubated with primary antibody in incubation buffer (0.05% Triton X100 and 1% BSA in 1 $\times$ PBS) for 4 hr at room temperature or overnight at 4°C. After rinsing 2 $\times$ 15 min and once for at least 30 min in wash buffer, samples were incubated with secondary antibody and 1:500 DAPI (Sigma-Aldrich) in incubation buffer for 4 hr at room temperature or overnight at 4°C. Samples were rinsed 2 $\times$ 15 min in wash buffer and stored in 1 $\times$ PBS at 4°C until imaging (at least overnight). **Microfluidics device.** Cells encapsulated within microfluidic devices were fixed with 4% paraformaldehyde (PFA) for 30 min. Samples for immunofluorescence imaging of CD31 and VE-Cadherin were prepared as described above. For experiments that illustrate F-actin only, samples were rinsed with 1 $\times$ PBS and then incubated with 1:1000 DAPI and 1:50 phalloidin in 1 $\times$ PBS (added to the side channels) for 1 hr at room temperature, followed by at least 2 $\times$ 15 min incubation in 1 $\times$ PBS. All samples were stored in 1 $\times$ PBS until imaging.

2.9 RNA sequencing

Total RNA was isolated using the RNeasy Kit (Qiagen) and included lysing in 350 μ L RLT lysis buffer and the optional DNase treatment. cDNA libraries were prepared from 50 ng of total RNA and indexed with Illumina's TruSeq RNA Sample Preparation Kit v2 (RS-122-2001 and RS-122-2002). Final indexed cDNA libraries were pooled with 12 uniquely indexed TruSeq cDNA libraries for a total of 6 samples per lane. Multiplexed samples were sequenced on an Illumina HiSeq 2500 with a single 51 bp read and a 7 bp index read. Base-calling and demultiplexing were performed using Casava (v1.8.2). Sequences were filtered and trimmed to remove low quality reads, adapters, and other sequencing artifacts. The remaining reads were aligned to 19084 Refseq genes extracted from the Illumina iGenomes reference, selecting only those with 'NM_' annotations. Bowtie (v0.12.9) was used for alignment, allowing two mismatches in a 28 bp seed [64]. Reads with more than 200 alignments were excluded from further analysis. RSEM (v1.2.3) was used to estimate isoform and relative gene expression levels (transcripts per million or "TPM") [65]. LOG-fold changes are calculated as $\text{LOG}_2[(\text{iPSC-EC TPM}+1)/(\text{iPSC TPM}+1)]$ to avoid calculation errors associated with samples that have zero expression. **Differential gene expression.** Differentially expressed genes between individual samples were calculated from RSEM expected read counts (EC) using EBSeq (v1.5.3) [66], with median quantile normalization of EC and a maximum False Discovery Rate of 0.005. Differentially expressed genes between individual samples were calculated in EBSeq using expected read counts and the False Discovery Rate cutoff was set at FDR = 0.005. **Gene Ontology analysis.** The DAVID Bioinformatics Database Functional Annotation Tool (v6.7) was used to identify Gene Ontology terms [67–70] from differentially expressed genes. DAVID analysis was done using the following settings: Gene Ontology category GOTERM_BP_FAT; Threshold options: Counts = 10, EASE = 0.001. GO lists presented in Supplemental Tables 2–3 were limited to the Top 25 GO terms and Benjamini corrected p-value = 0.05 (thus, some lists included fewer than 25 terms). **Code availability (EBSeq).** Details about the EBSeq algorithm were previously described in detail [66]. EBSeq download and documentation is available at: <http://www.bioconductor.org/packages/devel/bioc/html/EBSeq.html>

Additional files and instructions for downloading the user interface and instructions for installing the EBSeq toolshed for Galaxy are available at: <https://www.biostat.wisc.edu/~kendzior/EBSEQ/>

2.10 Statistical analysis

Statistical significance for data in Suppl. Fig. 5 was calculated using a one-way ANOVA with a post hoc Tukey-Kramer test for individual comparisons ($\alpha = 0.05$).

