Functionality of Endothelial Cells and Pericytes From Human Pluripotent Stem Cells Demonstrated in Cultured Vascular Plexus and Zebrafish Xenografts

Valeria V. Orlova, Yvette Drabsch, Christian Freund, Sandra Petrus-Reurer, Francijna E. van den Hil, Suchitra Muenthaisong, Peter ten Dijke,* Christine L. Mummery*

Objective—Endothelial cells (ECs), pericytes, and vascular smooth muscle cells (vSMCs) are essential for vascular development, and their dysfunction causes multiple cardiovascular diseases. Primary vascular cells for research are, however, difficult to obtain. Human-induced pluripotent stem cells (hiPSCs) derived from somatic tissue are a renewable source of ECs and vSMCs; however, their use as disease models has been limited by low and inconsistent efficiencies of differentiation and the lack of phenotypic bioassays.

Approach and Results—Here, we developed defined conditions for simultaneous derivation of ECs and pericytes with high efficiency from hiPSCs of different tissue origin. The protocol was equally efficient for all lines and human embryonic stem cells (hESCs). The ECs could undergo sequential passage and were phenotypically indistinguishable, exhibiting features of arterial-like embryonic ECs. Moreover, hiPSC-derived ECs formed an authentic vascular plexus when cocultured with hiPSC-derived pericytes. The coculture system recapitulated (1) major steps of vascular development including EC proliferation and primary plexus remodeling, and (2) EC-mediated maturation and acquisition of contractile vSMC phenotype by pericytes. In addition, hiPSC-derived ECs integrated into developing vasculature as xenografts in zebrafish. This contrasts with more widely used ECs from human umbilical vein, which form only unstable vasculature and were completely unable to integrate into zebrafish blood vessels.

Conclusions—We demonstrate that vascular derivatives of hiPSC, such as ECs and pericytes, are fully functional and can be used to study defective endothelia–pericyte interactions in vitro for disease modeling and studies on tumor angiogenesis. (Arterioscler Thromb Vasc Biol. 2014;34:177-186.)

Key Words: endothelial cells ■ induced pluripotent stem cells ■ pericytes

Human pluripotent stem cells (hPSCs) proliferate during long periods and can differentiate into many cell types of the body. Because they can be derived from any healthy individual or patient, they are an increasingly important source of human cells for tissue engineering, modeling human disease, drug discovery, and safety pharmacology. Efficient directed differentiation of hPSCs toward both vascular endothelial cells (ECs) and the adjacent mural cells (pericytes or mesenchymal stromal cells [MSCs]) and vascular smooth muscle cells (vSMCs) that provide vessels with stability is however crucial for some of these applications. Moreover, vascular differentiation of hPSCs could reveal early steps in endothelial specification during human development and tumor angiogenesis and, as a result, lead to better understanding of mechanisms that cause vascular defects, underlie various cardiovascular diseases, and contribute to vascular invasion of tumors. Investigating the molecular signals driving differentiation, their timing and impact on vascular function are therefore important in a broader context than just vascular development.

In vivo ECs, pericytes, and vSMCs exhibit remarkable heterogeneity, with the cells of arterial, venous, and lymphatic vessels as well as tissue-specific vascular beds each showing distinct features. Unfortunately, it has been difficult to derive and maintain ECs with the relevant vascular identity and defective communication between ECs and pericytes cause multiple vascular diseases. For example, various genetic vascular malformations such as hereditary hemorrhagic telangectasia, cerebral cavernous malformations, and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) are caused by mutations in either endothelial (hereditary hemorrhagic telangectasia, cerebral cavernous malformations) or vSMC-specific (CADASIL) genes. Furthermore, defective EC–pericyte interactions are

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BOECs</td>
<td>blood outgrowth ECs</td>
</tr>
<tr>
<td>ECs</td>
<td>endothelial cells</td>
</tr>
<tr>
<td>Fib</td>
<td>skin biopsy fibroblasts</td>
</tr>
<tr>
<td>Fib-iPSC</td>
<td>skin biopsy fibroblasts-derived iPSC</td>
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<tr>
<td>hiPSC</td>
<td>human-induced pluripotent stem cell</td>
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<tr>
<td>hPSC</td>
<td>human pluripotent stem cell</td>
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<tr>
<td>HUVEC</td>
<td>human umbilical vein EC</td>
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<tr>
<td>KDR</td>
<td>kinase insert domain receptor</td>
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<tr>
<td>MSC</td>
<td>mesenchymal stromal cell</td>
</tr>
<tr>
<td>PDGFR</td>
<td>platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>SM</td>
<td>smooth muscle</td>
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<tr>
<td>TGFB</td>
<td>transforming growth factor β</td>
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<tr>
<td>VEC</td>
<td>vascular endothelial Cathedrin</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>vSMC</td>
<td>vascular smooth muscle cell</td>
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a hallmark of tumor blood vessels. The limited availability of primary human tissues, poor expansion of ECs in vitro, and a lack of appropriate vSMCs are major drawbacks toward better understanding of the mechanisms of different cardiovascular diseases and pathological angiogenesis.

