Stem Cell Reports
Article

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A Genome-wide Analysis of Human Pluripotent Stem Cell-Derived Endothelial Cells in 2D or 3D Culture

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http://dx.doi.org/10.1016/j.stemcr.2017.02.014

SUMMARY

A defined protocol for efficiently deriving endothelial cells from human pluripotent stem cells was established and vascular morphogenesis was used as a model system to understand how synthetic hydrogels influence global biological function compared with common 2D and 3D culture platforms. RNA sequencing demonstrated that gene expression profiles were similar for endothelial cells and pericytes cocultured in polyethylene glycol (PEG) hydrogels or Matrigel, while monoculture comparisons identified distinct vascular signatures for each cell type. Endothelial cells cultured on tissue-culture polystyrene adopted a proliferative phenotype compared with cells cultured on or encapsulated in PEG hydrogels. The proliferative phenotype correlated to increased FAK-ERK activity, and knockdown or inhibition of ERK signaling reduced proliferation and expression for cell-cycle genes while increasing expression for "3D-like" vasculature development genes. Our results provide insight into the influence of 2D and 3D culture formats on global biological processes that regulate cell function.

INTRODUCTION

In vivo cell function depends on dynamic cues provided by a 3D microenvironment that includes soluble and sequestered biomolecules, an extracellular matrix (ECM), and other cell types (Nelson and Bissell, 2005). Thus, while most in vitro studies in cell biology use planar substrates such as tissue-culture polystyrene (TCP) plates, cellular models have increasingly relied on culture platforms such as reconstituted ECM gels to recapitulate features of native tissues (Simian and Bissell, 2016). In vitro vascular network formation was first reported for endothelial cells more than three decades ago (Folkman and Haudenschild, 1980), and early studies demonstrated the importance of the ECM for guiding capillary network formation (Maciag et al., 1982; Madri and Williams, 1983). Thus, vascular biologists were among the first to widely adopt naturally derived ECM materials such as collagen (Davis and Camarillo, 1996; Davis and Senger, 2005; Montesano et al., 1983), fibrin (Montesano et al., 1987; Nicosia and Madri, 1987), and Matrigel (Arnaoutova et al., 2009; Kubota et al., 1988) for investigating blood vessel formation.

Gels formed from reconstituted ECM proteins retain complex features of native tissue, such as fibrillar structure and cellular adhesion sites, but also introduce experimental uncertainties due to batch-to-batch variability and poorly defined compositions (Hughes et al., 2011; Yang et al., 2009). Limitations inherent to reconstituted protein gels have motivated efforts to design synthetic polyethylene glycol (PEG) hydrogels and other engineered materials that use modular chemistries to provide spatial and temporal control over the presentation of bioactive molecules (e.g., peptides) (Fairbanks et al., 2009; Khalil et al., 2014; Lutolf et al., 2003; Polizzotti et al., 2008; West and Hubbell, 1999); such materials have been used to investigate biochemical and mechanical factors that promote in vitro and in vivo vascularization (Belair et al., 2016; Chwalek et al., 2014; Kusuma et al., 2013; Moon et al., 2010; Nguyen et al., 2014; Phelps et al., 2009; Turturro et al., 2013; Zanotelli et al., 2016). However, while engineered culture platforms have gained acceptance within the biomedical engineering community, reconstituted ECM gels are still viewed as the "gold standard" for modeling tissue assembly in vitro.

The goal of this study was to understand how synthetic hydrogels influence global biological function compared with standard 2D or 3D cell-culture formats. Vascular morphogenesis was chosen as a model biological system,

and a bioinformatics approach was employed to provide genome-wide analysis for H1 embryonic stem cell (ESC)-derived endothelial cells (H1-ECs), primary human pericytes, and human umbilical vein endothelial cells (HUVECs) in 2D or 3D culture. Human pluripotent stem cells provide a scalable endothelial cell source for vascular modeling (Belair et al., 2015; James et al., 2010; Lian et al., 2014; Nguyen et al., 2016; Patsch et al., 2015; Prasain et al., 2014; Wang et al., 2007), and H1-ECs were derived in the present study with >90% efficiency without a sorting step by optimizing a previous protocol (Schwartz et al., 2015). PEG hydrogels formed via thiolene photopolymerization (Fairbanks et al., 2009) were chosen as a synthetic scaffold based on previous studies, which demonstrated that the only bioactive components necessary to promote vascular network formation by primary (Nguyen et al., 2014) and induced pluripotent stem cell (iPSC)-derived (Zanotelli et al., 2016) endothelial cells are matrix metalloproteinase (MMP)-degradable peptide crosslinks (Nagase and Fields, 1996) and pendant CRGDS cell adhesion peptide (Pierschbacher and Ruoslahti, 1984). RNA sequencing (RNA-seq) was used to generate global gene expression profiles (Table S1) for endothelial cells or pericytes in 2D or 3D culture, and differentially expressed genes were evaluated for functional characteristics using The DAVID Bioinformatics Database Functional Annotation Tool (v6.7) (Ashburner et al., 2000; Edgar et al., 2002; Huang et al., 2008). Our combined results provide valuable insight into roles for cellular and extracellular context when modeling cellular self-organization in vitro.

RESULTS

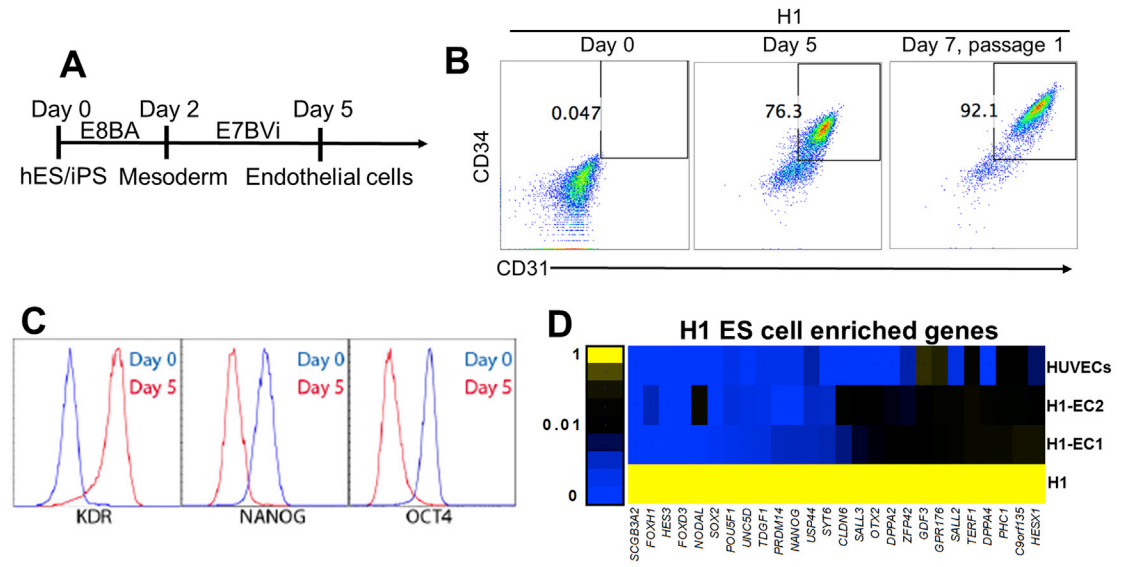
A Fully Defined, High-Efficiency Protocol for Generating Endothelial Cells from Human Pluripotent Stem Cells

