Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells

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The use of human pluripotent stem cells for in vitro disease modelling and clinical applications requires protocols that convert these cells into relevant adult cell types. Here, we report the rapid and efficient differentiation of human pluripotent stem cells into vascular endothelial and smooth muscle cells. We found that GSK3 inhibition and BMP4 treatment rapidly committed pluripotent cells to a mesodermal fate and subsequent exposure to VEGF-A or PDGF-BB resulted in the differentiation of either endothelial or vascular smooth muscle cells, respectively. Both protocols produced mature cells with efficiencies exceeding 80% within six days. On purification to 99% via surface markers, endothelial cells maintained their identity, as assessed by marker gene expression, and showed relevant in vitro and in vivo functionality. Global transcriptional and metabolomic analyses confirmed that the cells closely resembled their in vivo counterparts. Our results suggest that these cells could be used to faithfully model human disease.

Human pluripotent stem cells1–3 (hPSCs) have unlimited proliferation capacity and the potential to differentiate into all somatic cell types. Ideally, they can be used to generate an inexhaustible supply of cells for clinical and scientific applications. Patient-specific hPSCs promise to reveal the molecular and genetic basis of disease. However, a prerequisite for exploiting their potential to understand disease is the development of strategies for directing their differentiation into functional adult cell types4–6. In addition to being reproducible, simple and quick, ideal differentiation strategies would yield pure populations of cells in sufficient quantities to enable high-throughput screening and large-scale analyses. Thus, a major obstacle for using hPSCs to model disease remains the lack of reliable, efficient and scalable protocols to differentiate functionally mature adult cell types.

Blood vessels deliver oxygen and nutrients to all of the tissues and organs in the body. The two main cellular components of blood vessels are endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). Both ECs and VSMCs are required for vascular function, including blood pressure control, interactions with immune cells, and the uptake of nutrients. Consequently, these cells are involved in a variety of pathological dysfunctions, including the most common cardiovascular diseases, atherosclerosis. So far, there exist two commonly used methods to induce vascular cell differentiation from hPSCs: embryoid body formation7–9; and monolayer-directed differentiation10,11. Embryoid body formation results in differentiation of hPSCs into various cell types, including vascular cells, albeit inefficiently (1–5%; refs 7,11,12). Moreover, embryoid body differentiation is often time consuming, with peak expression of endothelial genes occurring after 10–15 days13. Current monolayer differentiation methods offer increased efficiencies (5–20%) but depend on undefined supplements, co-culture14,15, heterogeneous cell aggregates16, or conditioned medium17,18, or lack consistent yields of vascular cells19. Thus, improved methods would increase differentiation fidelity, efficiency and kinetics.

In mammalian development, vascular progenitors emerge from the lateral and posterior mesoderm19. Several studies describe the importance of canonical Wnt signalling in mesoderm commitment during embryogenesis20. For example, mice with impaired Wnt signalling lack mesoderm21,22. Canonical Wnt signalling in hPSCs induces mesendoderm23, cardiogenesis24 and the formation of vascular cells25. On the
basis of previous reports 25–27, we sought to develop a protocol for the differentiation of hPSCs to vascular cells. Here, we describe the rapid and efficient conversion of hPSCs into vascular cells using chemically defined conditions. Our protocol utilizes GSK3 inhibition and BMP4 treatment to convert hPSCs into mesodermal cells that when exposed to VEGF-A or PDGF-BB produced functional ECs or VSMCs.

RESULTS

Canonical Wnt activation and mesoderm induction by pharmacological inhibition of GSK3

Wnt signalling directs differentiation of hPSCs into mesoderm and GSK3β inhibition activates this pathway 16,23. However, small-molecule inhibitors of GSK3 can promote either self-renewal or mesendodermal differentiation of hPSCs (refs 16,28,29). We therefore attempted to identify selective GSK3β inhibitors that promoted efficient commitment of hPSCs towards mesoderm. A panel of GSK3 inhibitors was evaluated for their selectivity and potential to inhibit GSK3 and to activate Wnt signalling (Supplementary Table 1).

An in vitro competition binding assay against 96 protein kinases was performed to evaluate the specificity of GSK3 inhibitors, including 6-bromoindirubin-3'-oxime (BIO), CHIR99021 (CHIR; ref. 30), SB216763 (SB; ref. 31) and a Roche compound, CP21R7 (CP21; Supplementary Fig. 1A). CP21 and CHIR were the most selective GSK3 inhibitors (Supplementary Table 2). CP21 also showed the highest affinity to GSK3β followed by CHIR (Supplementary Fig. 1D). These findings indicate that CP21 and CHIR are high-affinity, selective GSK3β inhibitors.

To examine the capacity of these compounds to activate canonical Wnt signalling, a dose–response assay was performed using a reporter cell line 32 with the luciferase gene expressed by a TCF/LEF promoter (Supplementary Fig. 1B). CP21, BIO and CHIR were able to potentially activate canonical Wnt signalling with the highest activity at 3 μM (CP21, BIO) and 10 μM (CHIR). In contrast, SB, AR and MeBIO did not induce TCF/LEF luciferase expression (Fig. 1a). The increase in TCF/LEF–luciferase activation by GSK3 inhibitors was not due to global transcriptional activation as measured in β-gal–luciferase-responsive reporter cells (Supplementary Fig. 1C). Thus, CP21, CHIR and BIO were able to activate canonical Wnt signalling to similar levels, but given the toxicity of BIO we chose not to include this compound in subsequent experiments. Next, we analysed protein levels of β-catenin in hPSCs treated with CP21 and CHIR. Immunofluorescence staining revealed that CP21 and CHIR significantly increased total levels of intracellular β-catenin (Fig. 1b). This result was confirmed by gene expression analyses (Fig. 1c), wherein Wnt target genes were upregulated following CP21 and CHIR treatment of hPSCs. Thus, CP21 and CHIR potently and selectively inhibit GSK3 to activate canonical Wnt signalling.

We next investigated whether GSK3 inhibition with CP21 and CHIR in hPSCs would induce mesoderm. As BMP4 is also a potent inducer of mesoderm 33, we tested BMP4 alone or combined with Wnt activation by CP21 and CHIR. Gene expression analyses revealed that BMP4 treatment with Wnt activation led to upregulation of genes associated with mesoderm, such as T; MIXL and EOMES (Supplementary Fig. 2A). Immunostaining of CP21- and BMP4-treated cells showed high levels of BRACHURY (T) expression that peaked at day three (Fig. 1d). Conversely, OCT4 was downregulated. SOX17 was not detected, indicating that the cells were likely to be mesodermal (Fig. 1d). Similar results were obtained using CHIR and BMP4 (Fig. 1d). Thus, activation of Wnt signalling by GSK3 inhibition with CP21 or CHIR combined with BMP4 induced commitment of hPSCs towards mesoderm.