Our goal here was to develop a protocol for differentiation of hPSCs toward vascular cell lineages that is applicable to any hPSC line and results in well-characterized, functional ECs, pericytes, and vSMCs. The protocol described is based on fully defined media and timed addition of growth factors to monolayer cultures and does not require an aggregation step (embryoid body [EB] formation). It results in efficient generation of ECs from both hESC and human-induced pluripotent stem cell (hiPSC; either derived from skin biopsy fibroblasts [Fib] or blood outgrowth ECs [BOECs]) lines. In addition, the protocol facilitates simultaneous derivation of both ECs and pericytes, which have the potential to differentiate to more contractile vSMCs. On coculture, hiPSC-derived ECs and pericytes formed a vascular plexus in vitro that was responsive to contractile vSMCs. On coculture, hiPSC-derived ECs and pericytes, and vSMCs. The study thus provides human models of ECs and compared with more widely used human umbilical vein ECs. The protocol described is based on fully defined medium based on the previously published bovine serum albumin (BSA) polyvinylalcohol essential lipids protocol. The mesoderm differentiation protocol was optimized based on cardiac mesoderm induction from hPSCs, and vascular specification conditions found to be effective in a forced aggregation SpinEB protocol (Orlova, unpublished). A schematic representation of the final protocol is shown in Figure I in the online-only Data Supplement. We first tested combinations of the following growth factors from day 0 to 3 of differentiation: bone morphogenetic protein 4 (B; 30 ng/mL), vascular endothelial growth factor (VEGF; V; 50 ng/mL) in the presence of GSK3-kinase inhibitor (CHIR99021; CHIR, C; 1.5 μmol/L; BVC), or the same conditions with additional Activin A (ActA, A; 25 ng/mL; BVAC). The cultures were then refreshed on day 3 with bovine serum albumin (BSA) polyvinylalcohol essential lipids containing VEGF or VEGF and SB431542 (SB), selective TGF β1 type I receptor kinase inhibitor, at 5 μmol/L. Examination of cell surface expression of the hematopoietic–specific marker (CD45) and CD31 or the cardiovascular marker Kinase insert Domain Receptor (KDR; also known as VEGFR2) by flow cytometry demonstrated robust induction of expression by day 10 (Figure 1A) with either protocol in the hiPSC line shown. Interestingly, vascular endothelial (VE)-Cathedrin (VEC) was the earliest marker that was upregulated (Figure IIa in the online-only Data Supplement). The earliest ECs were observed at day 6 to 7 of differentiation based on the appearance of cells with cobblestone morphology (data not shown), which coincided with the appearance of nonhemogenic VEC+/CD73+ cells, as described previously. However, the percentage of mature ECs identified as VEC+/CD73+ was the highest on day 10 of differentiation (Figure IIb in the online-only Data Supplement). In addition, we investigated whether there was expression of the earliest human hematopoietic–specific marker CD43. Flow cytometric analysis revealed the presence of ≤1% of CD43+ cells, with majority of the CD34+/CD43+ cells being CD43 negative (Figure IIa in the online-only Data Supplement). Notably, addition of Activin A on days 0 to 3 resulted in more robust and reproducible differentiation among a range of hiPSC lines (Figure III in the online-only Data Supplement). In addition, supplementation with VEGF together with SB (10 μmol/L) resulted on average in a 30% increase in the yield of ECs, which was consistent among several cell lines tested and in line with findings by other groups. The defined differentiation conditions resulted in the generation of CD34+/KDR+ cells in a variety of hiPSC cell lines (on average 18±3%) we recently generated from both human adult skin fibroblasts (Fib-iPSC) and BOECs (BOECs-iPSC) as well as hESC (HESC-NL4). To confirm efficient mesoderm induction, we examined the expression of various early mesoderm genes, as well as EC-specific genes (Figure 1B). Downregulation of pluripotent stem cell markers, such as OCT4 and NANOG by day 4 of differentiation, was followed by the induction of primitive streak/mesoderm lineage markers, such as Brachyury T and platelet-derived growth factor receptor (PDGFR)α, over the same time period. The early endothelial-specific transcription factor (ETV2) was also upregulated at day 4, followed by robust induction of endothelial-specific genes by day 10 of differentiation (activin receptor-like kinase 1, CD105, KDR). Interestingly, we also

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Mesoderm Induction and Vascular Specification of hPSCs in Monolayer**

To induce mesoderm differentiation in hPSCs, we used defined medium based on the previously published bovine

**Specification of hPSCs in Monolayer**

**Mesoderm Induction and Vascular**

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**Mesoderm Induction and Vascular Specification of hPSCs in Monolayer**

To induce mesoderm differentiation in hPSCs, we used defined medium based on the previously published bovine
observed induction of early mesenchymal/pericyte cell markers such as PDGFRβ, CD146, and NG2 proteoglycan, but not of contractile SMC markers. Overall, we observed no differences in the expression of mesoderm and endothelial-specific genes between the different lines.

