Differential HDAC6 Activity Modulates Ciliogenesis and Subsequent Mechanosensing of Endothelial Cells Derived from Pluripotent Stem Cells

Highlights

- Cilia presentation and response to shear vary among hiPSC-derived endothelial cells (ECs)
- Lack of cilia presentation in hiPSC-ECs impacts initial stages of 3D morphogenesis
- Migration and calcium uptake are aberrant in hiPSC-ECs lacking cilia
- Inhibition of HDAC6 rescues cilia presentation and mechanosensitivity of hiPSC-ECs

Authors

Quinton Smith, Bria Macklin, Xin Yi Chan, Hannah Jones, Michelle Trempel, Mervin C. Yoder, Sharon Gerecht

correponдейnce

gerecht@jhu.edu

In Brief

Primary cilia, an antenna-like structure that protrudes from the apical cell membrane, is essential in endothelial cell (EC) shear responsiveness. Smith et al. show that cilia presentation varies among hiPSC-derived ECs and affects responses to shear, migration, and calcium uptake. Inhibition of HDAC6 restores cilia and mechanosensing of hiPSC-ECs.

Data Resources

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Differential HDAC6 Activity Modulates Ciliogenesis and Subsequent Mechanosensing of Endothelial Cells Derived from Pluripotent Stem Cells

Quinton Smith,1 Bria Macklin,1 Xin Yi Chan,1 Hannah Jones,1 Michelle Trempel,1 Mervin C. Yoder,2 and Sharon Gerecht1,3,4,*

1Department of Chemical and Biomolecular Engineering, Physical Sciences-Oncology Center and the Institute for NanoBioTechnology, Johns Hopkins University, Baltimore, MD 21218, USA
2Department of Pediatrics, Biochemistry, and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA
3Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD 21218, USA
4Lead Contact
*Correspondence: gerecht@jhu.edu
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SUMMARY

The role of primary cilia in mechanosensation is essential in endothelial cell (EC) shear responsiveness. Here, we find that venous, capillary, and progenitor ECs respond to shear stress in vitro in a cilia-dependent manner. We then demonstrate that primary cilia assembly in human induced pluripotent stem cell (hiPSC)-derived ECs varies between different cell lines with marginal influence of differentiation protocol. hiPSC-derived ECs lacking cilia do not align to shear stress, lack stress fiber assembly, have uncoordinated migration during wound closure in vitro, and have aberrant calcium influx upon shear exposure. Transcriptional analysis reveals variation in regulatory genes involved in ciliogenesis among different hiPSC-derived ECs. Moreover, inhibition of histone deacetylase 6 (HDAC6) activity in hiPSC-ECs lacking cilia rescues cilia formation and restores mechanical sensing. Taken together, these results show the importance of primary cilia in hiPSC-EC mechano-responsiveness and its modulation through HDAC6 activity varies among hiPSC-ECs.

INTRODUCTION

Endothelial cells (ECs) withstand a range of pressures and shear stress, mediating the exchange of oxygen, nutrients, and waste to surrounding tissue to support growth and tissue homeostasis. Developmentally, ECs arise from splanchnic mesoderm and self-organize as a vascular plexus in the absence of blood flow. Hemodynamic forces generated from contracting cardiomyocytes and viscous blood is critical to the maturation and morphogenesis of ECs as they extend into the somatic mesoderm by angiogenesis (a process of branching and sprouting of previously established vessels) (Davies, 2009; Lucitti et al., 2007; Moyon et al., 2001; Riha et al., 2005). Zebrafish studies have helped to identify primary cilia as a critical microtubule-based mechanosensory organelle (May-Simera and Kelley, 2012) that is indispensable for proper vascular development. Primary cilia, an antenna-like structure composed of nine outer microtubule doublets, protrude from the apical cell membrane and is present on ECs before the onset of flow. Once shear is introduced, cilia bend to changes in flow, resulting in the translation of physical forces to intracellular molecular signals (i.e., calcium influx), which acts to direct downstream vascular morphogenic events including angiogenesis (Goetz et al., 2014). Furthermore, EC primary cilia are necessary for the recruitment of mural cell populations in the developing vasculature (Chen et al., 2017). Vascular tissue homeostasis also relies on coordinated and deterministic cell migration, a process requiring dynamic cytoskeletal reorganization, and primary cilia presentation. Jones et al. (2012) demonstrate that primary cilia defects can result in endothelial dysfunction including the ability to directionally migrate. While the importance of primary cilia has been shown in vivo and in vitro, the role of cilia presentation in the mechanosensation of developing ECs from human induced pluripotent stem cells (hiPSCs) has yet to be elucidated.

hiPSCs, forged from somatic cells of varying origin, are re-programmed to an embryonic state through the introduction of pluripotent gene sets. This technology offers the potential to generate a renewable source of patient-derived cells, permitting the investigation of human development and the production of differentiated cell types for therapeutic application. A key challenge in the promise of implementing stem cell derivatives in a clinical setting, however, is a thorough evaluation of functionality of the derived population. Generating functional ECs from hiPSCs is a critical goal in vascularizing tissues either pre- or post-transplantation. Indeed, numerous studies demonstrated the derivation of ECs from hiPSCs that can integrate with host tissue (Hu et al., 2016; Park et al., 2014; Rufaihah et al., 2011). Our laboratory has developed a bi-potent differentiation protocol leading to early vascular cells (EVCs) that consist of platelet derived growth factor receptor beta positive/smooth muscle protein 22 alpha positive (PDGFRB+/SM22A+) pericytes and vascular endothelial cadherin (VECad)+/CD31+ expressing ECs (Chan et al., 2015; Kusuma et al., 2013). hiPSC-derived EVCs form three-dimensional vascular networks in natural and synthetic extracellular matrices (Chan et al., 2015; Kusuma et al., 2013; Macklin and Gerecht, 2017; Shen et al., 2016).
A

Aspect Ratio = L/W

Nuclei Alignment

θ

B

ECFCs
HUVECs
HUAECs

F-Actin Dapi

20 dynes/cm²

5 dynes/cm²

Nuclei Alignment (θ)

*** ***

C

HUVECs
ECFCs

Acetyl α-tub F-Actin Dapi

% Ciliated Cells

0 hr 24 hr

D

Acetyl α-tub F-Actin Dapi

1 μM Cytochalasin D

10 μM Nocodazole

10 μM Cyto-D

NS

Legend on next page
Promisingly, these vascular networks not only survive implantation, but are able to establish blood flow, integrating with host vasculature. In addition to this work, other more clinically relevant differentiation approaches, utilizing serum-free media (Smith et al., 2017) and the addition of glycogen synthase kinase 3 (GSK3) inhibitor CHIR 99021, have led to hiPSCs that can be efficiently differentiated to mesodermal progenitors capable of EC maturation (Bao et al., 2015; Lian et al., 2014; Orlova et al., 2014a, 2014b; Patsch et al., 2015). Furthermore, ECs with greater specificity can be derived from hiPSCs. For example, endothelial colony-forming cells (ECFCs), a population of ECs with high clonal proliferative potential and in vivo wound healing capacity, have been derived from various hiPSCs and have been shown to exhibit in vivo functionality (Prasain et al., 2014). Collectively, while ECs can be differentiated from varying sources using many techniques, in vitro characterization of hiPSC-EC mechanor-sensitivity is an additional parameter in EC functionality that extends beyond gene and surface marker expression. Toward this, we evaluated primary cilia presentation, shear response, and in vitro wound healing capacity of hiPSC-ECs differentiated with varying techniques and explored hiPSC-ECs from varying hiPSC origins.