Efficient differentiation of mesodermal progenitors to endothelial cells or vascular smooth muscle cells

We assessed the ability of hPSC-derived mesoderm to undergo vascular lineage commitment using a chemically defined protocol consisting of three steps. First, hPSCs were plated as single cells at a density of 37,000 cells per square centimetre with the Rho-kinase inhibitor Y-27632 (ref. 34); second, cells were differentiated to mesoderm using either CP21 or CHIR and/or BMP4; and third, they were treated with VEGF-A to induce ECs (ref. 16). At day five, EC differentiation was evaluated by analysing the expression of vascular endothelial cadherin (VE-cadherin, CD144). CD144+ cells were readily detected in CHIR- and CP21-treated cultures, but were absent in controls (Supplementary Fig. 2B). Flow cytometric analysis of CD144 expression revealed that only BMP4 and CP21 or CHIR combined were capable of inducing high levels of expression, whereas treatment with BMP4 alone led to fewer than 10% CD144+ cells (Supplementary Fig. 2C). Additional analysis identified CP21 as the most potent compound (Supplementary Fig. 2D) with a concentration of 1 μM yielding up to 35% CD144+ cells, whereas CHIR induced CD144 expression most efficiently at a concentration of 6 μM. When either GSK3 inhibitor was used at its optimal concentration the yield of CD144+ cells was equivalent (Supplementary Fig. 2E).

We next combined VEGF-A treatment with forskolin for two days, as protein kinase A activation by cyclic AMP leads to an increase in vascular development 35, followed by VEGF-A treatment alone for an additional four days (Supplementary Fig. 2F). We tested this strategy on several hPSC lines, including HUES9, SA001, BJ-RipPS and a commercially available induced PSC line 36,37. This protocol promoted the differentiation of hPSCs into endothelial cells with efficiencies between 61.8% and 88.8% (s.e.m. 3.1, n = 10 independent experiments) as assessed by flow cytometry of CD144+ cells (Fig. 2a,b and Supplementary Table 4A). CD144+ cells were further analysed for expression of EC-specific markers. Flow cytometry revealed that hPSC-derived ECs expressed KDR, CD31, CD34 and CD105. Expression of the haematopoietic lineage markers CD43 and CD45 were readily detected in CHIR- and CP21-treated cultures, whereas CHIR and/or BMP4 at a density of 37,000 cells per square centimetre with the Rho-kinase inhibitor Y-27632 (ref. 34); second, cells were differentiated to mesoderm using either CP21 or CHIR and/or BMP4; and third, they were treated with VEGF-A to induce ECs (ref. 16). At day five, EC differentiation was evaluated by analysing the expression of vascular endothelial cadherin (VE-cadherin, CD144). CD144+ cells were readily detected in CHIR- and CP21-treated cultures, but were absent in controls (Supplementary Fig. 2B). Flow cytometric analysis of CD144 expression revealed that only BMP4 and CP21 or CHIR combined were capable of inducing high levels of expression, whereas treatment with BMP4 alone led to fewer than 10% CD144+ cells (Supplementary Fig. 2C). Additional analysis identified CP21 as the most potent compound (Supplementary Fig. 2D) with a concentration of 1 μM yielding up to 35% CD144+ cells, whereas CHIR induced CD144 expression most efficiently at a concentration of 6 μM. When either GSK3 inhibitor was used at its optimal concentration the yield of CD144+ cells was equivalent (Supplementary Fig. 2E).

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Interestingly, immunostaining revealed that in addition to ECs a small fraction of cells expressed the vascular smooth muscle marker smooth muscle actin (αSMA; Supplementary Fig. 2H). We reasoned that by replacing endothelial inducive cues with factors that promote VSMC formation we might efficiently generate VSMCs. ActivinA and PDGF-BB have been shown to promote the formation of VSMCs (refs 38–40). Thus, we added ActivinA and/or PDGF-BB...
Figure 1: Canonical Wnt activation by GSK3β inhibitors and mesoderm induction. (a) Luciferase assay of the β-catenin promoter activity after treatment with increasing concentrations of GSK3β inhibitors. A 6-point threefold serial dilution of each compound was performed (10, 3, 1, 0.3, 0.1, 0.03 µM, last 2 concentration data not shown). Columns show means ± s.d. of 5 independent experiments. (b) Immunofluorescent localization of β-catenin in hPSCs after a 24 h treatment with either CP21 or CHIR. A representative image is shown of 3 independent experiments for 3 different wells per condition. Scale bars, 50 µM. (c) Quantitative PCR of β-catenin target genes on treatment of hPSCs with CP21 or CHIR. Results shown are means and s.e.m. of 3 independent experiments with 3 biological and technical replicates for each gene. (d) Immunofluorescence staining of hPSCs for markers of pluripotency, mesoderm and endoderm during the first 4 days of differentiation. A representative image is shown of 3 independent experiments for 3 different wells per condition. Scale bars, 50 µM.

following mesoderm induction. Strikingly, this modification resulted in the formation of almost exclusively CD140b+ (PDGFRB) cells with virtually no CD144+ cells detectable when ActivinA and PDGF-BB were used (Fig. 2a,b and Supplementary Fig. 2I,J). The differentiation of CD140b+ cells required either CP21 or CHIR and was not induced following treatment with BMP4 alone (Supplementary Fig. 2K). The cells also expressed other markers of VSMCs, such as αSMA and myosin IIB (Fig. 2d). The efficiency of differentiation of VSMCs was similar across hPSC lines, with on average 95.4% CD140b+ cells generated (s.e.m. 2.7, n = 16 independent experiments; Supplementary
Figure 2 VEGF-A and PDGF-BB-mediated differentiation of hPSCs into vascular endothelial or vascular smooth muscle cells. (a) Differentiation efficiency of hPSC ECs and hPSC VSMCs from four different hESC and induced PSC lines, (s.e.m. 3.1, n = 10 independent differentiation experiments) for hPSC ECs and (s.e.m. 2.7, n = 16 independent differentiation experiments) for hPSC VSMCs. Columns show means ± s.e.m. (b) Representative fluorescence-activated cell sorting plots from the differentiation experiments described in a: hPSCs (top panel), hPSC-derived ECs (middle panel) or hPSC-derived VSMCs (lower panel) stained for CD144 and CD140b. Day 0 (hPSC ECs) and 16 (hPSC VSMCs) independent differentiation experiments. (c) Immunostaining of EC-specific markers on hPSC ECs; vWF: Von Willebrand factor. All cells (100%) were expressing VE-cadherin and PECAM1 for both GSK3 inhibitors. 73.48% of the hPSC ECs differentiated with CHIR and 74.52% of the cells differentiated with CP21 express vWF. A representative image is shown of 3 independent experiments for 3 different wells per marker. Scale bars, 50 µM. (d) Immunostaining of VSMC-specific markers on hPSC VSMCs; αSMA: alpha-smooth muscle actin. For CHIR, 48% of cells expressed αSMA, 96.99% myosin IIB and 98.7% SM22a. For CP21, 62.08% of cells expressed αSMA, 92.75% myosin IIB and 100% SM22a. A representative image is shown of 3 independent experiments. Scale bars, 50 µM. (e) Schematic illustration of the EC differentiation strategy for hPSCs. (f) Schematic illustration of the VSMC differentiation strategy for hPSCs.
Table 4B). Thus, GSK3 inhibition and BMP4 treatment followed by ActivinA and PDGF-BB treatment rapidly and efficiently induced VSMCs from hPSCs (Fig. 2f).