Isolation and Expansion of hPSC-Derived ECs

Flow cytometry analysis of different hPSC lines on day 10 of differentiation showed robust induction of a CD31+ EC population, as well as CD31+PDGFRβ+ mesenchymal cells (Figure 2A). The differentiation efficiency was similar and consistent among different hPSC lines tested also using this assay, with average percentages 10% to 30% of CD31+/CD34+ cells, as well as ≈30% PDGFRβ+ mesenchymal cells (Figure 2B and 2C). We additionally examined the expression of other EC and mesenchymal cell markers on the endothelial CD31+/CD34+ population as well as PDGFRβ+ cells. Importantly, CD31+/CD34+ cells expressed reproducible and comparable levels of other EC-specific markers (VE-Cadherin, KDR, CD73, and CD105) and were negative for PDGFRβ. By contrast, the CD31−/CD34− population strongly upregulated PDGFRβ. Interestingly, expression of other mesenchymal stem cell markers (CD105 and CD73) in the CD31−/CD34− population was not observed on day 10 of differentiation (Figure 2D).

We considered CD31 as the most appropriate marker for positive selection of ECs because our differentiation protocol resulted in a large number of ECs, and not other CD31+ hematopoietic progenitors, as evidenced by flow cytometry analysis showing that all CD31+ cells expressed VEC and other endothelial-specific markers (Figure 2D).

The protocol facilitated isolation of ECs with average purities >95% after just 1 selection round (Figure 3A). This is particularly important for reproducible and specific functional and signal transduction studies in cell populations. Furthermore, ECs isolated from hESC, BOECs-iPSC, or Fib-iPSC cell lines expressed similar levels of endothelial-specific surface antigens such as CD31, CD34, VEC, CD73, and CD105 (Figure 3A). At the same time, these ECs lacked expression of hematopoietic markers CD43 and CD45, although CD14 expression was still detected at low levels, in agreement with previously published studies (Figure IV in the online-only Data Supplement).16

Immunofluorescent analysis revealed junctional localization of VEC and CD31, as well as prominent intracellular staining for von Willebrand factor (Figure 3B). Interestingly, hiPSC-derived ECs also exhibited heterogeneous expression of lymphatic associated markers lymphatic vessel endothelial hyaluronan receptor 1 (Figure 3B). hiPSC-derived ECs could be passaged ≥5× in EC-serum free medium with VEGF (30 ng/mL), basic fibroblast growth factor (20 ng/mL), and 1% bovine platelet poor plasma extract and cryopreserved using standard procedures (not shown).
Gene expression analysis revealed on average 2-fold higher expression of pan-endothelial markers such as CD31, VEC, and KDR than in HUVEC or human aortic ECs (Figure 3C). Interestingly, hPSC-derived ECs expressed significantly higher levels of the arterial marker Ephrin-B2 compared with human umbilical artery endothelial cells although expression of another more prominent arterial-specific marker Hey2, previously identified by gene expression profiling of primary human ECs, was lower compared with human umbilical artery endothelial cells. In addition, we observed lower levels of the
venous marker CoupTFII although EphrinB4 levels were comparable with HUVEC. hiPSC-derived ECs also expressed lymphatic vessel endothelial hyaluronan receptor 1 and VEGFR3. No expression of the lymphatic specific markers Podoplanin and Prox1 was observed (data not shown).

ECs derived from BOECs-iPSC and Fib-iPSC readily formed cord-like structures in the Matrigel tube formation assay as early as 6 hours postseeding (Figure Va in the online-only Data Supplement). Interestingly, the endothelial cords remained stable for ≤48 hours with prominent cord structures still evident ≤72 hours (data not shown). These results differ strikingly from HUVECs, where cord structures tend to regress rapidly after 24 hours both in our hands (unpublished) as well as in publications by other groups. Measurement of the total tube length and area covered by endothelial sprouts showed comparable values for BOECs-iPSC and Fib-iPSC-derived ECs (Figure Vb in the online-only Data Supplement).