First, using an established microfluidic platform (Abaci et al., 2014), we evaluated the role of primary cilia in both mature and progenitor ECs, and found shear responsiveness was largely correlated to cilia presentation. Microtubule destabilization leads to the loss of primary cilia and loss of ability to align to shear stress. When analyzing hiPSC-derived ECs, we found that while the derivatives with compromised cilia presentation were able to undergo in vitro vasculogenesis in collagen gels, they were unable to phenotypically align to shear stress, migrate coordinately in in vitro wound healing assays, or uptake calcium uniformly after shear application. We verified that differentiation procedure had marginal influence on cilia presentation and found genes relating to ciliogenesis were differentially expressed between ciliated and non-ciliated hiPSC-ECs. These findings motivated us to examine histone deacetylase 6 (HDAC6) activity, a cytoplasmically localized post-transcriptional regulator, which deacetylates cytoplasmic structures including cortactin, heat shock protein 90 (HSP90), and α-tubulin (Kaluza et al., 2017). We found that small-molecule inhibition of HDAC6 rescued the ciliated phenotype in non-ciliated hiPSC-ECs, as well as improved their mechanosensation. Collectively, these results demonstrate the putative role of HDAC6 activity in hiPSC-EC phenotype and mechanosensing. These findings have important implications in hiPSC-EC functionality and therapeutic prospective.

RESULTS

Cytoskeletal Integrity Regulates Cilia Presentation and Shear Response

In recognition that ECs exhibit functional heterogeneities (Aird, 2007), the dose-dependent shear responsiveness of primary progenitor (ECFCs) and mature (human umbilical vein endothelial cells [HUVECs] and human umbilical arterial ECs [HUAECs]) ECs were evaluated. We used a modified version of our previously described microfluidic platform (Abaci et al., 2012b, 2014) that allows continued cell culture under low and high shear stresses (Figure S1A; Figure 1A). Human ECFCs, HUVECs, HUAECs, and human cerebral microvascular EC (hCMEC)/D3 ECs were analyzed pre-arterial and post-arterial (20 dynes/cm²) and venous-mimicking (5 dynes/cm²) shear stresses (Figures S1B and S1C). Shear exposure to 20 dynes/cm² induced extensive nuclear alignment and cytoskeletal elongation oriented parallel to the applied flow as previously described (Abaci et al., 2014). After 24 hr of 20 dynes/cm², all cell types aligned with the direction of the flow. Quantification demonstrated that ECFCs, HUVECs, and HUAECs had similar aspect ratios (ARs) and nuclei orientation (Figure 1B). Evaluating responses to 5 dynes/cm², we found that while HUAECs and ECFCs oriented after 24 hr, HUVECs remained unaligned with a 1.2 ± 0.1 AR and 39 ± 1-degree orientation (Figure 1B). Overall, we show that ECFCs and HUAECs demonstrate plastic shear responsiveness under 20 and 5 dynes/cm² shear regimes in comparison with HUVECs, which did not respond after 24 hr of 5 dynes/cm² shear application. To this end, we focused our efforts on 20 dynes/cm², which resulted in robust alignment across multiple ECs after 24-hr exposure.

The platelet EC adhesion molecule CD31, adherens junction VEcad, and vascular endothelial growth factor receptor 2 (kinase insert domain receptor [KDR]) mechanosensory complex has been implicated as a key determinant of endothelial shear
responsiveness (Coon et al., 2015; Lagendijk et al., 2017; Tzima et al., 2005) and has been thoroughly characterized in mature and progenitor EC types (Kusuma et al., 2012). Here, we speculated that in addition to this necessary mechanosensory complex, the presence of primary cilia might serve as an additional mechanotransducer and account for variation in shear response behavior in vitro. To explore this notion, we first evaluated cilia prevalence in cell-cycle arrested cells following 24 hr of serum starvation. We found that HUVECs and ECFCs display primary cilia (Figure 1Ci) evidenced by acetylated α-tubulin staining (Figure 1Ci), with increasing frequency in the absence of serum (Figure 1Cii). HUAECs did not display acetylated microtubule extensions from their apical surface. Nonetheless, HUAECs aligned with the flow direction, suggesting that even in vitro, ECs, which experience high degrees of shear stress in vivo, do not necessitate a primary cilium.

Because primary cilia are linked to cytoplasmic microtubules through the basal body, we examined whether temporary disruption of microtubules in ciliated cells would result in their inability to sense the onset of shear. We accessed the effect of cytoskeletal disruption in serum-starved HUVECs and ECFCs incubated with either cytochalasin D, a potent inhibitor of F-actin polymerization, or nocodazole, a small molecule that disrupts microtubule assembly. We found that when actin polymerization was inhibited, evidenced by diffuse phalloidin staining and absence of stress fibers throughout the cell body, ciliary contents remained intact (Figure 1Di). When polymerization of microtubules was inhibited by nocodazole, on the other hand, we found that F-actin stress fibers remained intact; however, acetylated microtubules appeared unorganized with unidentifiable primary cilia (Figure 1Dii). We next investigated the consequences of cytoskeletal disruption and EC shear alignment. We found that the initial loss of actin polymerization in HUVECs did not prevent primary cilium assembly after serum starvation or cytoskeletal elongation after 20 dynes/cm² shear application (Figure 1Di and iii). When ECFCs were treated for 1 hr with 10 μM nocodazole after 24 hr of serum starvation, microtubule organization, identified with acetylated α-tubulin, appeared disorganized, and a cilium extending from the microtubule-organizing center could not be identified. When ECFCs were sheared after temporal microtubule disruption with nocodazole, we measured a lower AR with no change in nuclei orientation compared with sheared, untreated ECFCs (see Figure 1B). These sheared, nocodazole-treated ECFCs also exhibited a dense F-actin assembly at the cortical actin rim, lacking stress fibers throughout the cell body parallel to the applied shear (Figure 1Dii and iii). Collectively, these results demonstrate that microtubule integrity in ECs influence ciliogenesis and capacity to align to shear forces.

**hiPSC-BC1-Derived ECs Lack Primary Cilia and Shear Responsiveness**

Next, we evaluated the potential for stem cell-derived ECs to form primary cilia and respond to shear stress in vitro. In these studies, we used the hiPSC-BC1 cell line because of its integration-free reprogramming (Chou et al., 2011), reproducibility in our differentiation protocols (Chan et al., 2015; Kusuma et al., 2013; Wanjare et al., 2014), and full genetic characterization (Cheng et al., 2012). Generation of EVCs from BC1 resulted in the bi-potent generation of both ECs and pericytes. hiPSC-derived ECs exhibit classic functional hallmarks, including the ability to uptake acetylated low-density lipoprotein and express von Willebrand factor (vWF). Both EVCs and derived ECs from the BC1 line have been shown to generate functional vascular networks in vitro, using both three-dimensional (3D) natural and synthetic hydrogels, survive implantation, and integrate with host vasculature in vivo (Chan et al., 2015; Kusuma et al., 2013; Macklin and Gerecht, 2017; Shen et al., 2016).