To purify hPSC-derived ECs, we performed magnetic-associated cell sorting (MACS) at day six of differentiation. After MACS-mediated selection for CD144-expressing cells, we obtained virtually pure (on average 95.9%, s.e.m. 3.0, n = 19 independent experiments) EC cultures (Supplementary Fig. 2L and Supplementary Table 4C). As hPSC VSMC cultures were nearly homogeneous they required no further purification.

A step-by-step protocol describing the culture of hPSCs and the differentiation of hPSC ECs (http://dx.doi.org/10.1038/protex.2015.055) and hPSC VSMCs (http://dx.doi.org/10.1038/protex.2015.056) can be found at Nature Protocol Exchange.

**hPSCs-derived ECs and VSMCs exhibit a transcriptional signature similar to primary vascular cells**

To evaluate hPSC-derived vascular cells and monitor differentiation dynamics on a molecular level, we performed gene expression profiling at seven time points during differentiation. A projection of the complete expression profiles onto the first two principal components following principle component analysis revealed that differentiating cells cluster until day 4 with hPSCs. However, on induction into ECs or VSMCs, the cells became highly similar to their corresponding primary cells as shown by principal component projections after two weeks of differentiation (Supplementary Fig. 3A). This similarity is also reflected by the Pearson correlation coefficient for all expressed genes between primary and hPSC-derived ECs (r = 0.93) or primary and hPSC-derived VSMCs (r = 0.88). Of note, cultured primary cells do not perfectly mimic their in vivo counterparts.

To examine mesoderm commitment a time course of gene expression was plotted for representative genes from pluripotent cells, mesoderm, neuroectoderm, endoderm, endothelium and regionalization of the primitive streak (Supplementary Fig. 3B). These analyses revealed that pluripotent cell markers such as NANOG, UTF1 and SOX2 were rapidly downregulated. OCT4 expression was less rapidly downregulated and decreased by day 10. We observed a transient activation of the genes found in mesoderm, such as MIXL1, T (also known as BRACHYURY), FGF4 and EOMES. With the exception of the endodermal marker SOX17, which was highly expressed at day five of EC differentiation, expression of markers associated with neuroectoderm or endoderm was not observed. Expression of SOX17 mimics the in vivo pattern found in the developing and adult vasculature. We did not observe differentiation of trophoderm cell or visceral endoderm, as ESX1 and SOX7 were undetectable. These data support the differentiation of hPSCs through a mesodermal intermediate to ECs or VSMCs.

We found that 2,955 genes were differentially expressed by at least tenfold between hPSCs, differentiated ECs, VSMCs and primary cells (Fig. 3a). These genes were hierarchically clustered and analysed on the basis of Gene Ontology (GO) terms using the DAVID Bioinformatics Resource. The most prominently enriched gene sets included annotations for blood vessel development (GO:0001568, \( P < 10^{-10} \)) and cell adhesion (GO:0007155, \( P < 10^{-13} \)) for ECs, extracellular matrix organization (GO:00030198, \( P < 10^{-11} \)) and muscle organ development (GO:0007517; \( P < 10^{-8} \)) for VSMCs, and cell projection organization (GO:0030030; \( P < 10^{-9} \)), cell adhesion (GO:0007155, \( P < 10^{-13} \)) and the cell cycle process (GO:0007049; \( P < 10^{-6} \)) for hPSCs (Fig. 3b).

We performed gene set enrichment analysis to find within all differentially expressed genes, over- or under-represented gene sets representative for biological processes. Only gene sets passing significance thresholds with a \( P \) value of 0.001 and a false discovery rate of 0.05 were selected as biological processes significantly over- or under-represented in hPSC-derived ECs, VSMCs, and primary cells versus hPSCs. Top over-represented biological processes for hPSC-derived and primary ECs included endothelial cell differentiation (GO:0045446), angiogenesis (GO:0001568), blood vessel development (GO:0001568) and blood vessel development (GO:0001568). For all VSMCs, the top upregulated biological processes were extracellular matrix (GO:0030198), collagen fibril organization (GO:0003199), regulation of smooth muscle cell migration (GO:0014911) and proliferation (GO:0048660). The most significantly downregulated processes in hPSC-derived ECs and VSMCs were gene sets for mitosis (GO:0007067) and chromosome segregation (GO:0007059) (Supplementary Table 3 and Supplementary Fig. 3C).

In summary, transcriptome-wide expression analysis demonstrated that hPSC-derived ECs and VSMCs were highly similar to their respective primary cells.

**hPSC-derived ECs and VSMCs exhibit a metabolomic profile similar to primary vascular cells**

We performed metabolomic analysis by liquid chromatographymass spectrometry of hPSC-derived vascular and primary cells. Of 106 metabolites, we detected 66 intracellularly (Supplementary Table 5). There was a strong correlation between metabolite levels in primary ECs and in hPSC-derived ECs (Spearman coefficient \( r = 0.96 \), HCAEC versus hPSC EC = 0.96, Fig. 3c,d), and also between metabolite concentrations in primary VSMCs (UASMCs) and in hPSC VSMCs (Spearman coefficient = 0.92, Fig. 3c,d). These similarities were seen across metabolite classes, including amino acids, amino-acid derivatives, nucleosides, organic nitrogenous compounds, aromatic acids, amine oxides, and other organic acids (Supplementary Table 5). In contrast, hPSCs had a distinct metabolic profile when compared with hPSC ECs (Spearman coefficient = 0.80) or hPSC VSMCs (Spearman coefficient AUSMC (contractile) versus hPSC VSMC (contractile) = 0.97, AUSMC (synthetic) versus hPSC VSMC (synthetic) = 0.97, Fig. 3c,d). Thus, these analyses indicate that hPSC-derived vascular cells have metabolic profiles that closely resemble primary ECs and VSMCs.

**hPSC-derived ECs and VSMCs exhibit mature functional properties**

We sought to analyse the functional capabilities of hPSC-derived vascular cells. To determine the barrier function of hPSC ECs we measured impedance using an xCELLigence RT-CA system. The formation of a tight monolayer was observed by an increase in impedance within four hours after plating. Monolayer impedance reached a plateau, which was maintained for several days without major fluctuations (Fig. 4a). Treatment with the vasoactive agent thrombin led to a rapid and reversible decrease in impedance. Analysis of trans-endothelial electrical resistance (TEER) revealed that hPSC ECs showed TEER values similar to primary ECs (HUVECs).
Treatment with the cytokines TNF-α, VEGF-A and IL1β led to disruption of the barrier tightness as revealed by a decrease in the TEER values (Fig. 4b). We then analysed further in vitro functional features of hPSC ECs. The capacity of lipid uptake was monitored using an in vitro functional assay of endothelial cells (ECs) treated with fluorescently labelled acetylated LDL (Fig. 4c).