Isolation and Expansion of hPSC-Derived Pericytes

Plating the CD31− fraction resulted in the expansion of a homogenous population of cells that expressed pericyte and MSC markers (PDGFRα, CD146, NG2, CD73, CD44, CD105; Figure 4A). In addition, supplementation of medium with 10% fetal bovine serum or fetal bovine serum plus the growth factors TGFβ3 (1 ng/mL) and platelet-derived growth factor-BB (2 ng/mL) for 4 days resulted in downregulation of PDGFRα and upregulation of early contractile SMC markers, such as smooth muscle (SM)22 and Calponin (CNN1) as observed by real-time polymerase chain reaction gene expression (Figure 4B) and immunofluorescent staining (Figure 4C). However, we did not observe expression of the definitive SMC marker SM myosin heavy chain (SMMHC) by immunofluorescent staining (data not shown), and only slight upregulation was observed by real-time gene expression analysis in hESC-derived mesenchymal cells (Figure 4B). The serum-free conditions resulted in the generation of cells with a similar morphology to those obtained in serum-based media (Figure VI in the online-only Data Supplement). Thus, we created a renewable source of pericytes/MSCs cell types that are the principle components of the vascular wall, from hPSCs simultaneously with ECs.

Modeling Endothelia–Pericyte Interactions in Cultured Vascular Plexus In Vitro

Next, we established a 2D coculture system in vitro consisting of hiPSC-derived ECs and pericytes (Figure 5A). The protocol was optimized and adapted to the BD Pathway 855 imaging system, based on a method initially described for the coculture of HUVECs and vSMCs. On day 7 of coculture, hiPSC-derived ECs had organized and formed sprouts on top of the mesenchymal cells, as evidenced by immunofluorescent staining with the EC-specific CD31 marker (Figure 5B).

Then, we examined the functionality of BOECs-iPSC and Fib-iPSC-derived ECs in the endothelia-pericyte coculture sprouting assay. Interestingly, both BOECs-iPSC− and Fib-iPSC− derived ECs had organized sprouts at day 7 of coculture (Figure VII in the online-only Data Supplement). The TGFβ signaling pathway is indispensable for the formation of stable vasculature because it is crucial for regulating EC proliferation, pericyte/vSMC function, and endothelia–pericyte interactions. Therefore, we first determined whether TGFβ signaling is important for the formation of vascular sprouts in the cocultures of hiPSC-derived ECs and pericytes. Notably, the activin receptor-like kinase 5 inhibitor SB enhanced sprout formation and resulted in an increase in the total area covered by endothelial sprouts (Figure 5C; Figure VII in the online-only Data Supplement). It also caused an increase of EC proliferation as determined by immunofluorescent staining with Ki67 nuclear proliferation marker (Figure VIII in the online-only Data Supplement).

The TGFβ and NOTCH signaling pathways are known to play important roles in promoting differentiation and acquisition of the contractile phenotype by mural cells. In addition, paracrine interactions between ECs and mural cells can...
by SM22-positive cells in basal coculture conditions. However, addition of SB and N-3,5- difluorophenyl acetyl-L-alanyl-2-phenylglycine-1,1-dimethyl ester together had no effect on the expression of SM22 in pericytes/MSCs cultured without ECs (Figure IXa and IXb in the online-only Data Supplement). Therefore, we conclude that SB regulates SM22 levels via NOTCH-dependent heterotypic cell–cell interactions. In summary, supplementation with SB results in increased EC proliferation, and an increase in the substrate surface area covered by ECs; this resulted in potentiation of heterotypic cell–cell contacts mediated by NOTCH. However, blocking the NOTCH pathway had no effect on the total areas covered by EC sprouts (Figure 5C). To further strengthen our data, and to exclude the possibility that the increase of SM22 is via paracrine factors, we also performed experiments where EC-conditioned medium, with or without SB, was used. EC growth medium or EC-conditioned medium gave similar outcomes on SM22 expression on SB addition (Figure X in the online-only Data Supplement). These data indicate that heterotypic cell–cell interactions and not paracrine factors play an important role in the EC-mediated induction of SM22-positive cells.

**Vascular Competence of hPSC-Derived ECs in Zebrafish Xenograft Model**

Next, we examined the engraftment potential of Fib-iPSC and BOECs-iPSC–derived ECs in vivo. Initially, hiPSC-derived ECs were injected into early stage blastula (265 out of 512 cells) stage embryos (Figure 6A). The CD31+ cells derived from either Fib-iPSC or BOECs-iPSC were highly proficient in forming vessel-type structures within the zebrafish embryo (Figure 6B and 6C). Importantly, zebrafish embryos developed normally, and no abnormalities or edema were observed (Figure XI in the online-only Data Supplement). Examination of human-specific proliferation markers (Ki67; Figure XII in the online-only Data Supplement), as well as the apoptosis marker Caspase-3, (Figure XIII in the online-only Data Supplement) revealed the presence of proliferative human EC, with few apoptotic ECs. To confirm that ECs retain their characteristics on injection into zebrafish, we performed immunofluorescent staining with a human specific antibody against platelet endothelial cell adhesion molecule-1 (huPECAM1). The entire vessel formed by the human ECs was also labeled with human specific antibody against platelet endothelial cell adhesion molecule-1 (Figure XIV in the online-only Data Supplement).