Here, we found that the ECs contained diffuse F-actin staining with little to no stress fiber presentation, whereas pericytes demonstrated robust cytoskeletal architecture (Figure 2A).

When evaluating primary cilia presentation, we could not detect cilia in VECad⁺ cells in the EVC population, even under serum starvation conditions (Figure 2Bi and ii). Using our microfluidic platform, we exposed EVCs to both 5 and 20 dynes/cm² laminar shear stress and found that VECad⁺ cells did not align in the direction of shear after 24 hr, maintaining an AR similar to cells under static conditions (Figure 2C). Interestingly, it should be noted that under 20 dynes/cm² shear stress, SM22A⁺ pericytes elongated and oriented perpendicular to the applied flow (Figure 2C).

Because EVC-VECad⁺/CD31⁺ cells did not possess primary cilium following serum starvation and did not align or elongate in response to shear stress, we next examined hiPSC-ECs. We followed our established protocol to sort and expand VECad⁺ cells. Sorted VECad⁺ cells (i.e., passage 0) demonstrated cytoplasmic punctate expression of vWF and cytoplasmic endothelial nitric oxide synthase, while absent for perivascular marker SM22A⁺ (Figures S2A and S2B). With continued passage, we found that the expression of VECad and CD31 remained consistently high, coinciding with a lack of perivascular PDGFRβ marker expression (Figures S2C and S2D). Nonetheless, acetylated α-tubulin staining remained disorganized, with very few ciliary projections originating from the microtubule organizing center (Figure S2E). We next examined the shear responsiveness of hiPSC BC1-ECs and found slight changes in the AR, with retained static nuclei orientation following exposure to 20 dynes/cm² for 24 hr (Figure 2D). Once again, the isolated perivascular population from hiPSC-BC1 contained primary cilia and phenotypically aligned perpendicular to the applied shear (Figures S2F–S2H).

**hiPSC Line, Not Differentiation Protocol, Dictates Shear Responsiveness**

To determine whether the lack of primary cilia and shear responsiveness in hiPSC-BC1 EC derivatives were consequential to our differentiation protocol, we evaluated ciliogenesis and morphological changes of BC1-ECs to shear stress with a slightly modified differentiation scheme using serum-free mesoderm induction (E6) media (Smith et al., 2017). BC1-ECs derived using E6 did not align in response to shear (Figures S3A and S3B). We next found that BC1-ECs derived using E6 with GSK-3β inhibitor CHIR-99021 during mesoderm induction (E6-CHIR) were also unable to elongate in response to shear even when exposed for 96 hr (Figures 3Ai, 3Di, and 3Dii). In addition, we observed that BC1-ECs lacked primary cilia extensions and cytoskeletal stress fibers (Figures 3Ai, 3Dii, and...
3Div). In addition to cell-cycle-induced ciliogenesis, microtubule stabilization and primary cilia formation can occur independent of cell proliferation. For example, the disruption of actin-polymerization with cytochalasin D increases the pool of soluble cytoplasmic tubulin, leading to the polymerization of long primary cilia (Sharma et al., 2011). Cytochalasin D is a strong positive effector on the length and ability for cells to form primary cilia, and has been demonstrated in many systems for studying the effects of cytoskeletal regulation and ciliogenesis (Keeling et al., 2016; Kim et al., 2010; Pitaval et al., 2010). From our results, we hypothesized that inhibition of F-actin polymerization with cytochalasin D would be sufficient to induce cilia in the BC1-ECs. However, we did not observe an increase in primary cilia prevalence when treated with cytochalasin D (Figure S3C). Next, we differentiated another hiPSC line (C12) using the E6-CHIR protocol. We compared the

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**Figure 2. Responsiveness to Shear of EVCs and ECs Derived from hiPSC-BC1 Line**

(A) (i) Immunofluorescence staining of EVCs showing both ECs and pericytes. (ii) Quantification of traced F-actin stress fibers in VECad+ and VECad− cells. (B) (i) EVCs stained for cilia 0 and 24 hr after serum starvation. (ii) Quantification of EVC cilia before and after serum starvation (n = 883 control; n = 258 serum starved). Statistical significance and analysis were determined using one-way ANOVA with Bonferroni post hoc tests. White arrowheads show primary cilia. (C) (i) EVCs seeded in microfluidic device before and after 5 or 20 dynes/cm² shear application. (ii) Nuclei alignment and aspect ratio of VECad+ and SM22a+ cells quantified from Ci. (D) (i) Cilia staining for hiPSC-BC1 ECs. (ii) hiPSC-BC1 ECs 24 hr after 20 dynes/cm² shear application. (iii) Quantification of nuclei alignment and aspect ratio in comparison with unaligned static controls (blue dotted line). Shear stress and immunostaining were conducted in at least two biological replicates. Aspect ratio analysis was conducted on n ≥ 50 and nuclei alignment on n > 200 cells. All graphical data are reported as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.
expression of the key EC mechanosensory complex (CD31/VECad/KDR) on day 8 of differentiation, as well as arterial or venous specification once ECs were isolated, and did not detect significant differences between the BC1 and C12 E6-CHIR derivatives (Figures S3D and S3E). We also examined the fully characterized ECFCs derived from hiPSCs (Prasain et al., 2014), as an early developmental state of ECs. Surprisingly, C12-ECs formed cilia projections, aligned in response to shear stress in 24 hr, and contained stress fibers throughout the cell body (Figures 3Bi, 3Bii, and 3D). Moreover, hiPSC-ECFCs also aligned to shear stress, formed long primary cilia extensions, and contained stress fibers organized throughout the cell body (Figures 3Ci, 3Cii, and 3D). We found that lysates from serum-starved BC1-ECs had reduced acetylated α-tubulin protein expression via western blot (Figure 3E), consistent with our immunofluorescence images when compared with the other derived ECs. Overall, these results suggest that F-actin destabilization is unable to rescue cilia prevalence in BC1-ECs, and that iPSC derivatives from different sources have the capacity to form primary cilia and phenotypically align to shear stress.

**Effects of Cilia Presentation in Vascular Morphogenesis of hiPSC-ECs**

After establishing BC1-ECs were unable to align to shear stress, we explored additional functional consequences of lacking...

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**Figure 3. Shear Responsiveness, Cilia, and Stress Fibers in hiPSC-ECs**

(A–C) BC1 E6-CHIR (A), C12 E6-CHIR (B), and hiPSC-ECFCs (C) were assessed for (i) shear responsiveness (acetylated α-tubulin in red, VECad in green, and nuclei in blue) and (ii) cytoskeletal organization (F-actin in red, acetylated α-tubulin in green, and nuclei in blue).