To determine whether hPSC ECs respond to pro-inflammatory cytokines with a pro-adhesive phenotype, we challenged the hPSC ECs with TNF-α and IL-1β. Immunofluorescence analysis showed an increased expression of intracellular adhesion molecule-1 (ICAM1) on TNF-α treatment (Fig. 4d). Flow cytometric analysis results confirmed the upregulation of ICAM1 on TNF-α and IL-1β stimulation (Fig. 4e). We co-cultured hPSC ECs with leukocyte-like HL60 cells to examine cellular adhesion molecule-mediated tethering and capture. Remarkably, adhesion of HL60 cells was significantly enhanced when hPSC-derived ECs were stimulated with TNF-α (Fig. 4f). Application of ICAM1-specific antibodies reduced adhesion of HL60 cells in a concentration-dependent manner suggesting that leukocyte adhesion was indeed mediated by ICAM1 (Fig. 4g).

Next, we analysed the contractility of hPSC VSMCs by stimulation with vasoconstrictive drugs or cytokines (Fig. 4h and Supplementary Fig. 4A). Calcium imaging revealed that endothelin1 and carbachol induced an increase in intracellular calcium levels. Treatment with atropine prevented carbachol-mediated calcium influx. Using a three-dimensional collagen contractility assay, hPSC VSMCs contracted similarly to primary VSMCs when exposed to a vasoconstrictor (U46619; Fig. 4i and Supplementary Fig. 4B). Thus, hPSC-derived VSMCs are capable of responding to vasoconstrictive stimuli exhibiting a key functional in vivo characteristic.
**Figure 4** *In vitro* characterization of hPSC ECs and hPSC VSMCs.  
(a) Impedance-based monitoring of hPSC EC monolayer culture. Thrombin treatment (blue, see inset) induced a rapid decrease in impedance compared with the control (red). One of the 4 independent experiments is depicted ($n=8$ technical replicates per experiment). Points represent mean ± s.d.  
(b) TEER properties of human umbilical cord endothelial cells (HUVECs) and hPSC ECs either untreated or treated with 100 ng ml$^{-1}$ TNF-α, 100 ng ml$^{-1}$ VEGF-A or 100 ng ml$^{-1}$ IL1β; $n=3$ wells of 3 independent experiments ($n=9$ wells in total except for untreated hPSC ECs $n=7$ in total). Columns show mean ± s.e.m. Student’s $t$-test; $^{*}P=5.56 \times 10^{-6}$; $^{*}P=1.61 \times 10^{-5}$.  
(c) Uptake of fluorescently labelled acetylated LDL (Ac-LDL) by hPSC ECs. Scale bar, 50 µM. Representative image of 3 independent experiments.  
(d) Expression analyses of the adhesion molecule ICAM1 on treatment of hPSC ECs with proinflammatory cytokines. Representative images and fluorescence-activated cell sorting plot from 3 independent experiments (3 technical replicates per experiment).  
(e) Immunofluorescence staining reveals upregulation of ICAM1 by TNFα treatment. Scale bars, 50 µM.  
(f) Quantification of ICAM1 expression on TNFα or IL1β stimulation.  
(g) Dose-dependent blockage of HL60 cell adhesion by anti-ICAM1 antibody pretreatment. Columns show mean ± s.d. of 3 independent experiments (except for treatment with control antibody concentrations 0.1, 1.3, here $n=1$).  
(h) Calcium imaging of SC VSMCs at day 13 of differentiation. Stimulation with vasoconstrictive reagents resulted in an increase in intracellular calcium. Fold-change RFU to $t=1$ (before treatment) at $t=50$ s (maximum peak). Columns show mean ± s.d. of 3 independent assays and data were evaluated using Student’s $t$-test; $^{*}=P=0.03$.  
(i) Contractility assay on UASMCs and hPSC VSMCs with U46619 48 h after treatment. This graph shows one single experiment where control = 1 well and test = 2 wells per conditions.

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Deposition of extracellular fibronectin was analysed following treatment of hPSC VSMCs with increasing concentrations of TGF-β (Supplementary Fig. 4C). Interestingly, fibronectin deposition could be detected even in untreated cultures. As VSMCs produce TGF-β this is possibly due to autocrine TGF-β signalling. Treatment with exogenous TGF-β led to a slight increase in fibronectin production. The addition of small molecules inhibiting TGF-β signalling (SB525334 or JQ1) prevented fibronectin production in the presence and absence of exogenous TGF-β. Thus, fibronectin deposition of hPSC VSMCs is mediated by TGF-β.

In summary, these findings show that hPSC-derived ECs and VSMCs demonstrate key mature functional properties.

hPSC ECs form vascular structures in vitro and in vivo after transplantation

Tube formation assays were performed to determine the angiogenic potential of hPSC ECs. Formation of vascular network-like structures was observed within 24 h and could be perturbed by treatment with sulphoraphane and an anti-VEGF monoclonal antibody (Fig. 5a,b). Remarkably, when hPSC ECs were co-cultured with primary human brain vascular pericytes (hBVPs) both cell types arranged in highly organized structures with hBVPs tightly associated to endothelial tubes (Fig. 5c). hBVPs cells seemed to envelope hPSC EC tubes (Fig. 5d). These results demonstrate that hPSC ECs have angiogenic potential in vitro. To evaluate this potential in vivo, we used fibrinogen grafts in immunodeficient NOD-SCID mice. We implanted either hPSC ECs alone, HUVECs and MSCs, hPSC ECs and MSCs, or hPSC ECs and hPSC VSMCs into the dorsal side of NOD-SCID mice and recovered the grafts for analysis after 14 days (except for hPSC ECs alone = 7 days) (Supplementary Fig. 5). Haematoxylin and eosin staining showed vessel-like networks in implants containing HUVECs and MSCs, hPSC ECs and MSCs, and hPSC ECs and hPSC VSMCs (Fig. 5g,i,k). Of note, vessels contained numerous circulating red blood cells. Staining with an antibody against human CD31 revealed vessel-like structures throughout the implants (Fig. 5h,j,l). Thus, hPSC ECs are capable of contributing to patent, blood-containing vessel-like networks in vivo.

DISCUSSION

We report the rapid and efficient differentiation of hPSCs to ECs and VSMCs. GSK3β inhibition combined with BMP4 treatment enabled the commitment of hPSCs to mesoderm. Subsequently, differentiation was directed by treatment with VEGF-A for ECs or PDGF-BB and ActivinA for VSMCs. This in vitro differentiation pathway showed similarities to vascular development in vivo. For instance, Wnt signalling is required to induce primitive streak formation, which then becomes committed lateral plate mesoderm by BMP4 signalling. These events were also observed during our in vitro differentiation protocol. Genome-wide expression analysis revealed transiently expressed gene clusters linked to mesoderm formation and further regionalization into lateral plate mesoderm. Subsequently, EC and VSMC formation was indicated by increased expression of genes associated with vasculogenesis. Importantly, our protocol produced ECs and VSMCs that closely resembled their primary counterparts. Comprehensive functional characterization of hPSC-derived vascular cells indicated a bona fide vascular phenotype. Taken together, these experiments confirm
the identity and maturity of the hPSC-derived ECs and VSMCs and suggest that the cells could be used to faithfully model human disease.