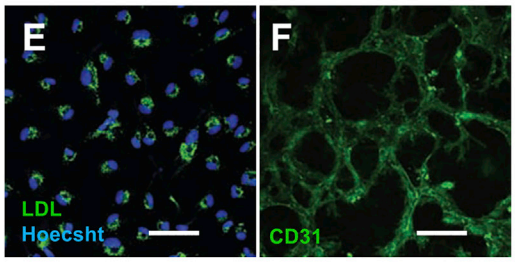
The defined protocol chosen for the present study was previously used to derive endothelial cells from H1-ESCs, which successfully vascularized model neural tissues in vitro (Schwartz et al., 2015). Here, we extended our previous work by deriving endothelial cells from both human ESCs (Thomson et al., 1998) and iPSCs (Takahashi et al., 2007; Yu et al., 2007) to demonstrate the robustness of the protocol and by thoroughly characterizing the endothelial phenotype of H1-ECs. The 5-day protocol proceeds through a mesoderm intermediate step (Yu et al., 2011) and uses defined components that include recombinant vitronectin-coated plates and modified E8 media formulations (Figure 1A) (Chen et al., 2011). H1 ESCs generated 74% ± 2% endothelial cells after 5 days of differentiation and purity was increased to 90% ± 1% with an additional passage (day 7, Figure 1B). Modification of the protocol to include CHIR 99021 (a Wnt agonist) in the mesoderm differentiation medium increased purity to 85% ± 3% and 96% ± 3% for H1-ECs at day 5 and day 7, respectively (Figure S1). The modified protocol also yielded 59%–95% endothelial cells from five additional human PSC lines (Figure S1), and further passage of the DF19-11 (the lowest yield) and PBMC-3-1 (the highest yield) iPSC-derived endothelial cells increased purity to 71% and 99%, respectively.

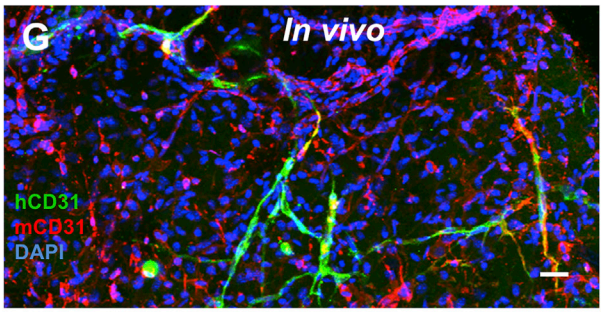
H1-ECs were characterized by upregulated vascular endothelial growth factor receptor 2 (VEGFR-2/KDR) and downregulated NANOG and OCT4 by fluorescence-activated cell sorting (FACS) analysis (Figure 1C), and reduced expression of H1 ESC enriched genes by RNA-seq (Figure 1D; "H1-EC1," E8BA protocol, −CHIR; "H1-EC2," E8BAC protocol, +CHIR). H1-ECs also took up low-density lipoprotein (LDL) (Figure 1E) and formed capillary networks in Matrigel in vitro (Figures 1F and S1) and in vivo (Figure 1G). Spearman's rank correlation analysis demonstrated that H1-EC gene expression was more closely correlated with primary HUVECs than H1 ESCs or pericytes (Figure S2). Finally, differential gene expression analysis via the R package EBSeq (false discovery rate [FDR] $\leq$0.005) (Leng et al., 2013) demonstrated that genes upregulated for H1-ECs and HUVECs over control H1 ESCs were associated with gene ontology (GO) (Ashburner et al., 2000; Edgar et al., 2002; Huang et al., 2008) categories relevant to endothelial phenotypes (Figure 1H and Table S2). These results demonstrate that the defined protocol efficiently produced endothelial cells with characteristic gene expression profiles, protein expression, and functional properties.

H1-ECs and Pericytes Cocultured in PEG Hydrogels or Matrigel Were Characterized by Similar Gene Expression Profiles

Cocultured H1-ECs and pericytes (H1-EC-PC) formed interconnected vascular networks and activated similar patterns of expressed genes in PEG hydrogels with 35%–60% crosslinking density (Figures 2A–2C and S3) or in Matrigel (Figure 2D). H1-EC-PC cocultures were characterized by Spearman's coefficients (ρ) of $\geq$0.98 for all comparisons between PEG hydrogels and Matrigel on the same day (Figure S2), demonstrating that global gene expression profiles were similar for matrices with widely varying properties. EBSeq analysis (Leng et al., 2013) demonstrated that the total number of differentially expressed genes was higher for H1-EC-PC cocultures when comparing PEG hydrogels and Matrigel (on the same day) than for comparisons between PEG hydrogels with different crosslinking densities (Table S3), but that $\geq$85% of the differentially expressed genes for H1-EC-PCs in Matrigel relative to H1 ESCs were also identified in PEG hydrogels on each day (Table S3, "DE Lists"). Genes upregulated by H1-EC-PC cocultures over H1 ESCs in both materials were similarly enriched







H

Top 10 GO terms (H1-ECs > H1 ES cells)	H1-ECs	HUVECs
GO:0001944~vasculature development	61	60
GO:0001568~blood vessel development	60	60
GO:0048514~blood vessel morphogenesis	50	51
GO:0042127~regulation of cell proliferation	97	91
GO:0001525~angiogenesis	37	38
GO:0040012~regulation of locomotion	38	35
GO:0051270~regulation of cell motion	38	38
GO:0007155~cell adhesion	79	89
GO:0022610~biological adhesion	79	89
GO:0032101~regulation of response to external stimulus	34	27

Figure 1. Protocol for Deriving Endothelial Cells from Human Pluripotent Stem Cells