(D) Quantification of (i) nuclei alignment and (ii) aspect ratio after arterial shear exposure compared with unaligned static controls (dotted blue line). In addition, (iii) percentage of ciliated cells and (iv) number of stress fibers per cell were quantified.

(E) Protein expression of cytoplasmic (C) and nuclear (N) fractions of acetylated α-tubulin with GAPDH and HDAC1 as endogenous controls, respectively. Cilia and stress fiber quantification are shown from at least three independent differentiations, using five fields of view for image quantification (BC1-E6 CHIR: n = 813 serum starved; C12 E6-CHIR: n = 1428 serum starved; hiPSC-ECFCs: n = 197 serum starved). Statistical significance and analysis were determined using one-way ANOVA with Bonferroni post hoc tests. Shear stress experiments are shown from at least biological duplicates. Aspect ratio analysis was conducted on n ≥ 50 and nuclei alignment on n > 200 cells.

All graphical data are reported as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001. White arrowheads show primary cilia.
primary cilia amidst the different stem cell-derived ECs. We first evaluated the effects of EC cilia phenotype and the ability to form vascular networks. Work from Norden et al. (2016), using early-stage developing zebrafish, describes how endothelial polarization events and subsequent lumen formation rely on tubulin acetylation and its regulation through HDAC6 expression (Kim et al., 2013). We examined these findings by utilizing an in vitro 3D relevant model using collagen gels (Davis and Senger, 2005, 2008). Here, we focused on comparing BC1 with C12 E6-CHIR ECs, as we confirmed similar shear-related mechanosensory protein expression (CD31/VECad/KDR) and transcript activity denoting arterial or venous specification (see Figures S3D and S3E). We began by monitoring self-assembly kinetics with time-course evaluation of vacuole growth, a necessary step in lumen formation. Although we found that the ability to form primary cilia affects early-stage vacuole formation in iPSC-ECs (Figures 4A–4C), vascular network characteristics were unaltered after 2-day encapsulation in collagen gels (Figure 4D).

**Aberrant Cell Migration and Shear Induced Calcium Uptake in Non-ciliated hiPSC-ECs**

The ability for coordinated EC migration in response to a wound has been shown to be a factor of stress fiber organization and cilia presentation (Jones et al., 2012). To this end, using an in vitro scratch wound healing assay, we investigated whether the lack of primary cilia in BC1-ECs could impair directional cell migration in comparison with C12-ECs derived using the same differentiation protocol and hiPSC-ECFCs. We found that wound closure was impaired in BC1-ECs, evidenced by the inability to fully close the wound (Figure 5A) and lack of directional migration toward the site of injury (Figures 5B and 5C).

We next evaluated whether calcium uptake during shear stress served as an additional functional readout to compare ciliated and non-ciliated hiPSC-ECs. Previous studies have demonstrated that the loss of EC primary cilia leads to a reduced influx of cytosolic calcium levels (Nauli et al., 2008), a phenomenon we observed in our mature controls (Figure S4). hiPSC-ECs seeded within our microfluidic platform were loaded with Fluo-4, AM and imaged while exposed to a step waveform of shear, allowing a relaxation after 20 dynes/cm² shear application (Figure 5D). Increased cytosolic calcium levels in ECs exposed to shear stress were measured by normalizing fluorescence signal in cells statically cultured before shear application (Figure 5E). BC1-ECs demonstrated large heterogeneity in calcium uptake as a function of shear stress in comparison with C12-ECs and hiPSC-ECFCs. This observation of reduced calcium uptake at the onset of shear was consistent with immortalized hCMEC/D3 cells, non-ciliated brain ECs (data not shown), and HUVECs treated with nocodazole (Figure S4).
Transcriptional Variance between hiPSC Source Unveils PLK-1/HDAC6 Imbalance in Non-ciliated ECs

To gain insight into the phenotypic differences in primary cilia assembly among the lines, we performed cDNA microarray analysis to transcriptionally profile hiPSC-ECs deprived of serum after 24 hr. This array was specifically designed to evaluate differentiation state, endothelial identity, and relative expression of genes responsible for cilia assembly and disassembly. Suppression of cell-cycle progression through serum starvation acts to reduce mitogenic re-entry into the cell cycle, a process requiring the activation of mitotic Aurora-A kinase and polo-like kinase 1 (PLK-1). We found markers related to cilia resorption (Figure 6A) were reduced in BC1-ECs compared with C12-ECs and hiPSC-ECFCs. hiPSC-ECFCs consistently had higher levels of cilia assembly and disassembly machinery, namely, AKT1, IFT88, and AURKA and PLK1, respectively. No conclusive differences could be determined from the relative expression levels of pluripotent, endothelial, and cytoskeletal markers between ciliated and non-ciliated hiPSC-ECs (Figure S5). Interestingly, we observed centrosomal-rich localization of PLK-1, a key regulator in primary cilia disassembly before mitotic entry, accompanied by primary cilia in C12-ECs and hiPSC-ECFCs, while in BC1-ECs, PLK-1 co-localized with tubulin bodies (Figure 6B). In addition, we found that cilia length varied among the different hiPSC-ECs, suggesting that in addition to cilia formation, primary cilia length may also contribute to their mechanosensing capacity (Figure 6C).

We thus speculated that the PLK-1 pericentriolar material accumulation in BC1-ECs, evidenced by co-localization with tubulin bodies, resulted in primary cilia disassembly through activation of HDAC6, a tubulin deacetylase implicated in cilia resorption. This hypothesis was examined by measuring HDAC6 expression using western blot. Whereas acetylated α-tubulin levels were decreased in BC1-ECs (see Figure 3E above), we found higher levels of HDAC6 expression in BC1-ECs and C12-ECs compared with hiPSC-ECFCs (Figure 6D). Taken together, our results suggest an hiPSC-specfic sensitivity in transcriptional regulation of primary cilia machinery. Here, imbalance between PLK-1 accumulation and HDAC6 expression in BC1-ECs dictated acetylated α-tubulin levels and primary cilia phenotype.

Cilium Presentation in hiPSC-ECs Corresponds to HDAC6 Levels

Because acetylated α-tubulin serves as a substrate for HDAC6, we speculated that although HDAC6 levels appeared similar
between BC1-ECs and C12-ECs, its accumulation at the primary cilium along with PLK-1 might lead to its resorption and length restriction. To study the role of HDAC6 in cilium presentation in BC1-ECs, we performed serum starvation with varying concentrations of Tubastatin A, a potent and selective inhibitor of HDAC6 deacetylase activity. Tubastatin treatment led to increased levels of acetylated α-tubulin among all the hiPSC-ECs (Figure S6A) and rescued the ability for BC1-ECs to form primary cilia, by decreasing HDAC6 protein expression through reducing the deacetylation of α-tubulin (Figures 7A and 7B).