Compared with other protocols that induce vascular cells from hPSCs, our approach provides several advantages. First, it occurs under chemically defined conditions in monolayer culture with low variability. Although a recently published report uses chemically defined conditions and starts with a monolayer culture, they observed considerable variation between experiments and starting cell lines and achieved efficiencies of only 10–30% (ref. 18). Second, our protocol allows the formation of ECs and VSMCs in a process closely resembling in vivo development. Last, our method allows rapid vascular differentiation with high efficiency. We obtained from 1 million hPSCs approximately 25–30 million ECs or VSMCs within 6 days, cutting differentiation with high efficiency. We obtained from 1 million hPSCs considerably variation between experiments and starting cell lines and fined conditions and starts with a monolayer culture, they observed variability. Although a recently published report uses chemically de-

methods

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper

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AUTHOR CONTRIBUTIONS

C.P., L.C.-M., E.C.T. and E.U. designed and performed experiments, analysed and interpreted data and wrote the manuscript. S.G., F.G.K., L.S., K.C., Y.X., M.H.C.F., M.P., D.K., T.H. and W.H. contributed to description of online methods.

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METHODS

Human PSC culture and differentiation. The human ESC line SA001 (ref. 36) was obtained from Celltrek AB and the human Gibco Episomal hiPSC line was from Life Technologies. Other cell lines used in this study are hESC Hues9 (ref. 49) and hiPSCs (all passage 4). All cell lines were grown in the presence of 4% paraformaldehyde (PFA) for 10 min. The cells were then blocked with 10% normal donkey serum in PBS (Blocking buffer) for 45 min. When probing for an intracellular antigen, 0.1% Triton-X was included in the blocking buffer. The cells were then incubated with the primary antibodies (see Supplementary Table 8A) in 5% normal donkey serum in PBS overnight at 4 °C. After three washes with PBS, cells were incubated in 1% BSA in PBS containing secondary antibodies (see Supplementary Table 8B) and DAPI. Cells were washed three times with PBS and images were captured using a fluorescence microscope (Axiovert 200) and images were analysed using ImageJ.

For flow cytometry analysis, cells were dissociated with Accutase. Single-cell suspensions were subsequently incubated in growth medium at 37 °C for 1 h before staining. All FACS antibodies were directly labelled and obtained from BD Pharmingen. Stainings were performed according to the manufacturer’s instructions and analysed using a Guava easyCyte flow cytometer (Millipore). The FACS antibodies used in this study are listed in Supplementary Table 8C.

Genome-wide gene expression analysis. Total RNA from cells was extracted using the RNeasy Mini kit combined with DNase treatment on a solid support (Qiagen). RNA quality assessment and quantification was performed using microfluidic chip analysis on an Agilent 2100 bioanalyzer (Agilent Technologies). Five micrograms of total RNA was reverse transcribed using the Roche cDNA Synthesis System. After silica-adsorption column purification, double-stranded cDNA was labelled with Cy3 using the Roche NimbleGen One Color DNA Labeling Kit. NimbleGen 12 × 135 K gene expression microarrays (100718_HG18_0pt_expr) were hybridized with 4 μg of Cy3-labelled cDNA for 16 h at 42 °C and were washed and dried according to the manufacturer’s instructions. Microarray data were collected by confocal scanning using the Roche NimbleGen MS200 Microarray Scanner at 2 μm pixel resolution (Roche NimbleGen). NimbleGen probe intensities were subjected to robust multi-array analysis (RMA). Background correction and quantile normalization as implemented in the NimbleScan Software, version 2.6 (Roche NimbleGen). Probe level signal intensities were summarized into gene calls and log2-transformed. Gene Ontology (GO) term analysis of gene lists was performed using DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov). Gene Set Enrichment Analysis was applied to the data on the basis of the BROAD Institute algorithm (http://www.broadinstitute.org/gsea). Fold changes were used to rank the genes and to determine the enrichment of genes in gene sets derived from 3591 Gene Ontology (GO) terms (http://www.geneontology.org). For all GSEA analyses, 1,000 permutation analyses were performed to assess the significance of the enrichment. Gene expression heat maps were generated using Tibco Spotfire 3.1.0 (Tibco Software) or the R software for statistical computing and graphics (http://www.r-project.org). The raw data from this study have been deposited at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession numbers GSE52044 and GSE59326.

Metabolite profiling. HUVECs, UASMCs (Lonza), hPSC ECs and hPSC VSMCs (all passage 4) were seeded in 6-well plates and cultured in StemPro34 medium. Once confluent, cells were starved in basal medium. The medium was siphoned off and the plate immersed in high-performance liquid chromatography (HPLC)-grade water, the water decanted and aspirated, and then the culture plate carefully placed on the surface of liquid nitrogen in a liquid nitrogen-resistant basin for 15 s to quench cellular metabolic activity. Next, 2 ml of extraction medium (75% 9:1 methanol/chloroform, 25% H2O) was poured into each well. After 15 s, 1.5 ml was aspirated into respective labelled sample tubes, and 0.5 ml was aspirated from each well into collective pooled lysate tubes. Lysates were then centrifuged (16,873g, 4 °C for 10 min), and 200 μl of the supernatant was aliquoted into conical Eppendorf tubes and labelled with isotope standards (L-phenylalanine-d4, and L-valine-d3) were added to the supernatants. Samples were then dried down on a speedvac concentrator (Thermo Scientific) and resuspended in 100 μl of acetonitrile/water (50:50 v/v) before injection. Sample injection volume was 10 μl.

Liquid chromatography–tandem mass spectrometry (LC–MS) data were acquired using a 4000 QTRAP triple quadrupole mass spectrometer (Applied Biosystems/Sciex) that was coupled to a multiplexed LC system comprised of a 1200 Series pump (Agilent Technologies) and an HTS PAL autosampler (Leapp Technologies) equipped with two injection ports and a column selection valve, as previously reported46. The pump was configured for hydrophilic interaction chromatography (HILIC) using a 150 × 2.1 mm Atlantis HILIC column (Waters) and a 30 × 2.1 mm Atlantis HILIC column (Waters) was switched using a T-piece (Waters). The mobile phase was 10 mM ammonium formate (pH 4.0) and 0.1% formic acid, v/v. Mobile phase B: acetonitrile with 0.1% formic acid, v/v. Multiplexing was used to enable the measurement of 106 metabolite transitions. The samples were injected directly onto a 150 × 2.1 mm Atlantis HILIC column (Waters) that
vascular pericytes (hBVPs) were obtained from ScienCell Research Laboratories. Co-culture with primary human brain vascular pericytes was observed. The tube formation capacity was parameterized by the total segment length per three or more segments. Large convex cell areas were classified as area of confluence. The network was classified into linear segments and nodes, defined as the convergence of metabolites. Formic acid, methanol, chloroform, ammonium acetate, LC–MS-grade solvents and L-valine-d8 were purchased from Sigma-Aldrich. L-phenylalanine-d8 was purchased from Cambridge Isotope Laboratories.

MultiQuant software (Version 2.1.1; Applied Biosystems/Sciex) was used for automated peak integration and metabolite peaks were manually reviewed for quality of integration and compared against a known standard to confirm identity. For all isotope measurements using these LC–MS methods, peak areas were greater than two orders of magnitude above the lower limit of quantification (as defined as a discrete peak tenfold greater than noise) and fell well within the linear range of the dose–response relationship. Data were normalized relative to pooled plasma reference samples that were analysed in the same queue after sets of 10 study samples. All experiments were done in triplicate.