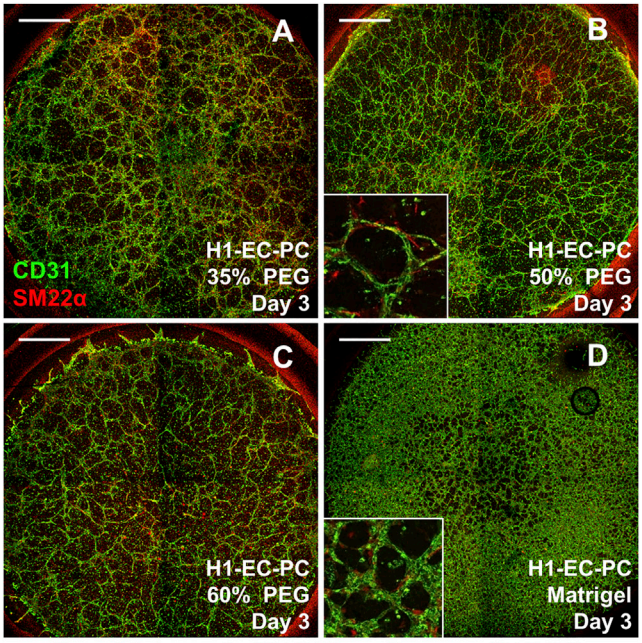
(A) Human PSCs were differentiated into endothelial cells via a mesoderm intermediate (Figure S1 provides representative data for the modified "E8BAC" protocol).

(B) Representative yields ($CD34^+CD31^+$ cells) by flow cytometric analysis for ESC-derived endothelial cells (H1-ECs) after 5 days of differentiation followed by one passage (to day 7).

(C and D) H1-ECs (C) upregulated KDR and downregulated NANOG and OCT4 by FACS analysis and (D) downregulated pluripotent genes by RNA-seq. H1-EC1, original differentiation protocol; H1-EC2, modified differentiation protocol; HUVECs, human umbilical vein endothelial cells.

(E–G) H1-ECs also (E) took up LDL and formed capillary networks in Matrigel (F) in vitro (CD31, green) and (G) in vivo (human CD31, green; mouse CD31, red). For the in vivo condition, 5×10^5 endothelial cells were suspended in 100 μL of E7V medium and 200 μL of Matrigel, injected subcutaneously into the neck of nude mice, and harvested for immunostaining after 2 weeks. Scale bars, 50 μm.

(H) The top ten gene ontology (GO) terms identified using the DAVID functional annotation database for upregulated genes by H1-endothelial cells relative to H1 ESCs cultured on TCP surfaces. Upregulated genes for HUVECs are shown in the same GO categories. Undifferentiated H1 ESCs treated with the same antibodies were used as gating controls for FACS experiments illustrated in (A) and (B). See Table S2 for full DE gene lists.



E Upregulated genes for H1-EC-PC cocultures over H1 ES Cells

GO Category	Both	PEG	Matrigel
GO:0001944~vasculature development	68	71	68
GO:0001568~blood vessel development	67	70	67
GO:0048514~blood vessel morphogenesis	56	59	56
GO:0001525~angiogenesis	42	44	42
GO:0042127~regulation of cell proliferation	97	114	99
GO:0001501~skeletal system development	56	63	57
GO:0007155~cell adhesion	86	97	88
GO:0022610~biological adhesion	86	97	88
GO:0051270~regulation of cell motion	42	46	43
GO:0030334~regulation of cell migration	38	42	39

Figure 2. A Comparison of H1-ECs Cocultured with Pericytes in PEG Hydrogels or Matrigel

(A–D) H1-ECs were cocultured with primary human pericytes (PCs) in (A–C) polyethylene glycol (PEG) hydrogels with 35%–60% MMP-degradable peptide crosslinks or (D) Matrigel. Scale bars, 1000 μm. (E) GO terms for genes upregulated by H1-EC-PC cocultures over H1 ESCs (FDR $\leq$ 0.005) were identified using the DAVID functional annotation database. The top ten GO terms are shown for upregulated genes that overlapped for H1-EC-PC cocultures in both PEG hydrogels and Matrigel on each of the first three days of culture ("Both"). The number of upregulated genes that overlapped on days 1–3 for H1-EC-PC cocultures in PEG hydrogels alone ("PEG") or Matrigel alone ("Matrigel") for the same GO categories are also shown. See Table S3 for full lists of DE genes and GO terms.

within the GO category vasculature development (GO:0001944) and others relevant to vascular network formation (Figure 2E and Table S3, "GO Terms"). These upregulated genes included basement membrane (e.g., *COL4A1*, *COL4A2*, *LAMA4*, *LAMB1*, *LAMB3*) and other ECM components (e.g., *COL1A1*, *COL1A2*, *COL3A1*, *FN1*, *CSPG4*, *HSPG2*), adhesion and cell-cell junction molecules (e.g., *ITGA1*, *ITGA2*, *ITGB3*, *CLDN5*, *CDH5*), and regulators of proteolysis (e.g., *MMP1*, *MMP14*, *TIMP1*, *TIMP3*), Notch signaling (e.g., *DLL4*, *NOTCH4*, *GJA4*), and lumen formation (e.g., *RASIP1*, *CD99*) (Herbert and Stainier, 2011) (Table S3). Similarities between H1-EC-PC cocultures in PEG hydrogels and Matrigel were also evident based on heatmaps for normalized expression of genes associated with vasculature development (Figure 3), ECM components, and cell adhesion (Table S3, "Heatmaps"). Genes upregulated by monocultured endothelial cells in PEG hydrogels or Matrigel over H1 ESCs were also similarly enriched within GO categories relevant to vascular function (Table S2, "GO Summary"). Thus, similar global gene expression patterns are activated for 3D vascular models that use PEG hydrogels or Matrigel.

Differential Gene Expression Provides Insight into Distinct Roles for H1-ECs and Pericytes during Vascular Assembly

A comparison of H1-EC or pericyte monocultures in PEG hydrogels identified distinct patterns of functional genes for each cell type. Characteristic gene expression signatures for H1-ECs and pericytes cultured in PEG hydrogels were defined as genes differentially expressed by EBSeq (Leng et al., 2013) on each of the first 3 days of culture. In total, there were 636 upregulated genes for H1-ECs and 921 upregulated genes for pericytes that overlapped on days 1–3 of culture in PEG hydrogels (Table S4, "H1-EC1"). H1-ECs and pericytes were characterized by distinct patterns of expressed genes associated with vasculature development (Figure 3), cellular adhesion (e.g., integrins), ECM assembly (e.g., collagen, laminin, and fibronectin), and proteolysis (e.g., MMPs). For example, while H1-ECs were characterized by upregulated expression of *PDGFB*, *KDR*, and *CXCR4*, pericytes expressed the complementary genes *PDGFRB*, *VEGFA*, and *CXCL12* (similar results for HUVECs, Table S4, "HUV1"), which is consistent with growth-factor-receptor interactions known to guide blood vessel formation and stabilization (Gaengel et al., 2009).