The CD31− control cells did not form any vessel-type structures (data not shown). Furthermore, there was no significant difference in vessel formation between ECs from the Fib-iPSC or BOECs-iPSC. The length of the vessels formed within the yolk of 30 embryos in which vessels were located in the yolk was compared. There was no significant difference in vessel length when ECs were derived from either the Fib-iPSC or the BOECs-iPSC lines (Figure 6F).

Furthermore, when the hiPSC-derived ECs were injected into the blood stream of 48 hpf embryos, the CD31+ cells were capable of integrating into the vascular system that had already developed (Figure 6D). Interestingly, HUVECs did not integrate into the embryonic zebrafish vasculature at all but rather attached to the vessels or migrated throughout the embryo (Figure 6E and 6G). Together, these observations
demonstrated that hPSC-derived ECs were able to integrate functionally into embryonic zebrafish vasculature.

**Discussion**

Several groups have described induction of ECs and pericytes from hPSC. However, most of the published protocols are based on serum-containing media or require coculture with stromal cell lines. More recently published protocols on differentiation in defined media required EB formation or forced aggregation in which hPSC are centrifuged to form compact spheroids or spin EBs. These protocols are efficient (on average 20% to 30% ECs in the differentiated cultures) but are more difficult to adapt for scaling up EC production. In addition, for the spin EB system, hPSC lines usually have to be adapted to single cell, enzymatic passaging in bulk culture from culture as colonies, which can be difficult.
and time consuming. This also might not be convenient if working with the multiple diseased hiPSC lines at the same time. We also noticed rather high line-to-line variation in the spin EB system when using hiPSC (Orlova, unpublished).

The protocol developed here has high line-to-line reproducibility and is suitable for use on hiPSCs growing in simple, feeder-free culture conditions, such as mTeSR1 medium on a Matrigel substrate. In addition, and in contrast to previously published protocols, it does not require the presence of stromal cells. As far as we are aware, it is the first description of simultaneous derivation of ECs and pericytes from hiPSC in defined conditions.

hiPSC-ECs exhibited a typical EC morphology and expressed distinctive EC markers such as CD31, VEC, and von Willebrand factor. Based on gene and protein expression of both arterial and venous markers, as well the presence of lymphatic endothelial markers such as lymphatic vascular endothelial hyaluronan receptor 1 and VEGFR3, we concluded that they resemble embryonic ECs. The question of whether hiPSC-ECs can be matured further toward various endothelial subsets in vitro remains open and requires additional research. By contrast, hiPSC-pericytes expressed common pericyte/MSCs markers, such as CD146, CD73, CD105, CD44, PDGFRβ, an NG2, and could differentiate further into more contractile SMCs in the presence of TGFβ and platelet-derived growth factor-BB; they also acquired expression of early contractile markers such as SM22 and CNN1.

We further tested the functionality of hiPSC-derived ECs and pericytes in 2 assays: the first was based on coculture of ECs and pericytes in monolayer and facilitated study of primary vascular plexus formation by ECs, as well as endothelia–pericyte interactions. hiPSC-derived ECs formed well-organized vascular sprouts on top of mesenchymal cells. In contrast to HUVECs, hiPSC-derived ECs were highly sensitive to TGFβ. Interestingly, the activin receptor-like kinase 5 inhibitor SB significantly increased the number of endothelial sprouts. This was most likely because of the more immature embryonic-like phenotype of hiPSC-derived ECs because sensitivity to TGFβ-mediated growth inhibition is typical of embryonic ECs.

Furthermore, heterotypic cell–cell interactions, between ECs and pericytes, are known to promote acquisition of more contractile vSMC markers by pericytes. Unexpectedly, supplementation of the culture medium with SB increased the expression of the contractile SMC marker SM22 in pericytes in coculture. It has been shown previously that endothelium-derived TGFβ is a more potent inducer of SM maturation than the growth factor added exogenously. Therefore, the observed effect is most likely because of increase of total number of ECs that resulted from loss of the growth inhibitory effect of endogenous TGFβ. In addition to TGFβ, Notch signaling has been shown to be important in heterotypic cell–cell interactions and promoting differentiation of SMCs. In line with this, blocking NOTCH signaling prevented acquisition of SM22 by pericytes.