Calcium imaging. At day 11 of differentiation, VSMCs were seeded on collagen-coated wells at 50,000 cells cm⁻² in N2B27 containing 2 µg ml⁻¹ heparin and 2 ng ml⁻¹ ActivinA. After 48 h, cells were pretreated with 1 mM atropine (Sigma) or an equal amount of ethanol (atropine solvent) for 45 min at 37 °C. Then, cells were incubated for 15 min with calcium dye and calcium imaging was performed. Relative fluorescence units (RFU) were measured every second. After 15 s, cells were treated with vehicle, 1 mM carbachol (Sigma) or 0.1 µM endothelin-1 (Sigma). For calculation of fold change, average values of the first 14 data points were used as reference (10). Fibronectin production. At day 12 of differentiation, hPSMCs were seeded in N2B27 containing 10 ng ml⁻¹ PDGF-BB at 37,500 cells cm⁻² on gelatin-coated wells. After 24 h, the medium was changed to N2B27 with 10 ng ml⁻¹ PDGF-BB supplemented with DMSO (control), 10 µM SB525334 (Tocris), or 10 µM JQ1 (Biovision) and indicated concentrations of TGF-β (Peprotech). After 24 h, cells were washed with PBS, fixed with 4% PFA for 10 min at room temperature, and immunofluorescence staining of deposited Fibronectin (no permeabilization step) was performed.

Tube formation. Four hundred microliters of Matrigel (BD Matrigel Basement Membrane Matrix Growth Factor Reduced, Phenol Red Free) was aliquoted into each well of a 12-well plate and incubated for 30–60 min at 37 °C to allow the gel to solidify. HPSC ECs pre-stained with CellTracker (Molecular Probes/Life Technologies) and 80,000 ECs were then seeded onto the matrix and cultured for 24 h at 37 °C until image acquisition. To test the impact of the angiogenesis inhibitors L-sulphoraphane (Sigma S6317) and anti-VEGF monoclonal antibody (human VEGFₐ antibody; R&D Systems) on tube formation, the ECs were pre-incubated with inhibitors at indicated concentrations for 45–60 min at 37 °C. Eighty thousand cells were then added to the Matrigel-coated well and cultured for 24 h at 37 °C. Cells were fixed with 2% PFA for 10 min and stained with Diff-Quick II (Medion Diagnostics) for 2 min. To measure the extent of tube formation, micrographs covering an area of 32 mm² were acquired in each well using an inverted microscope (Axiovert 200, ×5 objective). The acquired images were analysed using a custom-developed image analysis algorithm (see below).

Quantitative image analysis of tube formation. The capacity to form tubular structures on a two-dimensional Matrigel surface was quantified using a custom-developed image analysis algorithm implemented in the Developer XD software package (DST). Briefly, areas in the image containing cells were identified from background by multiple threshold segmentation. The segmented cell area was skeletonized to obtain the central line of the tubular structure. The resulting linear network was classified into linear segments and nodes, defined as the convergence of three or more segments. Large convex cell areas were classified as area of confluence. The tube formation capacity was parameterized by the total segment length per image (in pixels) and the number of nodes per image. No large area of confluence was observed.

Co-culture with primary human brain vascular pericytes. Human brain vascular pericytes (hBVPs) were obtained from ScienCell Research Laboratories.

The hBVPs used for the experiments were between passage 3 and 4. All cultureware (BD Falcon) was coated with rat tail collagen type I solution (BD Biosciences) at 10 µg cm⁻². hBVPs were grown in Pericyte Medium (ScienCell Research Laboratories). For the functional tube formation and association assays, the HPSC ECs and the hBVPs were grown using an in vitro angiogenesis kit from AMS Biotechnology. Immediately before seeding for functional assays, hBVPs and HPSC ECs were stained red and green, respectively, using CellTracker dyes (Molecular Probes/Life Technologies) according to the manufacturer’s instructions. Cells were grown in StemPro34 SFM (Life Technologies) supplemented with 50 ng ml⁻¹ rhVEGF-A (Peprotech). For the experiment, 2 × 10⁵ hBVPs cm⁻² and 2 × 10⁴ hPBGF (R&D Systems), for 24 h in the incubator.

Uptake of Dil-Ac-LDL. Cells were incubated with 2.5 µg ml⁻¹ Dil-Ac-LDL–Alexa597 (Molecular Probes/Life Technologies) in StemPro34 medium for 4 h at 37 °C. Thereafter, cells were washed three times with PBS and fixed with 4% PFA for 10 min. DNA was counterstained with DAPI. Cellular uptake of Dil-Ac-LDL–Alexa597 was visualized with a fluorescence microscope (Axiovert 200).

Real-time impedance measurement and thrombin stimulation. Cell growth behaviour was continuously monitored every 15 min for 2 days using a Real Time Cell Analyzer (xCELLigence, Roche). For time-dependent cell response profiling, 100 µl of cell culture medium was added to the fibrocartin-coated 96-well E-plates to obtain background readings followed by the addition of 100 µl of cell suspension. The E-plates containing the cells (20,000 cells cm⁻²) were incubated at room temperature for 10 min and placed on the reader in the incubator for continuous recording of impedance as reflected by cell index (CI). The cells were monitored every 15 min before and every 2 min after thrombin addition. The CI curves are shown as the average of 5 replicates ± s.d. Cells were treated with 20 U ml⁻¹ thrombin. All data were normalized to the first impedance background measurement.

Trans-endothelial electrical resistance (TEER). HUVECs and HPSC ECs were seeded at a density of 1 × 10⁵ cells ml⁻¹ on 24-well cell culture inserts (0.4 µm pore size, Falcon) coated with fibronectin. Cells were grown for 72 h in EGM-2 medium (Lonza). The medium was then changed to starvation medium (basal EBM medium + 0.5% FBS; Lonza) with or without vascular permeability factors (100 ng ml⁻¹ TNF-α and IL-1β; Peprotech). 100 ng ml⁻¹ VEGF-A (Aldevron) for 30 h. Trans-endothelial electrical resistance was recorded using the Endothelial for 6 mm Culture Cup (World Precision Instruments). This experiment was performed in triplicates for each condition and repeated 3 times. Results are depicted as the steady-state TEER values with blank filter subtracted.