Although gap closure remained the same, Tubastatin A treatment improved BC1-EC directional migration in the in vitro scratch wound healing assay (Figures 7C and 7D; Figure S6B). To stay consistent with the media composition in our shear stress assays, we next tested whether HDAC6 inhibition could circumvent serum starvation and promote ciliogenesis in HUVEC growth media. We found that cilia could be recovered, but BC1-EC migration potential after Tubastatin A treatment in wound healing assays led to minimal recovery (Figures S6C–S6E). Promisingly, we found that exposing Tubastatin A-treated BC1-ECs to shear stress (20 dynes/cm²) for 24 hr resulted in perpendicular alignment, indicative of partial recovery of shear sensitivity (Figures 7E and 7F). Overall, these data suggest that differential HDAC6 deacetylase levels in BC1-ECs correlate to primary cilia instability, and that small-molecule inhibition of HDAC6 activity rescues cilia formation in BC1-ECs, partially recovering their mechanosensation to shear stress.

**DISCUSSION**

Cardiovascular development and homeostasis rely on endothelial mechanotransduction of shear forces and is a critical parameter when accessing the functionality of stem cell-derived endothelium and potential for therapeutic application. The staple phenotypic response of endothelium to laminar shear stress involves microtubule, intermediate filament, and actin reorganization, resulting in a directionally aligned and elongated cell, transitioning from a cobblestone-like shape present in static culture (Dartsch and Betz, 1989). Using an engineered in vitro microfluidic platform, we evaluated the shear responses of a wide array of ECs including progenitor ECFCs and mature HUVECs, HUAECs, and hiPSC-ECs from varying sources. Among the mature and progenitor cell sources that were evaluated in this study, we found that all aligned and elongated to arterial (20 dynes/cm²) flow regimens after 24 hr of shear exposure.
Acetyl CD in vitro shear stress has been shown to rapidly disassemble cilia which are exposed to oscillatory low shear forces, are highly contribute to the degree of ciliogenesis within the vasculature. anatomical differences between arterial and venous ECs in endothelial biology. Previous work has demonstrated that responsive antenna-like structure, has been under-evaluated can be attributed to many mechanisms, the presentation of further explored. Consistent with these reports, we found that coping vasculature, the homology to human cells should be robust insight in the consequence of ciliary defects in the devel-

Even though the variability in EC shear responsiveness can be attributed to many mechanisms, the presentation of microtubule-based primary cilia, a cytoskeletonally linked shear responsive antenna-like structure, has been under-evaluated in endothelial biology. Previous work has demonstrated that anatomical differences between arterial and venous ECs contribute to the degree of ciliogenesis within the vasculature. Venous ECs or atheroprone regions within the vasculature, which are exposed to oscillatory low shear forces, are highly ciliated in vivo. Comparably, only ~25% of arterial ECs, which are narrow and elongated, have primary cilia, because high shear stress has been shown to rapidly disassemble cilia in vitro (Davies, 2009; Hierck et al., 2008; Iomini et al., 2004; Van der Heiden et al., 2008). Moreover, studies utilizing zebra-fish show that depletion of primary cilia or its constituents led to vascular defects affecting endothelial morphogenesis (Goetz et al., 2014) and recruitment of supporting perivascular cells (Chen et al., 2017). Although these systems have demonstrated robust insight in the consequence of ciliary defects in the developing vasculature, the homology to human cells should be further explored. Consistent with these reports, we found that shear response. Through drug studies we demonstrated that temporal inhibition of actin polymerization with cytochalasin D treatment permits ciliogenesis and phenotypic shear response (i.e., alignment and elongation). Temporal microtubule disruption with nocodazole led to the inability of ECFCs to form primary cilia and elongate in response to shear stress. In line with previous results showing both microtubule stabilization with paclitaxel (Taxol) and destabilization with nocodazole prevent endothelial alignment to shear stress (Malek and Izumo, 1996), we confirmed that microtubule dynamic instability is critical to cytoskeletal remodeling under mechanical stimulation. To this end, it is not surprising that other downstream pathways in planar cell skeletal remodeling under mechanical stimulation. To this end, it is not surprising that other downstream pathways in planar cell

primary HUAECs did not display primary cilia (data not shown) but had the ability to align to shear stress in vitro. In addition, we found that hCMEC/D3 ECs, which did not undergo alignment after shear application, lacked primary cilia (data not shown).

We did observe primary cilia in both HUVECs and ECFCs, and used these stable phenotypes for further characterization of the role of cilia presentation in
tissue (Kusuma et al., 2013; Shen et al., 2016). Using this established differentiation protocol, we found that although pericytes in the EVC population contained primary cilia, the ECs did not, and were unable to elongate in response to shear stress. After isolation and expansion, BC1-ECs derived using a variety of differentiation protocols were still unable to form primary cilia, stress fibers, or align to shear stress, even though they exhibited stable endothelial identity after several passages. In contrast, hiPSC C12-ECs and hiPSC-ECFCs underwent ciliogenesis and aligned to 20 dynes/cm² shear stress after 24 hr. With this work, we are able to mimic human vascular development, employing controlled differentiation approaches that lead to naive endothelium that lack a defined arterial or venous specification. Although shear is a known maturation cue in vascular development, the mechanisms in which maturing endothelium sense and respond to shear has not been fully elucidated. With this observation of variation in the mechanosensing capacity of hiPSC-ECs, we sought to understand the underlying mechanism of this phenomenon.

We explored other functional differences in ciliated and non-ciliated hiPSC-ECs using migration and calcium uptake assays. BC1-ECs lacked directional migration and wound healing capacity with in vitro scratch assays compared with ciliated hiPSC-ECs. Because this flow-induced calcium signaling has been demonstrated to be controlled through a primary cilia-mediated process (Nauli et al., 2008), we speculated that ciliated and non-ciliated hiPSC-ECs would have varied profiles of cytosolic calcium levels when exposed to a step increase in shear stress. Calcium uptake, which regulates transcriptional events and vasodilation through relaxing smooth muscles cells (Ando and Yamamoto, 2011), also drives endothelial alignment. For example, when ECs are sheared in calcium chelated media, they remain unaligned and fail to form actin stress fibers after shear exposure (Malek and Izumo, 1996). Here, we found that individual ciliated hiPSC-ECs had uniform cytosolic calcium uptake in comparison with non-ciliated ECs, which exhibited erratic calcium uptake, a phenotype that can be implicated to their downstream inability to align after prolonged shear exposure.

Serum starvation induces ciliogenesis through gene-level activation of AURKA (Pugacheva et al., 2007) and protein-level localization of PLK-1, which may also directly interact with HDAC6 in a phosphorylative capacity (Lee et al., 2012). Work from Resnick and Hopfer (2007) describes a mechanical model from experimental data, where increased primary cilia length amplifies drag forces imposed by shear stress, effectively increasing a cell’s mechano-sensitivity to physical forces. In line with this mechanical model, we find that developing hiPSC-derived ECs with long primary cilia projections display mechano-sensitivity to shear forces, while non-ciliated hiPSC derivatives do not. Through a combination of western blot analysis, confocal imaging, and small-molecule inhibition, we found that BC1-ECs have truncated cilia and decreased protein expression of acetylated α-tubulin, a phenotype that can be rescued by directly inhibiting HDAC6 expression. On the contrary, hiPSC-ECFCs or C12-ECs differentiated with the E6-CHIR protocol have primary cilia, with normal functional outputs of calcium uptake in response to shear and phenotypical alignment and directional migration. Although we do not know the exact mechanism of how increased Tubastatin treatment reduces the degree of primary cilia in the exposed BC1-ECs, we do expect that there is a concentration dependency. Other studies have demonstrated in mature ECs that treatment with HDAC6 inhibitors leads to a reduction in mobility and angiogenic capacity (Kaluza et al., 2011). We suspect from experimental treatment that 0.5 μM Tubastatin A brings HDAC6 activity within its normal regulatory range, whereas 2 μM is inhibitory, although cilia can be detected in BC1-ECs (see Figures S6C–S6E).