Both H1-ECs and pericytes cultured in PEG hydrogels expressed ECM genes associated with basement membrane assembly, including collagen IV and laminin isoforms, similar to ECM components previously identified for vascular networks in collagen (Stratman and Davis, 2012) and PEG hydrogels (Moon et al., 2010). However, pericytes were characterized by upregulated expression of several structural ECM genes compared with H1-ECs, including collagen I and collagen III isoforms (*COL1A1*, *COL1A2*, and *COL3A1*). Genes implicated in the assembly of collagen I and III matrices were also upregulated by pericytes, including fibronectin (*FN1*) and α11-integrin (*ITGA11*) (Svendsen et al., 2009; Velling et al., 2002). Finally, pericytes were characterized by enriched α10-integrin (*ITGA10*) and several α10-integrin receptors, including isoforms for laminin-1 (*LAMA1*) and collagen VI (*COL6A1*,

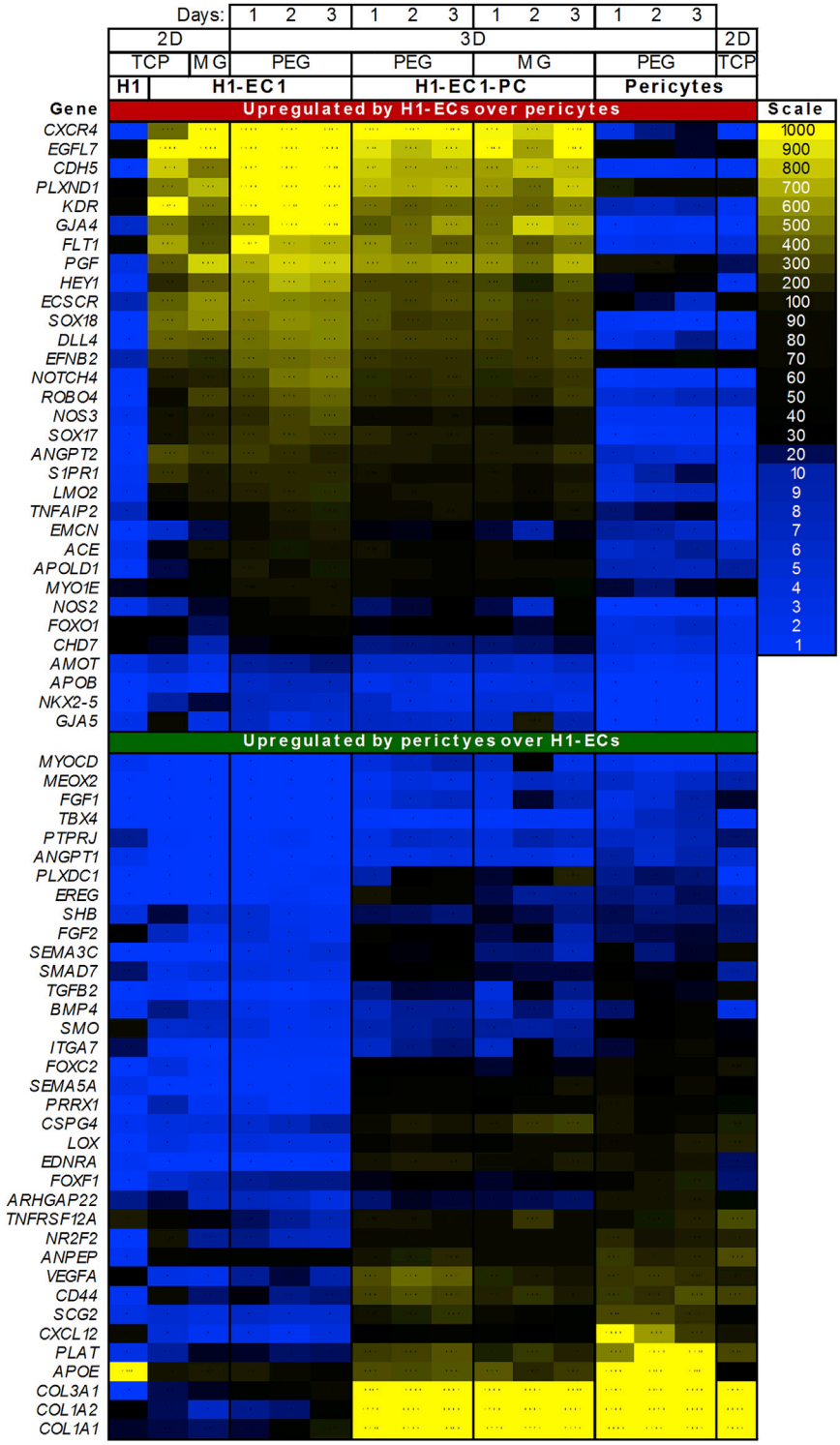


Figure 3. Vasculature Development Genes Robustly Expressed by H1-ECs or Pericytes

Upregulated genes (FDR $\leq$ 0.005) for H1-ECs (top) or pericyte (bottom, reverse order) that overlapped on days 1–3 of culture in PEG hydrogels (data from RNA-seq experiment #1). Genes were ranked within the GO category "vasculature development (GO:0001944)" based on average normalized expression (TPM) for H1-ECs or pericytes in 3D culture on days 1–3. H1 ESCs, 2D monocultured H1-ECs, pericytes, and H1-EC-PC cocultures in PEG hydrogels or Matrigel are shown for comparison. See Table S4 for comparisons of normalized expression for H1-ECs and HUVECs from RNA-seq experiment #2.