Adhesion molecule expression and adhesion of leukocyte-like cells to ECs. Adhesion molecule expression was also determined in a cell-based ELISA. Here, hPSMC ECs (2 × 10⁵ cells per well) were seeded and grown in HC-06 well microplate in 100 µl of EGM-2 medium (Lonza) until confluence (2–3 days). They remained either unstimulated or were stimulated with TNF-α (0.1–1 nM) for 24 h at 37 °C. Afterwards cells were washed with PBS and fixed with 2% PFA dissolved in PBS (pH 7.5). After a blocking step with sheep serum (20 min), cells were incubated for 1 h at 37 °C with a mouse anti-human VCAM1 (clone 1.4C3, Sigma), mouse anti-human ICAM1 (clone R&D Systems) or mouse anti-human E-selectin (BBIG-E4; R&D Systems) diluted in PBS + 0.01% BSA. A biotinylated sheep anti-mouse IgG (Amersharm) was used for the detection of the bound primary antibody, followed by the addition of a preformed streptavidin-biotinylated horseradish peroxidase complex. Peroxidase activity was detected using TMB (KPL, a seraCare Company, Cat. 50-76-00) substrate solution. Attenuance was measured at 450 nm with the use of a 96-well microtiter plate reader (Molecular Devices, Versa max). To analyse the adhesion of HL60 cells to ECs, Calcine-AM (Life Technologies)-labelled HL60 cells were added at a dilution of 150,000 cells per well were co-incubated with the ECs for 1 h at 37 °C. After non-adherent HL60 cells were removed by washing with PBS, the cells were either fixed with PFA for immunofluorescence staining or lysed with NP-40 (Fluka) and the fluorescence was measured in a spectrophotofluorometer (Spectravm Gemini XS, Molecular Devices). In some experiments the ECs were pre-treated with neutralizing anti-ICAM-1 monoclonal antibody (clone 15.2; SouthernBiotech) 15 min before TNF-α stimulation.

Fibrinogen plug assay. Animal procedures were performed in accordance with an IACUC-approved protocol reviewed by the Subcommittee on Research Animal Care (SARAC) at Massachusetts General Hospital and consistent with the NIH guidelines for laboratory animal usage. Twenty male (5 per condition) 8-week-old C57BL/6J were used. The implants were prepared following the protocol described in ref. 48. The matrix was prepared by dissolving 2.5 mg ml⁻¹ of bovine fibrinogen (Sigma-Aldrich) in basic EGM-2 (Lonza) and filtered through a 0.22 mm syringe filter (Millipore). HUVECs or hPHSC ECs were
mixed in a 1:1 ratio with primary MScs (Lonza) or hPSC VSMCs and resuspended in the fibrinogen solution at a final concentration of 10 million cells ml$^{-1}$. Mice were anaesthetized with 1 g of 2,2,2-tribromoethanol alcohol dissolved in 1 ml of tert-amyl alcohol and diluted in 19 ml of PBS. Then two sites of injections were shaved, sterilized with betadine (Thermo Fisher Scientific) and washed with alcohol. Right before injection, 5% PBS and 6 µl of a thrombin solution (50 µU ml$^{-1}$; Sigma-Aldrich) were added to 300 µl (= 3 million cells) of the fibrinogen–cell mixture. This solution was then immediately injected subcutaneously into the dorsal flank of the mouse. Two implants were injected per animal. Mice were returned to their cage for recovery about 5 min after injections to ensure proper polymerization of the implants. Three million hPSC ECs without any stromal cell type per implant were injected as the control. Experimenters were not blinded to the different experimental conditions. Implants were recovered after 14 days, except for the negative control, which was collected on day 7. Explants were placed in individual wells of a 12-well plate and imaged with an Axio Zoom V16 stereomicroscope (Zeiss). They were then fixed in 4% PFA for 1 h and then 0.4% PFA overnight. Both incubations were done at 4°C. Samples were then washed and kept in PBS at 4°C until they were embedded in paraffin and cut in 5 µM sections by C. MacGillivray at the HSCRB Histology Core facility. Haematoxylin and eosin staining was performed on even-numbered slides following a standard protocol. Odd-numbered slides were stained with a human specific CD31 antibody. Paraffin sections were rehydrated according to a standard protocol and boiled for 45 min in citrate buffer for antigen retrieval. After a quick rinse in distilled water, slides were treated with 3% hydrogen peroxide for 15 min and rinsed again. The primary antibody (HRP-conjugated anti-mouse) diluted 1:500 was added for 30 min at room temperature. After 4 × 15-min-long PBS washes, slides were incubated for 30 min with the ABC solution (DAB kit, Dako), washed again and incubated with DAB substrate following the manufacturer’s instructions. Slides were then mounted and imaged using a Dual head microscope (Olympus) and the Cellens software (Olympus).

**Statistical analysis.** Statistical analyses were performed using Student’s t-test and data were reported as mean ± s.d. or standard error of the mean. Statistical significance was assumed when $P < 0.05$. Spearman’s rank correlation coefficient was used to detect statistical dependence between cell metabolite levels for the non-parametric metabolomic data. No samples or data were excluded from the analysis. No power calculation was performed to determine the number of samples required to achieve statistical significance. No randomization was used to determine how mice or samples have to be allocated in different experimental groups. No experiment was done blinded to the experimenter.

**Reproducibility of experiments.** The luciferase assay was repeated 5 times independently at Roche. HPSCs ECs and hPSCs VSMCs were conducted 10 and 16 times respectively (Fig. 2a) and the resulting cells were used for the experiments described in Figs 1–5 and Supplementary Figs 1–5. All immunostainings (Figs 1b–d, 2c,d, 4c,d and 5a,c–i and Supplementary Figs 1E, 2A,F, 4B,C and 5A–D) were repeated 3 times in 3 different wells of a 24-well plate. The qPCR in Fig. 3c shows the mean of 3 independent experiments with samples from 3 wells of a 12-well plate each time. The microarray experiment (Fig. 3 and Supplementary Fig. 3) was performed once with one biological replicate for endothelial differentiation at day 0–10, and one biological replicate for HSVECs, HAVSMCs and HPVSMCs. For the other microarray samples three biological replicates were analysed. The metabolomics experiments (Fig. 3) was conducted only one time with biological triplicates for each cell type. The impedance-based monitoring of hPSC ECs monolayer culture (Fig. 4a) was repeated 4 times in the laboratory with 8 technical replicates each. The TEER experiment (Fig. 4b) was repeated 3 times independently for a total of 9 Transwells per condition except the untreated one where $n = 7$ Transwells only. Adhesion assay was repeated 3 times in the laboratory (Fig. 4c–g). The contractility experiment shown in Fig. 4h was repeated 3 times independently in the laboratory whereas the one in Fig. 4i was conducted only once with 2 wells per condition (except control $n = 1$ well). The tube formation assay in Fig. 5a was repeated 10 times and the effect of anti-angiogenic molecules was measured in 3 independent experiments (Fig. 5b). Co-culture experiments of hPSC ECs with hBVPS were repeated 3 times. The in vivo experiment was conducted once with 5 NOD-SCID mice per condition and 2 implants per mouse (=10 implants per condition).

**Data access.** The microarray data from this study have been deposited at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.gov/geo) under the accession numbers GSE52044 and GSE59326.