Overall, we propose that elevated HDAC6 activity in BC1-ECs impaired their ability to form cilia, resulting in diminished mechanotransduction that is partially rescued through HDAC6 inhibition. It has yet to be established whether the transcriptional and functional differences presented here are a direct result of the variation of stem cell origin prior to EC specification or the differentiation protocols used to induce their phenotype. Importantly, future studies exploring genomic stability and chromatin accessibility, coupled with gene manipulation, will lead to critical insights if somatic origin and/or reprogramming method guides downstream endothelial ciliogenesis and functionality.

In conclusion, this study reports the presentation and mechanosensing impact of primary cilia in hiPSC-ECs. Our approach mimics human developmental cues, where ECs undergo maturation first in the absence of shear stress. Our findings of HDAC6 dysregulation and its modulation on ciliogenesis in hiPSC-ECs derived from the BC1 line suggest that a hiPSC-EC source, regardless of how they are differentiated, inherently lack the capacity to form primary cilia, resulting in a series of functional consequences including the inability to align to shear stress. Remarkably, we can attribute this phenotypic lack of cilia formation to a post-transcriptional machinery that can be inhibited with small-molecule Tubastatin A. Given the relationships disclosed in this study, in regard to cytoskeletal architecture and its role in cell migration, calcium uptake, and shear-induced alignment, we believe this work will set the groundwork for further investigations of cilia in differentiated EC populations.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.06.083.

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AUTHOR CONTRIBUTIONS

Q.S., B.M., X.Y.C., and S.G. conceived the study, designed experiments, interpreted the results, and wrote the manuscript. M.C.Y. performed experiments and analyzed data. M.C.Y. provided differentiated cells, interpreted data, and edited the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


### STAR METHODS

**KEY RESOURCES TABLE**

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Sharon Gerecht (gerecht@jhu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Primary EC Culture
Human umbilical arterial ECs (HUAEcs) passages 10-20 and human vein ECs (HUVECs) passages 2-5 (PromoCell, Hielberg, Germany) were cultured in EC growth medium (ECGM; PromoCell) supplemented with 2% fetal bovine serum (FBS). Endothelial colony forming cells (ECFCs; Lonza, Walkersville, MD) were cultured on 50 μg/mL type I collagen coated plates (BD Biosciences, Franklin Lakes, NJ) in endothelial growth media-2 (EGM2; Lonza) containing VEGF and 10% FBS. The hCMEC/D3 cell line passages 25-35 were cultured on collagen I coated plates and grown in ECGM with 2% FBS. For all cell types, medium was changed every 2-3 days. Cells were passaged at approximately 80% confluency with 0.05% trypsin/EDTA (ThermoFisher Scientific, Waltham, Massachusetts) and maintained in a humidified incubator at 37°C/5% CO₂ atmosphere. For serum starvation experiments, cells were cultured in DMEM (ThermoFisher Scientific, Waltham, Massachusetts) for 24 hours, then fixed for immunofluorescence staining.

hiPSC Expansion and Differentiation
BC1 (Cheng et al., 2012; Chou et al., 2011), and C12 hiPSC (Wen et al., 2014) line were maintained either on an inactivated mouse embryonic fibroblast (MEF) feeder layer (MTI-Globalstem, Frederick, Maryland) supplemented with 80% ES-DMEM/F12...
EVCs we followed well-established protocols (Chan et al., 2015; Kusuma et al., 2013; Smith et al., 2017). In brief, pluripotent colonies were dissociated, strained with a 40 μm strainer (BD Biosciences) and seeded with 10 μM Y-27632 on collagen IV (R&D) coated plates at 1x10^6 cells/cm^2 in differentiation media containing 10% Hyclone FBS (GE Healthcare-HyClone), α-MEM (Invitrogen Carlsbad, CA), and 0.1 mM β-mercaptoethanol. Differentiation media was replaced every other day for six days. On day 6, cells were collected with TrypLE (Invitrogen) and seeded on fresh collagen IV plates at 3x10^4 cells/cm^2 in ECGM (PromoCell) supplemented with 10% Hyclone FBS (GE Healthcare-HyClone) and 0.1% penicillin-streptomycin (GIBCO; thereafter, known as EC Diff Media). Media was changed every other day for an additional six days.

For CHIR differentiation, hiPSCs were cultured in Essential 8 Medium (Thermofisher Scientific) on Vitronectin coated plates, routinely passaged at 80% confluence. To induce differentiation, hiPSCs grown to 60%–80% confluency on Vitronectin were changed daily for 48 hours to Essential 6 Medium (GIBCO) supplemented with 6 μM CHIR (STEMCELL Technologies). At day 2 of differentiation, cells were digested in TrypLE Express, seeded on Collagen IV coated plates at 2x10^4 cells/cm^2 in EC Diff Media containing 10 μM Y-27632. After 24 hours Y-27632 was removed and media was changed every other day for six additional days (Lian et al., 2014). hiPSC-ECFCs (passages 16-20) differentiated from the hiPSC-DF19-9-11T line, were expanded and characterized as previously described (Prasain et al., 2014).

**METHOD DETAILS**

**Isolation and Expansion of hiPSC Derived ECs**

CD31 or VECad expressing cells were isolated via magnetic activated cell sorting (MACS; Miltenyi Biotec) following the manufacturer’s protocol on day 12 of EVC differentiation. Briefly, after washing one time with 1x phosphate buffered saline (PBS, Sigma), EVCs were harvested with TrypLE dissociation buffer (Invitrogen), re-suspended in MACS buffer (0.5 EDTA and 0.5% BSA in PBS) and incubated with 10 μl of PE-conjugated anti-human VECad (BD Biosciences) for 10 minutes at 4 °C. After incubation, unbound primary antibody was removed by washing with MACS buffer. Next, 20 μl of anti-PE microbeads (Miltenyi Biotec) were added to 80 μl of cells suspended in MACs buffer and incubated for an additional 15 minutes at 4 °C. Cells were washed three times with MACs buffer and separated using the MS MACS separation column (Miltenyi Biotec). Following separation VECad enrichment was confirmed using flow cytometry. Finally, VECad+ cells were seeded on type IV collagen coated plates and maintained in EC differentiation media.