COL6A2, and *COL6A3*) (Tulla et al., 2001), which is a collagen subtype that was previously shown to bind von Willebrand factor (*VWF*) within the vascular subendothelium (Engvall et al., 1986; Hoylaerts et al., 1997). Genes upregulated for H1-ECs relative to pericytes included α6-integrin (*ITGA6*) (Bauer et al., 1992) and heparin sulfate proteoglycan (*HSPG2*), which interact with VEGFA to guide vascular branching (Ruhrberg et al., 2002). H1-ECs were

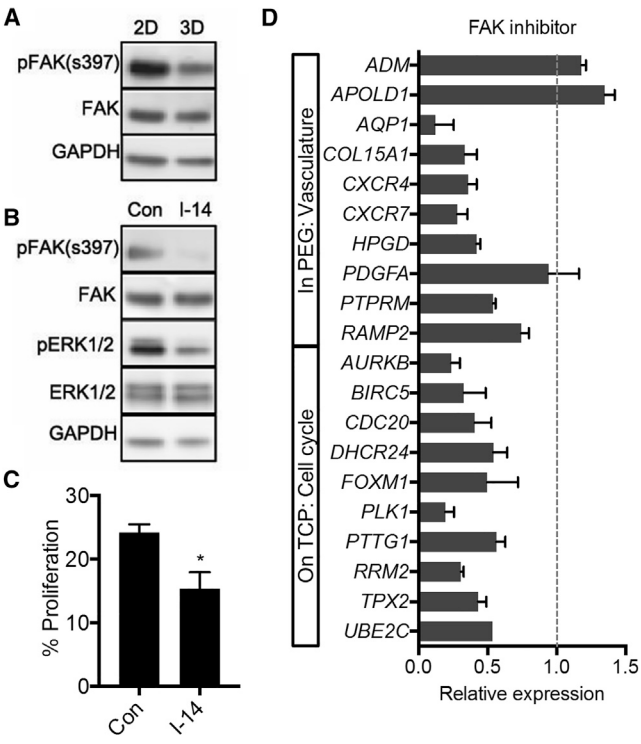


Figure 4. FAK Signaling and Differentially Expressed Genes for H1-ECs Cultured on TCP Surfaces or in PEG Hydrogels

(A and B) Western blot analysis for H1-ECs cultured on TCP surfaces or in PEG hydrogels. (A) Total and phosphorylated FAK expression by H1-ECs cultured on TCP (2D) or in PEG hydrogels (3D). (B) Total and phosphorylated FAK or ERK1/2 expression by H1-ECs cultured on TCP surfaces after treatment with 0 μM (untreated control) or 20 μM Inhibitor-14 (I-14, pFAK inhibitor). Protein was collected 1 day after encapsulation for H1-ECs in PEG hydrogels and 2 days after plating on TCP surfaces (cells were 100% confluent).

(C) I-14 treatment for H1-ECs in 2D culture resulted in decreased proliferation (FACS analysis of 5-ethynyl-2′-deoxyuridine [EdU] expression). Data are presented as mean ± SD (n = 3 independent experiments; *$p < 0.05$).

(D) qRT-PCR was used to investigate changes in gene expression for H1-ECs cultured on TCP surfaces in response to treatment with pFAK inhibitor (I-14). Gene expression was normalized to untreated control (dashed line, RE = 1). Data are presented as mean ± SD (n = 4 technical replicates; triplicate samples prepared from separate cryopreserved vials of the same differentiation were pooled for analysis; $p < 0.05$ for all genes except *ADM* and *PDGFA*). RNA-seq was used to identify differentially expressed genes upregulated by H1-ECs cultured in PEG hydrogels (top, associated with AmiGO term "GO:0001944: vasculature development") or on TCP surfaces (bottom, from the AmioGO term "GO:0007049: cell cycle"), as described in Supplemental Experimental Procedures.

See also Figure S3 and Table S5.

also characterized by upregulated α9-integrin (*ITGA9*), which is an integrin isoform that plays a role during angiogenesis by binding VEGFA and thrombosponin-1 (*THSD1*, also upregulated by H1-ECs) (Staniszewska et al., 2007; Vlahakis et al., 2007). Many differentially expressed genes for H1-ECs and pericytes cultured in PEG hydrogels were also identified for comparisons between HUVECs and pericytes (Table S4). Thus, gene expression patterns for endothelial cells and pericytes cultured in PEG hydrogels are consistent with reciprocal signaling that guides blood vessel formation and stabilization in vivo (Davis and Senger, 2005; Gaengel et al., 2009; Niland and Eble, 2012).

H1-ECs Cultured on Tissue-Culture Polystyrene Surfaces Were Characterized by a Proliferative Phenotype that Was Regulated by FAK-ERK Activity

Upregulated genes for H1-ECs cultured in PEG hydrogels were associated with functional characteristics such as vascular development, cell adhesion, and cell migration compared with cells on TCP surfaces, while downregulated genes (higher expression on TCP) were predominantly associated with the cell cycle and mitosis (Table S5). HUVECs and pericytes were also characterized by upregulated expression of functional genes in PEG hydrogels and cell proliferation genes on TCP surfaces (Table S5), and similar results were previously reported for iPSC-ECs (Zanotelli et al., 2016). Outside-in signaling through focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK/ERK) is important for diverse mechanisms of blood vessel morphogenesis, including proliferation, angiogenesis, and tube formation (Ilan et al., 1998; Niland and Eble, 2012; Yang et al., 2004). Therefore, we investigated potential roles for FAK (Figure 4) and ERK (Figure 5) in mediating the differences in gene expression for H1-ECs cultured in PEG hydrogels compared with cells cultured on TCP surfaces. H1-ECs cultured on TCP were characterized by increased phosphorylation for both FAK (pFAK, Figure 4A) and ERK1/2 (pERK1/2, Figure 5A) compared with cells cultured in PEG hydrogels. Phosphorylated VEGF-receptor 2 (VEGFR2/KDR) expression for H1-ECs cultured on TCP was similar compared with cells cultured in PEG hydrogels (Figure 5A), which indicates that elevated FAK-ERK activity was not correlated with VEGF/VEGFR2 signaling. Inhibition of FAK (I-14; Figures 4B and 4C) or MEK/MAPKK (UO126; Figures 5B and 5C) (Favata et al., 1998), an upstream regulator of ERK phosphorylation (Ahn et al., 1991) each corresponded to reduced pERK expression and decreased proliferation. Therefore, FAK-ERK activity regulated the proliferative phenotype for H1-ECs cultured on TCP surfaces.