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Supplementary Figure 1 Canonical Wnt activation by GSK3β inhibitors and mesoderm induction. (A) Molecular structure of different GSK3β inhibitors. (B) Schematic illustration of the luciferase reporter assay used in this study to detect the expression of β-catenin. (C) Cell viability assay upon treatment with different concentration of GSK3β inhibitors. A 6-point 3-fold serial dilution of each compound was performed (10, 3, 1, 0.3, 0.1, 0.03 µm, last 2 concentration data not shown). Columns show mean +/- SD of 5 independent experiments. (D) Binding constant (Kd) determination for the GSK3β inhibitors tested in this study. An 11-point 3-fold serial dilution of each test compound was prepared in 100% DMSO at 100x final test concentration and subsequently diluted to 1x in the assay (final DMSO concentration = 2.5%). Representative curve of four independent experiments is shown. (E) Bright field pictures of hESCs treated with increasing concentration of the GSK3β inhibitor BIO illustrating its cell toxicity. Representative images of one experiment are shown.
Supplementary Figure 2 VEGF and PDGF-BB-mediated differentiation of hPSCs into vascular endothelial or smooth muscle cells. (A) BMP4-dependent expression of mesoderm markers T, MIXL, and EOMES at day 4 of differentiation. Columns show mean of 3 technical replicates of a single well of a single experiment. (B) Comparison of the ability of different GSK3β inhibitors to induce endothelial cell differentiation as shown by immunostaining for the endothelial marker VE-Cadherin. Representative images of 3 independent experiments. (C) Effect of BMP4 on hPSC-ECs differentiation. 3 wells per conditions of a single experiment. (D) Potency of different GSK3β inhibitors to induce hPSC-ECs differentiation. Mean values +/- SD of 3 independent experiments are shown. (E) Differentiation efficiency of hPSCs on day 6 after sorting for CP21 and CHIR when used at their optimal concentration (defined in D). 3 wells per conditions of a single experiment. (F) Representative FACS plots showing the improvement of differentiation efficiency when BMP4 and forskolin are added to the media. This experiment was done once and 3 wells per conditions were analyzed. (G) FACS analysis of CD144+ hPSC-ECs on day 10 showing the expression of ECs-specific markers (KDR, CD31, CD34, CD105) and the absence of hematopoietic markers (CD43, CD45). Representative results of 5 independent experiments. (H) The endothelial cell differentiation protocol produces a small amount of alpha smooth actin positive cells as shown by immunostaining on day 6 before MACS sorting. Representative image of 5 independent experiments. (I) Role of ActivinA and PDGF-BB in the differentiation efficiency of hPSC-VSMCs. 3 wells per conditions of a single experiment. (J) Differentiation efficiency of hPSC-VSMCs for the two GSK3β inhibitors CP21 and CHIR when used at their optimal concentration (1μM and 6μM, respectively). 3 wells per conditions of a single experiment. (K) Effect of BMP4 on hPSC-VSMCs differentiation, n=3 wells of a single experiment. (L) Example of the efficiency of MACS sorting and the purity of the resulting hPSC-ECs population, representative result from 2 independent experiments.
Supplementary Figure 3 Global Transcriptome analysis during vascular wall cell differentiation. (A) Principal component projections of transcriptomes colored by sample type. The variability of the data set along Principal component 1 is 28.8 % and along Principal component 2 is 24.1 %. Note the clustering of precursor cells during the early time points and the clustering of differentiated vascular wall cells with their respective primary cells. (B) Dynamic gene expression of representative spatio-temporal regulated genes during the course of endothelial cell differentiation. The detection limit of the microarray platform is indicated by a dotted line; this signal is derived from 5000 random probes (60-mers of random nucleotides), which serve as a metric of non-specific annealing and background fluorescence. (C) Heat map of genes sets of biological processes (GO terms) significantly over- or under-represented in stem cell derived ECs, VSMCs, and primary cells in comparison to ESCs. Rows represent genes, and columns are samples. Row Z-score transformation was performed on log₂ expression values for each gene with blue denoting a lower and red a higher expression level according to the average expression level. Hierarchical clustering of genes and samples is based on average linkage and correlation distance.
**Supplementary Figure 4** *In vitro* characterization of hPSC-ECs and hPSC-VSMCs. (A) Calcium imaging of SC-VSMCs at day 13 of differentiation. Stimulation with vasoconstrictive reagents resulted in increase in intracellular calcium. Time course of calcium flux after treatment. RFU was measured every second and average values of three independent experiments are shown. (B) Example of collagen gel contraction assay after 48 hours with or without U46619. Gel surface areas were measured and further analyzed using ImageJ. Scale bars: 1cm (C) Fibronectin production of hPSC-VSMCs upon TGF-β treatment. Immunofluorescence staining of extracellular fibronectin depositions after 24 hours of TGF-β treatment in the presence of absence of TFG-β inhibitors. Scale bars: 50μM. Representative images of 3 independent experiments are shown.
Supplementary Figure 5 Whole mount view of fibrinogen implants. Representative pictures of whole mount implants. This experiment was conducted once with 5 mice per conditions and 2 implants per mice (=10 implants per conditions) (A) hPSC-ECs only (B) HUVECs + MSCs (C) hPSC-ECs + MSCs and (D) hPSC-ECs+hPSC-VSMCs. Scale bars = 500μM except A) scale bar = 5mm.
**Supplementary Table 1** Small molecule screening data. Overview over the parameters applied in the Wnt reporter assay (Figure 1A and S1C).

**Supplementary Table 2** Competitive protein kinase binding assay. One experiment: Selectivity Score or S-score is a quantitative measure of compound selectivity. It is calculated by dividing the number of kinases that compounds bind to by the total number of distinct kinases tested, excluding mutant variants. $S = \frac{\text{Number of hits}}{\text{Number of assays}}$. This value can be calculated using $\%\text{Ctrl}$ as a potency threshold (below) and provides a quantitative method of describing compound selectivity to facilitate comparison of different compounds. $S(65) = \frac{\text{number of non-mutant kinases with } \%\text{Comp} > 65}{\text{number of non-mutant kinases tested}}$ $S(90) = \frac{\text{number of non-mutant kinases with } \%\text{Comp} > 90}{\text{number of non-mutant kinases tested}}$ $S(99) = \frac{\text{number of non-mutant kinases with } \%\text{Comp} > 99}{\text{number of non-mutant kinases tested}}$

**Supplementary Table 3** gene set enrichment analysis (GSEA) Enriched gene ontology (GO) terms of 2955 deferentially expressed (>10 fold changes) genes, GO descriptions, GO identity, number of genes associated with this GO term, normalized enrichment scores and False discovery rate (FDR) q-values.

**Supplementary Table 4** FACS data of hPSC differentiation efficiencies. (A) hPSC-ECs before MACS separation. n=10. (B) hPSC-ECs after MACS separation. n=16. (C) hPSC-VSMCs after replating on fibronectin. n=19.

**Supplementary Table 5** List of the 66 intracellular metabolites found in primary and differentiated vascular cells. Of the 106 metabolites on the platform, 66 were detected intracellularly. These included: amino acids (e.g. proline, valine, leucine, beta-alanine, citrulline), amino acid derivatives (e.g. carnitine, carnosine, kynurenine), nucleosides (e.g. uridine, xanthine, adenosine), organic nitrogenous compounds (e.g. histamine), aromatic acids (e.g. anthranilic acid), amine oxides (e.g. trimethylamine-N-oxide), and other organic acids.

**Supplementary Table 6** QPCR primers sequences. List of primers used in this study to detect markers expression by QPCR. Sequences are given in the 5'-3' orientation.

**Supplementary Table 7** Taqman probes. List of Taqman probes used in this study to detect markers expression by QPCR. All probes can be purchased at Life Technologies using the catalog number provided in this table.

**Supplementary Table 8** Antibodies. (A) Primary antibodies used for immunofluorescence. (B) Secondary antibodies used for immunofluorescence. (C) antibodies used for Flow cytometric analysis.