**Flow Cytometry**

Cell differentiation was verified through flow cytometry, where FITC or PE-conjugated antigen specific antibodies outlined within the text, were incubated with live cells (Vo et al., 2010). To harvest the cells for flow analysis, residual serum was removed prior to adding TrypLE (Invitrogen) dissociation buffer, by washing with 1x PBS. After collection, cells were incubated in 100 μl of 0.1% bovine serum albumin (BSA; Sigma) in PBS with conjugated antibodies for 45 minutes to 1 hour on ice. Cells were washed twice with 0.1% BSA and passed through a 40 μm strainer (BD) to eliminate cell clumps. Flow analysis was conducted on a BD FACS Calibur flow cytometer. Following the manufacturer’s instructions, dead cell populations were gated out with forward-side scatterplots. To determine levels of expression, all analyses were conducted using IgG-PE or IgG-FITC (BD) isotype controls.

**Microfluidic Device and Cell Culture (Static/Flow)**

To study shear stress, we used our microfluidic platform with slight modifications. Polydimethylsiloxane (PDMS) microfluidic channels approximately 2.5 cm long, 250 μm tall, and 300 μm wide were fabricated and plasma bonded to glass slides as previously described (Abaci et al., 2012b, 2014). The shear profile within the microfluidic device was calculated as previously described (Abaci et al., 2012a, 2014). In brief, shear stress \((\tau) = 6\mu Q/wh^2\) where \(\mu\) is the viscosity of the media, \(Q\) the flow rate of the peristaltic pump, and \(w\) and \(h\) represent the width and height of the channels respectively. A bubble trap for high shear studies was fabricated and plasma bonded to the resulting glass/microchannel assembly. Briefly, PDMS blocks were cored with a circular punch, and cured to an additional PDMS slab for accommodating microfluidic tygon polyvinyl chloride tubing (.020 ID in, 0.60 OD; Small Parts). The resulting cylindrical reservoir allowed for bubble accumulation without entry into the microchannel. Microfluidic components were sterilized through ultraviolet light exposure within a biosafety cabinet for 15 minutes, followed by further sterilization with 100% ethanol. A glass media reservoir for prolonged shear experiments was sterilized by autoclaving. Prior to cell seeding, the single use microfluidic devices were rinsed with 1x PBS and coated overnight with 50 μg/mL fibronectin derived from human plasma (Sigma Aldrich, St. Louis, MO) at room temperature to promote endothelial attachment. ECs were grown to ~80 confluence in their respective media, collected and seeded in the microfluidic devices. After 4 hours of seeding, a complete monolayer was achieved by injecting approximately 2.5 million cells suspended in 250 μl of EGM media leading to an effective seeding density of ~10 million cells/mL. For all experiments, physiological levels of shear stress were circulated through the microfluidic device and media reservoir containing HUVEC media, using a peristaltic pumping system (Ismatec, Wertheim, Germany).
Disruption of Cytoskeletal Integrity
Cytochalasin D or Nocodazole dissolved in DMSO, was used to inhibit F-actin or microtubule assembly respectively. ECs which demonstrated cilia after serum starvation (HUVECs and ECFCs) were seeded on coverslips as described above but were incubated for an additional hour with either 10 μM Nocodazole or 1 μM Cytochalasin D diluted in DMEM. The cells were immediately fixed, stained for F-actin or acetylated α-tubulin expression and analyzed. For Cytochalasin D experiments in hiPSC-EC derivatives, 0.5 μM or 1 μM was supplemented in the absence of serum for the final 12 hours of starvation before fixation.

Immunofluorescence Staining and Imaging
Cells were cultured on coverslips, washed once with 1x PBS and fixed in 3.7% formaldehyde for 10 minutes. Formaldehyde was removed, then the samples were washed three times with 1x PBS. Following washing, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes. Samples were washed then incubated with 1% BSA solution for one hour at room temperature to block for non-specific binding. Following blocking, samples were washed with PBS and incubated with primary antibodies diluted in antibody diluent (DAKO) overnight at 4°C, then incubated with secondary antibodies in antibody diluent (DAKO) for 1 hour at room temperature. Samples were then washed three times with 1X PBS, incubated for 3 minutes with DAPI (Roche Diagnostics), washed and placed onto glass slides using mounting media (Dako, Carpinteria, CA).

For ciliogenesis assays, cells were serum starved in DMEM for 24 hours prior to fixation. Primary cilia were enumerated by acetylated α-tubulin staining, in images taken randomly using a 40/63x oil immersion objective (At least 5 fields of view from 3 independent biological replicates or differentiations).

Endothelial Alignment Quantification
To determine nuclei orientation (θ) pre and post microfluidic shear application, a custom image processing program was written in MATLAB (Natick, MA). Prior to preforming a global background subtraction to nuclei stains, microfluidic channel images were horizontally straightened. With the background fluorescence removed, the images were masked, and an ellipsoid was fit to each nucleus identified within the image. With the shape of the nucleus outlined, its corresponding orientation was identified by measuring the horizontal deviation of the major axis of the ellipsoid (n > 100 per experiment), relative to the applied shear stress (Figure S1). To denote cytoskeletal changes in response to shear stress, the AR pre and post-shear application was measured. In brief, the AR was determined manually through analyzing F-actin phalloidin stains with ImageJ. A line representing the major axis of the cell was drawn, measured, and divided by the corresponding measured minor cell axis. An AR = 1, denoted cells without a biased cytoskeletal elongation indicative of unaligned cells, whereas cells displaying an AR > 1 showed biased morphology indicating alignment. Using this quantification criterion, we found on average, the AR and nuclear orientation of static HUVECs within the microfluidic device were 1 and 40° (45° in randomly oriented cells) respectively and has been shown before (2015; Mack et al., 2017; Ye et al., 2014). These served as unaligned static control cells.

Cilia Length Quantification
To quantify the length of primary cilia, maximum intensity projections of confocal images, taken at 63x magnification, were reconstructed using ImageJ. Cilia were manually traced for measurement, using five fields of view per cell type.

Vasculogenesis Assay
Collagen gels were formed as previously described (Kusuma et al., 2014). In brief, to prepare 1mL of collagen gel solution, 800,000 cells (iPSC-ECs) were thoroughly re-suspended in 400 μL Medium 199 [1x], 40 μL of Medium 199 [10x], and 350 μL of 7.1 mg/mL Rat Tail Collagen Type 1. After decreasing the pH of the collagen gel solution with the addition of 10 μL of 1M NaOH, 56 μL of the mixture was added to wells of a 96 well plate. EC Growth Medium supplemented with 50 ng/mL of VEGF was added 30 minutes after the gel polymerized at 37°C.

Vacuole Quantification
To quantify vacuole formation, we used a well-established approach (Hanjaya-Putra et al., 2011). In brief, cells embedded in collagen gel solutions were added to 16 well glass bottom chamber slides. At 3 and 6 hours of incubation, cells were stained with membrane marker FM4-64FX by incubating on ice for 10 minutes. Following incubation, cells were fixed with 3.7% paraformaldehyde for 5 minutes. 55 cells per condition were analyzed for vacuole formation using confocal z stacks. Any area containing lumen occupying more than 30% of the cell’s area was considered to be a vacuole.