Inhibition of ERK Pathway Signaling Rescues Aspects of the 3D Phenotype for H1-ECs Cultured on TCP

Based on our combined results, we hypothesized that gene expression associated with the 3D phenotype could be rescued for H1-ECs cultured on TCP by reducing FAK-ERK

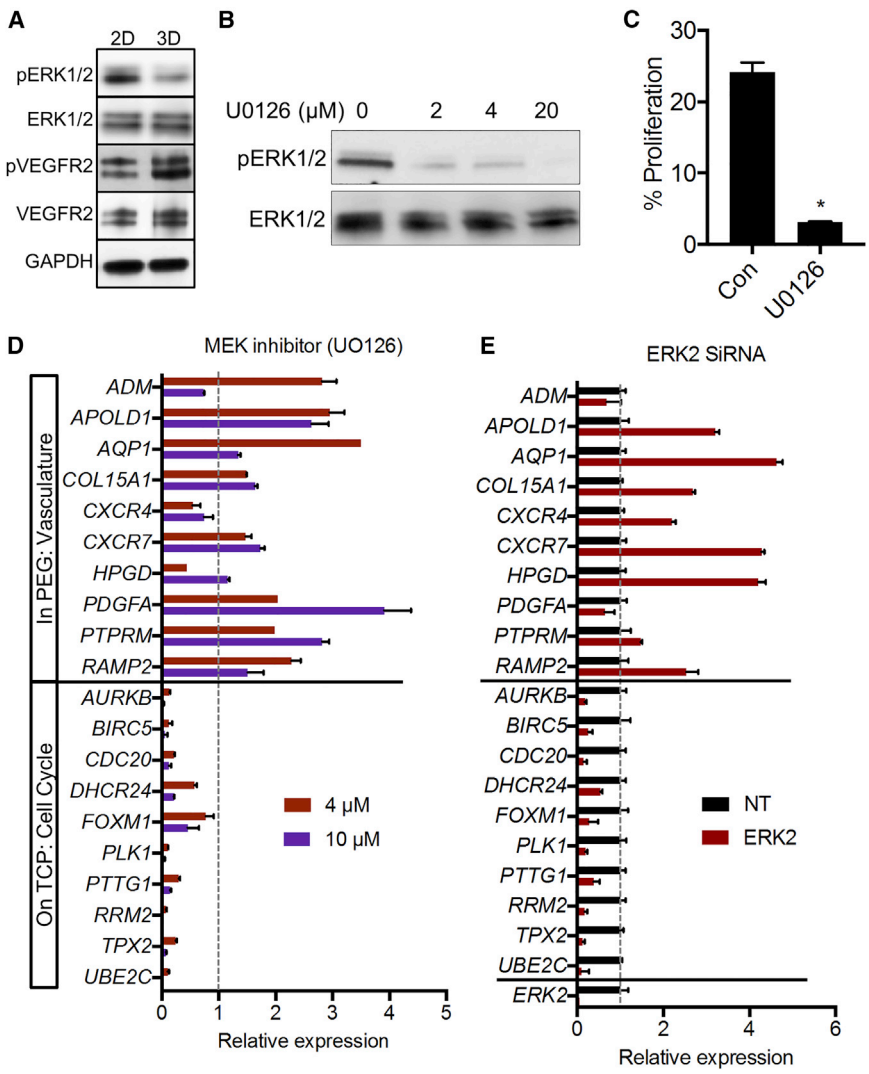


Figure 5. The ERK/MAPK Signaling Pathway Modulates H1-EC Phenotypes for Cells on TCP Surfaces or in PEG Hydrogels

(A and B) Western blot analysis for H1-ECs cultured on TCP surfaces or in PEG hydrogels. (A) Total and phosphorylated ERK and VEGFR2/KDR for H1-ECs cultured on TCP surfaces (2D) or in PEG hydrogels (3D). (B) Total and phosphorylated ERK expression as a function of MEK inhibitor concentration (U0126, μM) for H1-ECs cultured on TCP surfaces. Protein was collected 1 day after encapsulation for H1-ECs in PEG hydrogels and 2 days after plating on TCP surfaces (cells were 100% confluent).

(C) Click-it EdU assay demonstrates that 4 μM U0126 treatment decreases H1-EC proliferation. Data are presented as mean ± SD (n = 3 independent experiments; *$p < 0.05$).

(D and E) qRT-PCR was used to investigate changes in gene expression for H1-ECs cultured on TCP surfaces in response to (D) treatment with the MEK inhibitor U0126 or (E) knockdown of *ERK2* by siRNA. Gene expression was normalized to DMSO control for MEK inhibition and to a non-targeting (NT) control for *ERK2* knockdown by siRNA. Data are presented as mean ± SD (n = 4 technical replicates; triplicate samples prepared from separate cryopreserved vials of the same differentiation were pooled for analysis; $p < 0.05$ for all genes except 10 μM *CXCR4* and 4 μM *FOXM1* for MEK inhibition; $p < 0.05$ for all genes except *ADM* and *PDGFA* for *ERK2* knockdown). RNA-seq was used to identify differentially expressed genes upregulated by H1-ECs cultured in PEG hydrogels (top, associated with AmiGO term "GO:0001944: vasculature development") or on TCP surfaces (bottom, from the AmioGO term "GO:0007049: cell cycle"), as described in Supplemental Experimental Procedures.

See also Figure S3 and Table S5.

signaling. To test this hypothesis, we treated H1-ECs cultured on TCP with inhibitors of FAK and ERK expression, and RT-PCR was used to compare highly expressed cell-cycle genes for H1-ECs cultured on TCP and "3D-like" functional genes identified for cells cultured in PEG hydrogels (Figure S3). FAK inhibition reduced expression for both cell cycle and the 3D-like genes by H1-ECs cultured on TCP (I-14, Figure 4D), which is consistent with the importance of outside-in signaling for regulating diverse cellular functions relevant to blood vessel morphogenesis (Ilan et al., 1998; Niland and Eble, 2012; Yang et al., 2004). However, MEK inhibition (Figure 5D) and *ERK2* silencing by small interfering RNA (siRNA) (Figure 5E) each reduced expression of proliferative 2D genes while also increasing expression for 3D-like genes. Our combined results demonstrate that H1-ECs cultured on TCP adopted a proliferative phenotype that was regulated by FAK-ERK signaling, while 3D-like gene expression could be rescued by reducing ERK pathway signaling.