The second assay was based on a zebrafish xenograft model. Development of alternatives to mouse and in vitro models for disease modeling is of high importance for both basic and pharmacological/clinical research. Zebrafish has been widely used as a model for studying development, but they are also valuable for modeling human disease. One of their foremost advantages is optical transparency. Because of the small size of the embryos, they can be imaged throughout development and as whole-mount preparations. Critically, this preserves both the mechanical and the molecular integrity of the embryo. Zebrafish embryos have also recently been useful for studying vasculogenesis. We demonstrated that hiPSC-derived ECs were able to incorporate into developing as well as established vasculature. Notably, there was no functional difference between the Fib-iPSC– or BOECs-iPSC–derived vessels formed within the embryos. Both cell lines gave CD31+ differentiated vascular derivatives that were capable of forming vessel structures when injected at the blastula stage of development but in neither case did the CD31-derivatives integrate. Furthermore, these hiPSC-derived ECs performed much better than the widely used HUVECs. Our observation of hiPSC-derived ECs within the embryonic zebrafish illustrates a new method for confirming EC identity in vivo and represents a step forwards in validating hiPSC-derived ECs as human vascular disease models.

In summary, we have developed an efficient protocol for differentiation of hiPSCs toward ECs and pericytes. hiPSC-ECs derived in this protocol (1) exhibited an embryonic-like phenotype, with mixed arterial/venous identity and expression of subsets of lymphatic EC markers; (2) displayed remarkable functionality in multiple in vitro assays, and functional characteristics were similar in ECs derived from hiPSCs with different donor cell origin; (3) were able to integrate into host vasculature in a zebrafish xenograft model. hiPSC-derived pericytes expressed typical pericyte/MSCs markers and acquired a contractile phenotype on exposure to exogenous TGFβ or coculture with ECs. vascular differentiation of hiPSCs could reveal early steps in endothelial specification during human development and lead to better understanding of mechanisms that cause vascular defects and underlie different cardiovascular diseases. As importantly, our coculture model is capable of modeling specific properties of both ECs and pericytes/vSMCs as they form a bona fide vascular plexus that captures the cross-talk these cells would normally exhibit in vivo. This interaction model we have developed is quantifiable and mimics aspects of tumor vasculogenesis including expected responses to TGFβ. In the future, we expect that the generation of specific subsets of ECs will be useful in such cocultures for modeling human vascular diseases in vitro and for tissue engineering.

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Disclosures

None.

References

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**Significance**

We have derived the 3 principle cell types that form blood vessels from human pluripotent stem cells and shown in multiple assays that they are functional both in vitro and in vivo. Notably, endothelial cells derived from pluripotent stem cells were highly responsive to transforming growth factor β and formed an authentic vascular plexus when cocultured with pericytes also derived from the same stem cells. Strikingly, these endothelial cells could fully integrate into the vasculature of Zebrafish in the novel xenograft model. This contrasts with the more widely used human umbilical vein endothelial cells and sets the stage for using vascular derivatives of human pluripotent stem cells as a renewable source of endothelial cells and pericytes for modeling pathological tumor angiogenesis and genetic vascular diseases.
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Materials and Methods

Cell culture and differentiation

hiPSC lines were generated from Fib or BOECs using conditional lentiviral vectors containing the four transcription factors OCT4, c-MYC, KLF4 and SOX2, and characterized as described previously. hiPSCs and hESC (line NL-HES4) were routinely maintained on growth factor reduced Matrigel (BD, 354230) coated plates in mTeSR media (StemCell Technologies, 05850) and passaged mechanically. Differentiation was induced three days after passaging colonies by replacing mTeSR medium with differentiation media based on BPEL (without PVA) and timed addition of the following growth factors: 25ng/ml Activin A (Miltenyi, 130-095-547), 30ng/ml bone morphogenetic protein (BMP)4 (Miltenyi, 130-095-549), VEGF165 (R&D Systems, 293-VE) and the small molecule inhibitor CHIR99021 (Tocris, 4423). On day 3, and day 7 of differentiation, the medium was refreshed with BPEL containing VEGF and 10µM SB43152 (Tocris, 1614) only. A schematic diagram of the growth factor combinations and timing is given in Supplemental Figure I. Primary human umbilical vein and artery endothelial cells (HUVEC and HUAEC respectively) were purchased from PromoCell and cultured as previously described. Magnetic bead purification of ECs