3. RESULTS AND DISCUSSION

Induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) were encapsulated in peptidefunctionalized poly(ethylene glycol) (PEG) hydrogels to establish an *in vitro* vascular model using a uniform cell source [23] and a synthetic extracellular matrix (ECM) [36]. Thiol-ene photopolymerization was used to incorporate protease-degradable peptide

crosslinks [58] and cell adhesion peptides [60] into PEG hydrogels to provide a synthetic ECM permissive towards cellular remodeling (Fig. 1A) [36]. The iPSC-ECs were previously characterized by uniform purity between lots and functional characteristics that included thrombin-dependent barrier function, TNF- α responsiveness, and shear stress-induced alignment [23]. Here, calcein/ethidium homodimer staining (Fig. 1B–C) and time-lapse microscopy (Suppl. Fig. 1, Suppl. Movie 1) demonstrated that iPSC-ECs were viable and self-assembled into interconnected vascular networks during the first three days of culture in peptide-functionalized PEG hydrogels. After encapsulation, iPSC-ECs condensed into clusters, elongated, and extended protrusions to establish connections (Suppl. Fig. 1A, Suppl. Movie 2), which resembled vasculogenic sprouting *in vivo* [71, 72]. Sprouting from existing tubules played a dynamic role during iPSC-EC assembly (Suppl. Fig. 1B, Suppl. Movie 1), as sprouts often retracted before establishing new connections or formed tubules that later disassembled (Suppl. Movie 3). Vacuoles also appeared to play a role in tubule formation by iPSC-ECs (Suppl. Movie 4), such as previously observed *in vitro* and *in vivo* [73]. By day 3, capillary networks with polygonal organization were evident throughout the hydrogel spot (Suppl. Fig. 1C), which resembled mechanisms described for endothelial cells in naturally-derived matrices *in vitro* [15–17] and blood vessel development *in vivo* [71, 72].

RNA-Seq was used to analyze global gene expression for iPSC-ECs during vascular network formation in PEG hydrogels (Suppl. Table 1). Immunofluorescence imaging demonstrated that iPSC-EC networks in PEG hydrogels were CD31⁺ and VE-Cadherin⁺ by immunofluorescence (Fig. 2A–C), and both genes were highly expressed by RNA-Seq for 2D and 3D culture (Fig. 2D–E). Normalized gene expression was ranked for iPSC-ECs within the Gene Ontology (GO) categories vasculature development (GO:0001944) and biological adhesion (GO:0022610) (Suppl. Fig. 2), which are functional terms from the Gene Ontology Consortium [69, 70] chosen for their relevance to mechanisms that guide vascular morphogenesis [17, 74, 75]. Genes that were highly expressed by iPSC-ECs included many regulators of vascular function, such as cellmatrix and cell-cell adhesion genes (e.g., KDR/VEGFR-2, CD99, MCAM/CD146, CLDN5/claudin-5, integrins, etc.) and genes relevant to matrix remodeling (e.g., laminins, collagens, TIMPs, and MMPs; Suppl. Fig. 2) [17, 74]. Thus, iPSC-ECs were characterized by gene expression profiles and cell-cell adhesions that are characteristic of an endothelial phenotype, which agrees with previous results using the same cell line [23].

EBseq [66] was then used to identify differentially expressed genes for iPSC-ECs compared to undifferentiated iPS cells (Suppl. Table 2) or iPSC-ECs in 3D (PEG hydrogels) relative to 2D (TCP) culture (Suppl. Table 3). Differentially expressed genes were further analyzed for functional properties by identifying GO terms using the DAVID Functional Annotation Tool [67–70]. Genes upregulated by iPSC-ECs relative to iPS cells were enriched within GO categories that included vasculature development (Fig. 3), biological adhesion, cell motion (GO:0006928), and others that were relevant to vascular morphogenesis (see Suppl. Table 2 for full lists). EBSeq identified 23 upregulated and 16 downregulated vasculature development genes for iPSC-ECs in 3D relative to 2D culture (Fig. 4A; at least one time point). Genes that were upregulated by iPSC-ECs in 3D culture also included 20 regulation of locomotion genes (GO:0040012) and 19 response to hypoxia genes (GO:0001666), whereas genes upregulated in 2D culture were predominantly enriched within GO categories

