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# Human iPSC-derived endothelial cell sprouting assay in synthetic hydrogel arrays ☆

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ABSTRACT

Activation of vascular endothelial cells (ECs) by growth factors initiates a cascade of events during angiogenesis *in vivo* consisting of EC tip cell selection, sprout formation, EC stalk cell proliferation, and ultimately vascular stabilization by support cells. Although EC functional assays can recapitulate one or more aspects of angiogenesis *in vitro*, they are often limited by undefined substrates and lack of dependence on key angiogenic signaling axes. Here, we designed and characterized a chemically-defined model of endothelial sprouting behavior *in vitro* using human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs). We rapidly encapsulated iPSC-ECs at high density in poly(ethylene glycol) (PEG) hydrogel spheres using thiol-ene chemistry and subsequently encapsulated cell-dense hydrogel spheres in a cell-free hydrogel layer. The hydrogel sprouting array supported pro-angiogenic phenotype of iPSC-ECs and supported growth factor-dependent proliferation and sprouting behavior. iPSC-ECs in the sprouting model responded appropriately to several reference pharmacological angiogenesis inhibitors of vascular endothelial growth factor, NF-κB, matrix metalloproteinase-2/9, protein kinase activity, and β-tubulin, which confirms their functional role in endothelial sprouting. A blinded screen of 38 putative vascular disrupting compounds from the US Environmental Protection Agency's ToxCast library identified six compounds that inhibited iPSC-EC sprouting and five compounds that were overtly cytotoxic to iPSC-ECs at a single concentration. The chemically-defined iPSC-EC sprouting model (iSM) is thus amenable to enhanced-throughput screening of small molecular libraries for effects on angiogenic sprouting and iPSC-EC toxicity assessment.

**Statement of Significance**

Angiogenesis assays that are commonly used for drug screening and toxicity assessment applications typically utilize natural substrates like Matrigel™ that are difficult to spatially pattern, costly, ill-defined, and may exhibit lot-to-lot variability. Herein, we describe a novel angiogenic sprouting assay using chemically-defined, bioinert poly(ethylene glycol) hydrogels functionalized with biomimetic peptides to promote cell attachment and degradation in a reproducible format that may mitigate the need for natural substrates. The quantitative assay of angiogenic sprouting here enables precise control over the initial conditions and can be formulated into arrays for screening. The sprouting assay here was dependent on key angiogenic signaling axes in a screen of angiogenesis inhibitors and a blinded screen of putative vascular disrupting compounds from the US-EPA.



## 1. Introduction

Angiogenesis involves a complex interplay between the native extracellular matrix, vascular endothelial cells (ECs), and growth factors [1]. EC functions during angiogenesis have been extensively characterized *in vivo* [1–3], and models of angiogenesis that

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recapitulate multiple EC functions [4,5] can be used as screening tools for toxicity assessment and drug discovery *in vitro*. However, current EC functional assays lack chemically-defined substrates and are thus not well-suited for assessing key angiogenic signaling pathways. The most common *in vitro* models presently used to study EC function and screen for angiogenesis inhibitors can be broadly separated into proliferation [4], sprouting [6,7], and tubulogenesis [4,5] assays. EC proliferation models recapitulate initial EC activation after GF receptor engagement [4] but are not dependent on matrix metalloproteinase (MMP)-dependent activity [8] that drives angiogenic sprouting *in vivo* [6]. EC tubulogenesis models [9,10] recapitulate EC assembly into tubular structures [4,5]; however, EC tubulogenesis shows MMP-dependence only in three-dimensional (3D) contexts [11]. The lack of dependence on MMP activity is a limitation of two-dimensional configurations that are commonly used in small molecule screening applications [12,13]. Furthermore, the vascular endothelial growth factor (VEGF)-dependence is unclear in some tubulogenesis assay platforms [5,14], and this further limits comparisons of EC tubulogenesis to VEGF-dependent *in vivo* vascular formation [1].

EC sprouting models that recapitulate MMP-dependent and VEGF-dependent endothelial cell invasion provide a more physiologic context for modeling early stages of angiogenesis [6,7]. Such models can be used to evaluate the extracellular matrix (ECM) degradation and invasion characteristic of angiogenic sprouting *in vivo*. However, existing assays that use hydrogel-encapsulated, EC-coated microcarrier beads [15–18] or encapsulated EC aggregates [19–21] are limited in their ability to provide a chemically-defined, 3D microenvironment for EC sprouting behavior. Here we demonstrate a unique method to encapsulate ECs at a controlled cell density in cell-dense hydrogel spheres, which are surrounded by a synthetic ECM with defined composition. This approach allows for quantitative analysis of EC sprouting for enhanced-throughput screening in a chemically-defined sprouting model.