Purification of ECs was carried out on day 10 of differentiation with the CD31-labeled Dynabeads (Life Technologies, 1115D), as previously described. Briefly, differentiating cultures were washed once with phosphate buffered saline (PBS), and medium replaced by Dulbecco’s Minimum Essential Medium (DMEM)+0.1%BSA (Gibco). CD31-Dynabeads were washed twice with DMEM+0.1%BSA, and added drop-by-drop to the cells in DMEM+0.1%BSA. Dynabeads were incubated with the cells under gentle rotation (10-20rpm) for 20min at RT. After incubation, cells with the beads were washed once with the PBS, and detached by gentle enzymatic treatment for 5min at RT with 1x TrypLE Select (Life Technologies, 12563029). Beads and ECs were sequentially washed with the FACs buffer+10%FBS, followed by the FACs buffer alone then with DMEM+0.1%BSA to ensure good separation. After the last wash, ECs were re-suspended in human endothelial growth (serum free) medium (hEC-SFM) (Life Technologies, 11111) supplemented with 1% platelet poor plasma (BTI, BT-214), 30ng/ml VEGF and 20ng/ml basic fibroblast growth factor (bFGF) (R&D). ECs were then routinely grown on 0.1% gelatin coated plates, and passaged every 3-4 days with TrypLE Select.

Differentiation of iPSCs towards pericytes/mesenchymal cells

hiPSC-derived pericytes/mesenchymal cells were derived from the CD31- fraction emerging during the endothelial differentiation protocol. CD31- cells were plated on gelatin-coated plates in EGM-2 media (Lonza, CC-3162). After 4 days, medium was replaced with DMEM+10%FBS supplemented with TGFβ3 (2ng/ml, Peprotech) and PDGF-BB (4ng/ml, Peprotech). After 4 days medium was replaced with DMEM+10%FBS. iPSC-derived pericytes were routinely maintained on gelatin-coated plates in DMEM+10%FBS.
Flow cytometry (FACS) analysis

Cells were dissociated with TrypLE Select and washed once with the FACs buffer with 10% FBS, followed by the single wash with the FACs buffer. FACs lysing solution (BD, 349202) was used for the whole blood flow cytometry staining according to the manufacturer protocol. The combination of the following antibodies was used for the FACs staining: VE-Cadherin-A488 (eBiosciences, 53-1449-41, 1:100), CD31-APC (eBiosciences, 17-0319, 1:200), CD34-PerCP-Cy5.5 (BD Pharmingen, 347203, 1:100), KDR-PE (R&D Systems, FAB357P, 1:50), PDGFRb-PE (BD Pharmingen, 558821, 1:50), CD73-PE (BD Pharmingen, 550257, 1:50), CD105 A488 and PE (Life Technologies, MHCD10504, 1:200), NG2 (R&D, FAB2585P, 1:50), CD146-PE (BD, P1H12, 1:50), CD44-FITC (Biolegend, IM7, 1:50), CD14-APC-Cy7 (BD, 333951, 1:200), CD45-FITC (Sanquin, 1:10. M1491). The samples were acquired on LSRII (BD) with the following instrument settings Blue/488 FITC, A488: 505LP-530/30, PerCP-Cy5.5: 630LP-670/14; Yellow/561 PE: 570LP-582/15, APC: 635LP-660/20. In some experiment samples were analyzed with the MACSQuant VYB (Miltenyi) with the following instrument settings Blue/488 FITC, A488: 505LP-530/30, PerCP-Cy5.5: 630LP-670/14; Yellow/561 PE: 586/50; APC: 661/20, APC-Cy7: 750LP.

Immunofluorescent staining for endothelial and pericyte cell markers

ECs and pericytes were grown to confluence on fibronectin (FN)-coated glass coverslips (Sigma, F1141, 2ug/ml). Cells were fixed with 4% PFA and permeabilized with 0.05% TX-100 (Sigma). The following antibodies were used: VE-Cadherin (CellSignaling, 2158, 1:200), CD31 (Scbt, sc-1506-R, 1:200), vWF (Dako, A0082, 1:200), LYVE1 (ReliaTech, 102-PA50, 1:200), anti-SM22α (Abcam, Ab14106, 1:200), anti-SMA (Sigma, 1A4, 1:200), anti-CNN1 (Sigma, 2F5-1H4, 1:200).

Gene-expression analysis

Total RNA was isolated from cultured cells using the NucleoSpin® RNA II Kit (Macherey-Nagel) combined with Ambion® TURBO™ DNase treatment (Life Technologies). Total RNA purified from cultured cells was used to generate cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed using the CFX96™ Real-Time System (Bio-Rad) and data was analysed with Bio-Rad CFX Manager 3.0 software. For each reaction 200 ng cDNA was used in a 20 µL qPCR mixture containing 10 µM FW primer, 10 µM RV primer; 10 µL iQ™ SYBR Green Supermix (Bio-Rad). Samples were denatured for 3 min at 95 °C followed by 42 cycles of 15 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C. Melt-curve analysis was performed directly after the amplification protocol under the following conditions: 10 s denaturation at 95 °C and 0.5 °C increments of 5 s from 65 °C to 95 °C. Primers that were used for qPCR are shown in Supplemental Table I.

