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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 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.

Statement of Significance

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.

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

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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 prevalidation 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/cm^2 onto tissue culture plates treated with 3 μg/cm^2 fibronectin (Invitrogen) and passaged every 3–4 days with TrypLE (Invitrogen). The manufacturer's recommenced 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% CO_2 for all experiments.

2.1.1. 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 h (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 1X 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 1X 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 (KCG**GPQG*****IWGQ**GCK, GenScript; active sequence in bold; * = cleavage site) [58,59]. To promote cell adhesion [60], 2 mM C**RGDS** (GenScript, active sequence in bold) was incorporated as a pendant group through the terminal cysteine.