Cells Cultured on TCP Surfaces Adopt a Proliferative Phenotype Compared with Cells Cultured on Top of or Encapsulated in PEG Hydrogels and Gelled Matrigel

Cells cultured on TCP are exposed to a non-physiological planar surface and a modulus that is orders of magnitude higher than most tissues (Cox and Erler, 2011; Miyake et al., 2006) as well as the PEG hydrogels used here (~200–1,600 Pa for a similar range) (Zanotelli et al., 2016)

or Matrigel (values ranging from ~34 to 450 Pa have been reported) (Semler et al., 2000; Soofi et al., 2009). Therefore, we hypothesized that mechanical properties play an important role for inducing proliferative phenotypes when cells are cultured on TCP surfaces. Consistent with this hypothesis, the proliferation rate for H1-ECs cultured on TCP was higher than for cells cultured on top of (2D seeding) or encapsulated in (3D seeding) PEG hydrogels or Matrigel (Figure S3). Furthermore, EBSeq analysis demonstrated that endothelial cells cultured on TCP surfaces consistently upregulated cell-cycle genes compared with cells cultured on or encapsulated in PEG hydrogels or Matrigel (Tables S5 and S6). However, the proliferation rate was similar for H1-ECs cultured on top of or encapsulated in PEG hydrogels or Matrigel (Figure S3), and upregulated genes were not associated with a proliferative signature for H1-ECs or HUVECs cultured on PEG hydrogels compared with encapsulated cells (Table S6). Differential gene expression analysis did not identify GO terms for either condition when H1-ECs cultured on gelled Matrigel were compared with cells encapsulated in PEG hydrogels (Table S5), and gene expression profiles for H1-ECs cultured on Matrigel were better correlated with cells cultured in PEG hydrogels than cells cultured on TCP surfaces (Figure S3). We note that genes associated with the cell cycle and mitosis were identified for HUVECs cultured on top of gelled Matrigel compared with cells encapsulated in Matrigel (Table S6, see "DE HUV2" tab), although further investigation would be required to demonstrate that this result is due to the 2D environment rather than other factors (e.g., differences in nutrient diffusion). Taken together, our combined results strongly suggest that cells adopt a proliferative phenotype on TCP surfaces due to the stiffness of the substrate.

DISCUSSION

Matrix mechanical properties have been implicated in pathological conditions such as fibrotic diseases and cancer (Cox and Erler, 2011), and increased matrix stiffness has been linked to the disruption of epithelial organization and activation of a proliferative signature through increased FAK-ERK signaling (Provenzano et al., 2009). Here, H1-ECs cultured on TCP surfaces were characterized by increased proliferation, upregulated expression of cell-cycle genes, and increased FAK-ERK activity compared with cells encapsulated in PEG hydrogels. Inhibition or knockdown of ERK signaling for H1-ECs cultured on TCP resulted in reduced proliferation, downregulated expression of cell-cycle genes, and increased expression for "3D-like" vasculature development genes. Proliferation was similar for H1-ECs cultured on or encapsulated in PEG hydrogels, which suggests that the proliferative phenotype on TCP is not an inherent consequence of 2D culture. Importantly, EBSeq analysis demonstrated that cells cultured on TCP surfaces consistently upregulated cell-cycle genes compared with cells cultured on or encapsulated in hydrogels, which was true regardless of cell type (H1-ECs, HUVECs, or pericytes), culture platform (PEG or Matrigel), or surface coating (vitronectin, Matrigel, or poly-L-lysine). Based on these combined results, we speculate that cells cultured on TCP surfaces adopt a phenotype that is inappropriate for studying mechanisms of cellular self-organization due to a modulus that is orders of magnitude higher (Miyake et al., 2006) than PEG hydrogels (Zanotelli et al., 2016) or Matrigel (Semler et al., 2000; Soofi et al., 2009).

Reconstituted basement membrane gels such as Matrigel are widely used to investigate tubule formation by endothelial cells (Arnaoutova et al., 2009; Kubota et al., 1988), and the success of these assays has been attributed to the presentation of a complex mixture of growth factors and ECM components that regulate vascular function. Here, MMP-degradable crosslinks (Nagase and Fields, 1996) and CRGDS cell adhesion ligands (Pierschbacher and Ruoslahti, 1984) were the only bioactive components necessary to promote vascular network formation in PEG hydrogels, which is consistent with previous studies that have reported in vitro and in vivo vascularization using synthetic culture platforms (Belair et al., 2016; Chwalek et al., 2014; Kusuma et al., 2013; Moon et al., 2010; Nguyen et al., 2014; Phelps et al., 2009; Turturro et al., 2013; Zanotelli et al., 2016). Cocultured endothelial cells and pericytes remodel the ECM by producing basement membrane proteins during vascular network formation in collagen (Stratman and Davis, 2012) and PEG hydrogels (Moon et al., 2010), and RNA-seq results here identified gene expression patterns for H1-ECs and pericytes associated with similar ECM components. Therefore, when combined with previous studies (Moon et al., 2010), our results suggest that minimally complex PEG hydrogels provide the necessary cues for cellular self-organization, but that remodeling of the surrounding matrix plays an important role during vascular network formation.

Global gene expression patterns were comparable for H1-ECs and pericytes cocultured in PEG hydrogels with 35%–60% crosslinking density and Matrigel, which was surprising given the wide range of biochemical and mechanical properties tested. However, we note that caution is warranted when comparing culture platforms solely based on global gene expression profiles, since regulation of vascular morphogenesis likely differs at the protein modification level in different materials. For example, while H1-ECs cultured on TCP surfaces were characterized by higher ERK activation than cells in PEG hydrogels, total ERK expression was similar for both formats, and such

protein modification level differences likely exist for 3D culture materials with the wide range of biochemical and mechanical properties tested here. Nevertheless, our results demonstrate that synthetic hydrogels can be designed to regulate global gene expression patterns similarly to a "gold standard" material such as Matrigel by incorporating a minimal set of bioactive components. Taken together, our combined results offer insight into the influence of 2D and 3D cell-culture platforms on global gene expression programs that regulate vascular function.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for additional details.

Cell Maintenance

Human PSCs were maintained in Essential 8 (E8) medium on Matrigel-coated (BD Biosciences, #356230) tissue-culture plates and were passaged with EDTA, except for the 005B23.1 iPSC line, which was maintained on recombinant vitronectin-coated plates (Chen et al., 2011). HUVECs (Lonza) were maintained in EGM growth medium (Lonza) according to manufacturer's instructions. Human brain vascular pericytes (ScienCell) were maintained in Pericyte Medium (Catalog #1201, ScienCell) according to manufacturer's instructions. Purified or passaged ESC/iPSC-derived endothelial cells were cultured on recombinant vitronectin-coated plates (1 μg/cm^2; VTN-N, produced in house) (Chen et al., 2011) and maintained in E7V medium.