We designed a chemically-defined, quantitative assay of angiogenic sprouting behavior using human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) that were previously shown to exhibit stable angiogenic phenotype and function and vascular network formation *in vitro* [22,23]. Thiol-ene photopolymerization was used to rapidly encapsulate iPSC-ECs at high density in poly(ethylene glycol) (PEG) hydrogel spheres that were then embedded in a cell-free over-layer. PEG hydrogel arrays promoted iPSC-EC attachment *via* Cys-Arg-Gly-Asp-Ser (CRGDS) motifs and cell-mediated degradability *via* matrix metalloproteinase (MMP)-labile crosslinking motifs. Culture within the iPSC-EC sprouting model (iSM) supported iPSC-EC angiogenic phenotype and growth factor-dependent iPSC-EC proliferation and sprouting. We evaluated the effects of known angiogenesis inhibitors in iSM arrays and confirmed the role of key angiogenesis signaling pathways in iPSC-EC sprouting. Finally, in a blinded screen of 38 putative vascular disrupting compounds (pVDCs) from the U.S. Environmental Protection Agency's ToxCast library [24–29], we identified six compounds that specifically inhibited iPSC-EC sprouting at a single concentration in agreement with their predicted effects on vascular disruption.

## 2. Materials and methods

### 2.1. Poly(ethylene glycol) derivatization

Eight-arm poly(ethylene glycol) (PEG) terminated on each arm with hydroxyl groups was derivatized with norbornene using a modified procedure [30] and as previously described in detail [31]. All subsequent reagents were purchased from Sigma-Aldrich unless otherwise indicated. Briefly, 8-arm PEG ($M_n$ = 20,000; Jenkem) was reacted under inert gas with ten molar equivalents 5-norborne-2-carboxylic acid, five molar equivalents N,N'-dicycohexylcarbodiimide, half molar equivalent 4-diethylaminopyridine, and five molar equivalents of pyridine in dichloromethane. PEG derivatization with norbornene was verified with $^1$H nuclear magnetic resonance [30].

### 2.2. Generation of elastomeric stencils

iSM arrays were generated using an elastomeric stencil as previously described using a similar technique [32,33]. Briefly, polydimethylsiloxane (PDMS; Sylgard 184; Dow Corning) was prepared according to the manufacturer's instructions. After polymerization, circular wells were created using a 3 mm biopsy punch in 16 groups of 4 (Fig. 1B.i). After trimming to fit the dimensions of a standard microscope slide, PDMS stencils were cleaned in hexanes overnight using a Soxhlet extractor [34]. After cleaning, elastomeric stencils were stored at room temperature to remove residual solvent from the extraction, soaked in 70% ethanol in deionized (DI) water to sanitize, and air dried under aseptic conditions prior to use.

### 2.3. iPSC-EC culture

iPSC-ECs (Cellular Dynamics International, Inc.) were seeded at 4000 cells/cm$^2$ on flasks coated with fibronectin (Corning) and were expanded in Growth Medium (GM) containing 10 vol.% serum supplement (Cellular Dynamics International, Inc.), VEGF LifeFactors kit (LifeLine), penicillin/streptomycin (Gibco), and VascuLife (LifeLine) under normal culture conditions (37 °C, 95% relative humidity, 5% $CO_2$). The VEGF Life-Factors kit used here to formulate the GM contained recombinant human VEGF (5 ng/mL), fibroblast growth factor-2 (FGF-2) (5 ng/mL), insulin-like growth factor-1 (IGF-1) (15 ng/mL), epidermal growth factor (EGF) (5 ng/mL), L-Glutamine (4 mM), hydrocortisone hemisuccinate (1 μg/mL), heparin sulfate (0.75 U/mL), and ascorbic acid (50 μg/mL). iPSC-ECs were dissociated using TrypLE (Gibco) and were assayed between passages 5–8 under normal culture conditions.

### 2.4. Encapsulating iPSC-ECs in cell-dense hydrogel spheres

iSM arrays were formed in two steps on subsequent days (Fig. 1A). On day 0 of the experimental protocol, iPSC-ECs were harvested with Tryp-LE and suspended at $8 \times 10^7$ cells/mL in 0.1 wt.% Irgacure 2959 (Ciba) in phosphate-buffered saline (PBS; Fisher Scientific) and immediately diluted 1:1 in a hydrogel precursor solution for a final solution containing 4 wt.% 8-arm PEG-NB, 2 mM cell adhesion peptide with the sequence Cys-Arg-Gly-Asp-Ser (CRGDS) [35] (GenScript), 3.6 mM MMP degradable peptide crosslinker with the sequence KCGGPQGIWGQGCK (MMP-degradable peptide) [36] (GenScript), and 0.05 wt.% Irgacure 2959. Cells were then encapsulated by exposing 0.5 μL cell-dense spheres to 0.18 J/cm$^2$ UV (measured *via* ultraviolet radiometer) at the end of a 10 μL pipet tip (Fig. 1A#1). Hydrogels containing $4 \times 10^7$ cells/mL iPSC-ECs are hereafter referred to as 'cell-dense hydrogel spheres', and iPSC-EC cell-dense hydrogel spheres were plated into a 16 well ProPlate Slide Chamber (Grace Bio) with the 64-well PDMS stencil described above (creating 4 inner wells within 16 outer wells, Fig. 1B.i). iPSC-EC cell-dense hydrogel spheres were incubated in GM overnight (Fig. 1A#2) under normal culture conditions.

### 2.5. Double encapsulation of iPSC-ECs

On day 1 of the experimental protocol, iPSC-EC cell-dense hydrogel spheres were surrounded by a synthetic hydrogel