related to proliferation (e.g., 127 cell cycle genes) (Suppl. Table 3). Upregulated genes in 3D culture also included Notch signaling genes, adhesion genes, and others that regulate mechanisms such as tip and stalk cell specification and branching during angiogenesis (Fig. 4B; NOTCH4, GJA4, GJA5, CLDN5, UNC5B, etc.) [74, 76], which is consistent with observations by time-lapse microscopy.

Vascular morphogenesis is dependent on coordinated interactions between endothelial cells and the ECM, which includes proteolytic matrix remodeling, a balance of cell-cell and cell-matrix adhesions, and deposition of matrix components such as collagens and laminins (e.g., for basement membrane assembly) [16, 17, 74]. Genes that were upregulated by iPSC-ECs in 3D relative to 2D culture included COL1A1, COL1A2, COL6A3, and ITGA1, which encode the $\alpha 1$ and $\alpha 2$ subunits of type I collagen, the $\alpha 3$ subunit of type VI collagen, and integrin $\alpha 1$, respectively (Fig. 4, Suppl. Table 3). Integrin $\alpha 1$ is a cell adhesion receptor for interstitial collagens, as well as ECM components of the basement membrane (collagen IV and laminin-1 [77, 78]) and vascular subendothelium (collagen VI [79, 80]) [81]. Further, endothelial cells have been characterized by increased expression of collagen I during tube formation *in vitro* [82–85] and $\alpha 1$ integrin during angiogenesis *in vivo* [86, 87] and vascular morphogenesis *in vitro* [88]. Additional genes that have been implicated in ECM remodeling during vascular morphogenesis and were upregulated for iPSC-ECs in 3D culture included MMP9 (matrix metalloproteinase 9), TIMP3 (tissue inhibitor of metalloproteinase-3) and NID2 (nidogen-2) [17] (Suppl. Table 3), while other related matrix remodeling and cell adhesion genes were highly expressed in both 2D and 3D culture (Suppl. Fig. 2). Our combined results demonstrate that iPSC-ECs self-assemble in PEG hydrogels through mechanisms that are consistent with *in vitro* and *in vivo* vascular morphogenesis [16, 17, 71, 74].

Finally, vascular network formation was investigated for iPSC-ECs encapsulated in PEG hydrogels that were polymerized within a tri-channel microfluidic device (Figs. 5A) [55]. The passive flow microfluidic format uses standard pipetting to introduce cells and exchange media [55], and such “tubeless” designs can be arrayed and interfaced with automated liquid handlers for enhanced-throughput applications [89]. Vascular network formation was investigated within PEG hydrogels with shear moduli ranging from 183 ± 10 – 1612 ± 95 Pa, which were fabricated by maintaining a constant concentration for the PEG-NB backbone (2 mM 8-arm PEG-NB, 16 mM available norbornene groups) while varying the fraction of MMP-degradable peptide crosslinks (40–60% molar ratio thiol:norbornene) (Fig. 5B). Only limited tubule formation was evident after eight days when 1×10^7 iPSC-ECs per mL were encapsulated in 40–60% crosslinked hydrogels and cultured in basal medium (“Control”, includes 5 ng/mL VEGF, Fig. 5C,E,G). Vascular network formation was improved for each PEG formulation when iPSC-ECs were cultured in basal medium supplemented with 200 ng/mL VEGF (“200 VEGF”, Fig. 5D,F,H), and further optimization for iPSC-ECs encapsulated in 50% crosslinked PEG hydrogels demonstrated that vascular networks were most pronounced at intermediate VEGF concentrations (Suppl. Fig. 3). The optimal VEGF dose qualitatively increased when iPSC-ECs were encapsulated in PEG hydrogels at higher cell density (8.5×10^7 iPSC-ECs per mL), and vascular network formation was completely disrupted at the highest VEGF concentration (1000 ng/mL) (Suppl. Fig. 4). Importantly, capillary tubules with lumens were stable to at least 14 days in

the microfluidic device when 1×10^7 iPSC-ECs per mL were encapsulated in 50% crosslinked PEG hydrogels and cultured in medium that was supplemented with 100 ng/mL VEGF (Fig. 6). Thus, vascular network formation by iPSC-ECs depended on an optimal VEGF concentration that was cell density-dependent, which is consistent with the role for VEGF during vascular morphogenesis *in vitro* [17, 90] and *in vivo* [74, 91].