Vascular Network Quantification
Cells were fixed in 2% paraformaldehyde, permeabilized with 1% Triton-X, and blocked for non-specific binding with 10% bovine serum albumin. iPSC-EC networks were labeled with phalloidin and imaged by confocal microscopy. Network analysis was performed using the filament package in Imaris software from Bitplane.

Stress Fiber Quantification
To determine the degree of stress fiber formation in hiPSC-ECs, 20 x F-actin images were processed in ImageJ. First, the background intensity was identified by averaging the distribution of fluorescence intensities in confocal images containing regions without
cells. Next, the pixel by pixel fluorescence intensity along the minor axis of the cell was measured. Finally, cytoplasmic stress fibers were identified as pixel regions with fluorescence values greater than the determined background, as previously described (Wanjare et al., 2014).

**Confocal Fluorescence Measurement of Intracellular Calcium Concentration**

The Fluo-4, AM, cell permeant (ThermoFischer Scientific) calcium indicator was re-constituted in DMSO according to the manufacturer’s instructions. For live calcium influx studies, a monolayer of ECs seeded within the microfluidic device were incubated for 10 minutes at 37°C with 5 μM Fluo-AM buffered in EGM. The cells were then flushed with fresh EGM media and incubated for an additional 20 minutes to allow de-esterification. To monitor calcium influx in response to step changes in shear stress a syringe pump (Chemyx, Stafford, TX) was manually manipulated after the cells were loaded with the calcium indicator. Baseline signal was acquired using a spinning disk confocal (Zeiss AxioObserver Yokogawa CSU-X1), by imaging for 100 s without the application of shear. Following baseline measurements, flow was started to induce a constant shear (20 dynes/cm²) for 200 s. For re-stabilization of calcium levels, flow was suspended for 300 s before returning to a constant shear for an additional 200 s. After the addition of this second step in shear, calcium relaxation was achieved by suspending flow again for 200 s. To quantify changes in calcium signal to endothelial monolayers stimulated with shear stress, raw confocal videos were compressed to 20 frames per second (fps) avi files and imported into ImageJ. Using the Time Series Analyzer V3 plugin, cells were identified with a circular region of interest 20 pixels in diameter and monitored for temporal changes in fluorescence intensity (FI). Percent changes in FI was reported as Fl/FIo * 100 where FIo was the averaged baseline FI as previously described (Li et al., 2015) (30-50 cells analyzed per experiment, at least 2 independent experiments per cell type).

**Cell Migration/Wound Healing Assay**

hiPSC derived ECs were seeded at 53,000 cells/cm² on Collagen IV coated 12 well CellStar tissue culture plates in complete ECGM supplemented with 50 ng/mL VEGF and 10 μM TGFβ inhibitor. Cells were cultured to confluence for 48 hours, then manually scratched using the tip of a P200 pipette. Following wound application, media was changed to ECGM without further supplementation. Cell invasion was tracked over 25 hours using time-lapse microscopy (Zeiss Axiocure). Briefly, three random positions were imaged per seeded well, with a total of three separate wells imaged per cell type. Wound closure kinetics was quantified by measuring gap widths at the top, middle, and bottom of each scratch in 5-hour increments. Individual cell trajectories were acquired using the Manual Tracking plugin in ImageJ (25 cells per scratch assay were tracked). The following cell motility parameters were quantified as previously described (Hielscher et al., 2016): Gap width, mean squared displacement, and directionality (ratio between net displacement and total distance traveled).

**Quantitative Reverse-Transcription PCR**

Total RNA was extracted using TRIzol Reagent (Invitrogen) from stem cell derived ECs serum starved for 24 hours. Quality and quantity of extracted RNA was verified by spectrophotometry (NanoDrop 1000), prior to generating complementary DNA (cDNA) via reverse transcription using M-MLV reverse transcriptase and random primers (Promega) (Smith et al., 2017). qPCR was performed using 1 μg of high quality cDNA, on a custom gene expression array. Each measurement was carried out in triplicate using a real-time QuantStudio 12K flex system in a 20 μl reaction. 18sRNA, GAPDH and B-actin served as endogenous controls for global normalization to measure mRNA expression. Biological replicates were performed as follows: n = 3 for BC1-ECs, n = 2 for C12-ECs; n = 3 for hiPSC-ECFCs. Raw data presented in Table S1.

**Experimental Procedures for cDNA Microarray Analysis**

Raw cDNA microarray data acquired from the QuantStudio 12K flex system was directly imported into the ThermoFisher Cloud platform for analysis. In brief, global normalization from endogenous controls were used. Reference group for differential analysis was assigned to hiPSC-ECFCs, where fold change differences were calculated by the comparative Ct method.

**HDAC6 Inhibition**

Varying concentrations of Tubastatin A (Santa Cruz Biotechnology) was added to DMEM (serum starvation) or HUVEC media as described in the text. DMSO vehicle control samples for untreated groups were run in parallel. Shear experiments were performed following Tubastatin A treatment using HUVEC media.

**Western Blot**

Cell lysates were prepared in either RIPA buffer (ThermoFisher Scientific) or NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific) with 1 × Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific, MA). Protein isolated from cell lysates was quantified with the BCA assay (ThermoFisher Scientific). Protein (15 to 20 μg per well) was boiled at 95 °C for 5 min prior to being loaded into a 4%–12% Bis-Tris Protein Gels (ThermoFisher Scientific). Proteins were transferred to nitrocellulose membranes or PVDF membrane via dry transfer using iBlot2 (ThermoFisher Scientific), blocked in 3 percent BSA for 1 h, and incubated overnight at 4 °C (under constant shaking on a rocker) with primary antibody: rabbit anti-acetylated alpha tubulin (1:500; Cell Signaling Technology) or rabbit anti-acetylated HDAC6 (1:1,000; Cell Signaling Technology). Membranes were washed 6 times in
Tris-buffered saline containing 0.1% Tween 20 (TBST) for 15 min each, with a change of new Petri dish after the first 3 washes. And then, membranes were incubated with anti-rabbit horseradish peroxidase (HRP) (1:3000; Cell Signaling Technology) and HRP-conjugated GAPDH (1:20,000, Proteintech) or HRP-conjugated HDAC1 (1:1000; Cell Signaling Technology). Membranes were washed three times in TBST, developed with enhanced chemiluminescence (Pierce) and visualized using the ChemiDoc XRS+ System (Bio-Rad).

QUANTIFICATION AND STATISTICAL ANALYSIS

See figure legends for specification of ‘n’ samples analyzed and number of biological replicates. In brief, image analysis, flow cytometry, and shear assays were done in at least duplicate biological samples. Non-significant differences are labeled in figures as NS; while significant differences were reported as * p < 0.05, **p < 0.01, and *** p < 0.001, **** p < 0.0001. Statistical significance and analysis was determined using GraphPad Prism (v.6.0d, La Jolla, CA). All graphical data are reported as mean ± SEM.

DATA AND SOFTWARE AVAILABILITY

Data Resources
The accession number for the microarray data reported in this project is GEO: GSE115870.