Endothelial Cell Differentiation, Purification, and Culture

Human ESCs/iPSCs were passaged with EDTA 2 days before the differentiation to achieve maximum efficiency and consistent results. On the day of differentiation, human ESCs/iPSCs at 80%–90% confluency were dissociated with Accutase (Invitrogen) and plated 1:3 (~1 × 10^5 cells/cm^2) on vitronectin-coated plates (1 μg/cm^2). ESCs/iPSCs were differentiated into mesoderm by culturing for 44 hr in E8BA medium supplemented with 10 μM Y27632 for the first day to improve cell survival (note that 100% cell confluency at day 2 is critical to achieving high efficiency). Mesoderm was then cultured for 3 days in E7BVi medium. Addition of 1 μM CHIR 99021 (Lian et al., 2014) to the mesoderm differentiation medium (E8BAC) and one passage (to day 7) improved differentiation efficiency and eliminated the need for further purification (see Figure S1 and Supplemental Experimental Procedures). Endothelial cells were derived from H1 ESCs using E8BA mesoderm medium for RNA-seq experiment #1 (referred to as "H1-EC1") and from H1-FUCCI ESCs (Leng et al., 2015) in E8BAC mesoderm medium for RNA-seq experiment #2 (referred to as "H1-EC2").

Polyethylene Glycol Hydrogel Preparation

The procedure for fabricating synthetic PEG hydrogels using thiolene photopolymerization was modified from the original protocol (Fairbanks et al., 2009) and has been described in detail (Zanotelli et al., 2016). PEG hydrogels were formed by crosslinking 8-arm PEG-norbornene molecules (20,000 MW, JenKem, 8ARM (TP)-NB-20K) with an MMP-degradable peptide (GenScript, KCG**GPQGIWGQ**GCK, active sequence in bold) (Nagase and Fields, 1996), while cell adhesion was promoted using a pendant CRGDS peptide (GenScript) (Pierschbacher and Ruoslahti, 1984). Cell/monomer solutions were polymerized for 2 min under ~5–10 mW/cm^2 UV light centered at ~365 nm (top shelf of Exposure Stand for UVP XX-15L series UV lamp, Fisher). The gels were then immersed in medium for the duration of the experiment.

Matrigel Assays

3D Matrigel Tube-Forming Assay

A 10-μL monomer/cell solution (1.5 × 10^6 endothelial cells/mL in 5 mg/mL Matrigel) was spotted in a well of a 24-well plate and incubated for 5 min at 37°C for solidification and then cultured for 4 days before harvesting (Figure 1F).

In Vivo Matrigel Plug Angiogenesis Assay

This animal study was conducted with approval from the UW-Madison School of Medicine and Public Health Animal Care and Use Committee. A total of 5 × 10^5 endothelial cells were suspended in 100 μL of E7V medium and 200 μL of Matrigel. The 300-μL mixture was subcutaneously injected into the neck of nude mice and harvested 2 weeks after inoculation (Figure 1G).

RNA Sequencing

RNA-Seq Experiment #1

For further details, see Table S1. Encapsulated cells in PEG hydrogels (30 μL, 35%–50% crosslinking density, 0–2 mM CRGDS) or Matrigel (4.5 mg/mL) were maintained under hypoxic conditions (1.5% O_2) in E7V medium supplemented with 1% BSA. H1-ECs were cultured on top of gelled Matrigel (4.5 mg/mL) under normoxic conditions in E7V medium supplemented with 1% BSA. TCP surfaces were coated with Matrigel (H1 ESCs, "H1-ESC-1"), recombinant vitronectin (H1-ECs, "H1-EC1"; HUVECs, "HUV1"), or poly-L-lysine (pericytes, "PC"), and cells were cultured in maintenance medium (E7V + 1% BSA for H1-EC1) under normoxic conditions. Cells cultured on TCP were 100% confluent when RNA was collected. Samples were prepared using Illumina's TruSeq RNA sample preparation kit and followed a protocol previously described in detail (Zanotelli et al., 2016).

RNA-Seq Experiment #2

H1-ECs ("H1-EC2") or HUVECs ("HUV2") were encapsulated in or seeded on PEG hydrogels (50% crosslinking, 2 mM CRGDS) or gelled Matrigel (6.4 mg/mL) formed in multiwell plates or on TCP surfaces coated with a dilute solution of Matrigel. Samples were prepared followed a custom "LM-Seq" protocol previously described in detail (Hou et al., 2015).

RNA-Seq Data Analysis

The cDNA libraries were pooled and run on Illumina's HiSeq 2500. FASTQ files of nucleotide sequences were generated by CASAVA (v1.8.2) and reads were mapped to the human transcriptome with Bowtie (v0.12.8) (Langmead et al., 2009). Normalized gene expression (transcripts per million reads, or TPM) was calculated by RSEM (RNA-seq by expectation maximization) (v1.2.3) (Li and Dewey, 2011). EBSeq (v1.5.3) was used to calculate differentially expressed genes using the RSEM expected read counts (EC) with the maximum FDR set at 0.005 for all comparisons. EBSeq

for replicate samples was used for RNA-seq experiment #2. Details about the EBSeq algorithms were previously described in detail (Leng et al., 2013). GO terms were identified using the DAVID Bioinformatics Database (Resources version 6.7) Functional Annotation Tool (Ashburner et al., 2000; Edgar et al., 2002; Huang et al., 2008).

Previous Dataset Used in This Study

RNA-seq processing for EBSeq analysis and normalized gene expression for quadruplicate H1-ESC samples (Table S1, "H1-ESC-2") used a previously deposited dataset (Schwartz et al., 2015).

Statistical Analysis

Statistical significance was determined using Student's t test for all quantification except RNA-seq analysis. Data are presented as means ± SD for at least three replicate samples (see figure legends for additional details). Differential gene expression analysis via EBSeq for RNA-seq data is described in the section "RNA-Seq Data Analysis."

ACCESSION NUMBERS

RNA-seq data for this study are available through GEO Series accession number GEO: GSE93511 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE93511).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.02.014.

AUTHOR CONTRIBUTIONS

J.Z. and M.P.S. designed and performed experiments, analyzed data, and wrote the paper. Z.H. performed experiments and analyzed data. A.E., B.K.N., and J.B. performed experiments and processed data. Y.B., V.R., S.S., J.S., and R.S. analyzed data. H.A. performed experiments. W.L.M. and J.A.T. designed experiments. All authors edited the paper.

ACKNOWLEDGMENTS

Derivation and characterization of H1-ECs and RNA-seq experiments were supported by NIH in collaboration with the Defense Advanced Research Projects Agency and the US Food and Drug Administration as part of the Microphysiological Systems (MPS) program (NIH grants 1UH2TR000506-01 and 4UH3TR000506-03). PEG hydrogel development was supported by NIH R21EB016381-01, NIH RO1 HL093282, and the Environmental Protection Agency STAR Center grant 835737. W.L.M. is a founder and stockholder for Stem Pharm, Inc. and Tissue Regeneration Systems, Inc.

Received: September 7, 2016
Revised: February 15, 2017
Accepted: February 16, 2017
Published: March 23, 2017

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