Previous studies have demonstrated that endothelial cells will self-assemble into vascular networks within engineered matrices under static conditions, but these networks often regress within a few days in the absence of mural cells such as fibroblasts, mesenchymal stem cells, or pericytes [29–34]. The absence of pericytes in PDGF-B and PDGFR- β knockout mouse models leads to hyperplasia and other blood vessel abnormalities, but microvessel density, length, and branching are similar to wild type mice and the mutant animals survive into adulthood [92]. Thus, *in vivo* capillaries retain some level of function even in the absence of pericytes [92], which suggests that a lack of mural cells does not entirely account for regression of vascular networks *in vitro*. HUVECs form *in vitro* networks that are stable for at least five days in collagen when soluble factors are optimized [15], and up to 10 days in starPEG-heparin hydrogels that sequester VEGF, bFGF, and SDF1 α during encapsulation [34]. Here, iPSC-ECs in PEG hydrogels formed capillary tubules with lumens that were stable for at least 14 days in microfluidic channels when VEGF concentration was optimized (Fig. 6). Vascular network stability could also be improved for iPSC-ECs in PEG hydrogels in standard well plates by adding a second cell-free hydrogel layer to provide mechanical support after initial cellular selfassembly (Suppl. Fig. 5). These combined results demonstrate the value of engineering approaches for extending the stability of *in vitro* vascular networks using endothelial cell monocultures.

4. CONCLUSION

The aim of the present study was to develop a vascular model by encapsulating human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) [23] in a synthetic extracellular matrix (ECM) formed via thiol-ene photopolymerization [36]. Time-lapse microscopy, immunofluorescence imaging, and RNA-sequencing (RNA-Seq) demonstrated that iPSC-ECs encapsulated in poly(ethylene glycol) (PEG) hydrogels self-assembled into capillary networks through mechanisms that are consistent with *in vitro* and *in vivo* vascular morphogenesis. Capillary tubules with lumens were stable for at least 14 days when iPSC-ECs were encapsulated in PEG hydrogels that were polymerized within a passive flow microfluidic device. Thus, the *in vitro* model described here mimics important aspects of vascular morphogenesis by incorporating iPSC-ECs into chemically-defined PEG hydrogels using standard and microfluidic formats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance Statement

Human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) cultured in synthetic hydrogels self-assemble into capillary networks through mechanisms consistent with *in vivo* vascular morphogenesis.

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Figure 1. Synthetic extracellular matrix (ECM) formulations for culturing iPSC-ECs
(A) Peptide-functionalized poly(ethylene glycol) (PEG) hydrogels were fabricated as a synthetic extracellular matrix (ECM) permissive towards cellular remodeling. Thiol-ene photopolymerization chemistry was used to crosslink 8-arm poly PEG-norbornene molecules with cysteine flanked matrix metalloproteinase (MMP)-degradable peptide (active sequence in bold, cleavage site denoted by *) and to incorporate CRGDS cell adhesion peptide through the terminal cysteine. **(B)** Calcein (live cells, green) and ethidium homodimer (dead cells, red) staining (**Scale bars:** 200 μ m) and **(C)** the fraction of live cells (Mean $\pm$ S.D., 3 replicate experiments) for iPSC-ECs during the first three days of culture in 50% crosslinked PEG hydrogels.

Figure 2. Expression of characteristic adhesion components by iPSC-ECs cultured in PEG hydrogels

Maximum intensity z-projection images for iPSC-ECs after three days of culture in 50% crosslinked synthetic ECM illustrating (A) CD31 (green), VE-Cadherin (red), and DAPI (nuclei, blue) expression and single channels for (B) CD31 and (C) VE-Cadherin. **Scale bar:** 100 μ m. (D–E) Normalized gene expression (“transcripts per million” or “TPM”) for undifferentiated iPS cells (iPSC), iPSC-ECs in 2D culture on tissue culture polystyrene (TCP), or 3D culture in PEG hydrogels at different time points (Days 1, 2, 3, and 5): (G) PECAM/CD31 and (H) CDH5/VECadherin.

Figure 3. Differentially expressed vasculature development genes for iPSC-ECs relative to undifferentiated iPS cells
LOG2-fold changes in gene expression for iPSC-ECs relative to undifferentiated iPS cells. All differentially expressed genes from the GO category “vasculature development” (GO: 0001944) with an EBseq FDR = 0.005 for at least one time point were included. The iPSC-ECs were cultured on tissue culture polystyrene (TCP) or in PEG hydrogels at different time points (Days 1, 2, 3, and 5).

Figure 4. Differentially expressed vasculature development genes for iPSC-ECs in 2D and 3D culture

(A) Heatmap illustrating LOG2-fold changes in normalized expression for iPSC-ECs in synthetic ECM (3D) relative to cells cultured on TCP (2D). All differentially expressed genes from the GO category “vasculature development” (GO:0001944) with an EBseq FDR 0.005 for at least one time point were included. (B) Normalized gene expression (“transcripts per million” or TPM) for undifferentiated iPS cells (iPSC), iPSC-ECs in 2D culture on tissue culture polystyrene (TCP), or 3D culture in PEG hydrogels at different time points (Days 1, 2, 3, and 5). Select genes relevant to vascular morphogenesis that were

upregulated by iPSC-ECs in 3D culture are shown (* = FDR 0.005 relative to 2D culture on TCP).

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Figure 5. Vascular network formation as a function of crosslinking density for iPSC-ECs cultured in PEG hydrogels that were polymerized within a microfluidic device
(A) A passive pumping tri-channel microfluidic device was fabricated in polydimethylsiloxane (PDMS) (see Bischel et al, *Biomaterials* 34(5):1471–1477). 1×10^7 iPSC-ECs / mL were suspended in a PEG monomer and polymerized within the central channel of the device. **(B)** The PEG hydrogel shear modulus was tuned from 183 ± 10 Pa– 1612 ± 95 Pa by maintaining a constant PEG-norbornene concentration while varying the crosslinking density from 40–60% molar ratio thiol:norbornene. **(C–H)** Maximum intensity

projection confocal z-stacks illustrating phalloidin (F-actin, red) and DAPI (nuclei, blue) staining for iPSC-ECs encapsulated in (C,D) 40%, (C,D) 50%, or (C,D) 60% crosslinked PEG hydrogels. Encapsulated iPSC-ECs were cultured in (C,E,G) basal medium (Control, includes 5 ng/mL VEGF) or (D,F,H) basal medium supplemented with 200 ng/mL VEGF (200 VEGF). **Scale bars:** 250 μm .

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Figure 6. Capillary networks with lumens for iPSC-ECs after 14 days of culture in PEG hydrogels that were polymerized within a microfluidic device
 CD31 (green), VE-Cadherin (red) and DAPI (nuclei, blue) expression for 1×10^7 iPSC-ECs/mL that were suspended in a 50% crosslinked PEG hydrogel and polymerized in the central channel of a tri-channel microfluidic device. Cells were cultured for 14 days in basal medium supplemented with 100 ng/mL VEGF. (A) A maximum intensity z-projection (45 μ m thickness) in the central region of a microfluidic channel. (B) A 1 μ m thick z-slice within the boxed region shown in A. Slice View images through the XZ and YZ planes along the dashed lines are shown to illustrate the organization through the thickness of the channel (45 μ m thickness shown). (C) Zoomed image from region where dashed lines intersect in B illustrating lumen diameter for a larger capillary tubule. **Scale bars:** (A) 250 μ m; (B) 100 μ m.