Matrigel angiogenesis assay

The endothelial tube formation assay was performed as previously described with slight modifications. hiPSC-derived ECs were plated into 96-well plates at a density of 15,000 cells/well on the top of growth factor reduced Matrigel that had been solidified for 30 min at 37°C prior to plating of the cells. Endothelial SFM (Invitrogen) supplemented with 1% platelet poor plasma, VEGF and bFGF were used for the tube formation assay. In order to enhance visualization and facilitate quantification of tube formation, ECs were labeled with the general cytoplasmic labeling agent PKH67 according to the manufacturer’s protocol (Sigma, PKH67GL). Endothelial tubes were imaged with the BD Pathway 855 imaging system using the 4x objective and 2x2 Montage mode. Total area and total tube length of the endothelial sprouts were quantified using the Wimasis quantification method (www.wimasis.com).
Capillary-like network formation by ECs in co-culture with pericytes

The endothelial capillary-like network formation assay was carried out as previously described but with major modifications. These included using both ECs and pericytes derived from human hiPSC lines. ECs were labeled with the general cytoplasmic membrane labeling dye, PKH67 (Sigma, PKH67GL). For the sprouting assay, 12x10^3 ECs derived from each control line were plated with 5x10^4 control hiPSC-derived pericytes/well on 0.1% gelatin coated 96-well plates in EGM-2 (Lonza, CC-3162). Next day and on day 4 of the assay, medium was replaced with new EGM-2 or EGM-2 with additionally supplementation with SB431542 (Tocris, 10µM) or DAPT (Sigma, 10µM). Endothelial-pericyte co-culture was terminated on day 7 of the assay and ECs were stained for quantification with anti-CD31 antibodies, as follows: co-cultures were fixed with the 4% paraformaldehyde (PFA, Sigma), permeabilized with the 0.1%TX-100 and blocked with normal goat serum (Dako). Endothelial sprouts were visualized with the mouse anti-CD31 (Dako, JC70A, 1:200), pericytes were visualized with anti-SM22α (Abcam, Ab14106, 1:200), proliferating cell were visualized with anti-Ki67 (Abcam, Ab833, 1:50). The endothelial network was imaged with the BD Pathway 855 system using the 4x objective and 2x2 Montage mode or 10x objective and 3x3 Montage mode. Total area covered by the endothelial sprouts was quantified with the protocol developed within cell image analysis software CellProfiler (Broad Institute). High magnification images were acquired with the SP5 confocal microscope, using 20x DRY objectives.

Zebrafish blastula implantation

Zebrafish and embryos were raised, staged and maintained according to standard procedures in compliance with the local animal welfare regulations. The transgenic line Tg(fli1:GFP) was used in this study. hPSC-derived ECs were labelled with the fluorescent cell tracker CM-Dil (Invitrogen) according to the manufacturer’s instructions. Briefly, cells were grown to confluence in a T25cm² dish, and removed from the plate with TrypLE Select. Subsequently, cells were washed with PBS, transferred to 1.5 ml Eppendorf tubes and centrifuged for 5 min at 1200 rpm. Cells were re-suspended in PBS containing CM-Dil (4 ng/ul final concentration). Cells stained with CM-Dil were incubated 4 min at 37°C, 15 min at 4°C then centrifuged for 5 min at 1200 rpm, the supernatant discarded and cells re-suspended in medium, centrifuged again and washed twice with PBS. Cells were suspended in medium for injection into the zebrafish embryos. Approximately 100 cells (manually counted) were injected at the early blastula (256/512 cell) stage. After EC transfer, zebrafish embryos (including non-implanted controls) were maintained at 33°C, as previously described. For each cell line or condition, data representative of at least 2 independent experiments with at least 100 embryos per group were included.

Cell implantation in Zebrafish 48 hours post fertilization (hpf)

Fluorescently labelled cells were resuspended in PBS as single cell suspensions and kept on ice before implantation. Implantation occurred within 3 hours. Dechorionized 2 days post fertilization (dpf) zebrafish embryos were anesthetized with 0.003% tricaine (Sigma) and positioned on a 10cm Petri dish coated with 3% agarose. The cell suspension was loaded into borosilicate glass capillary needles (1mm O.D. x 0.78mm I.D.; Harvard Apparatus) and the injections were performed using a Pneumatic Picopump and manipulator (WPI). Fluorescently labelled cells were prepared as before. Approximately 400 cells (manually counted) were injected at approximately 60µm above the ventral end of the duct of Cuvier (DoC), where it opens into the heart. After implantation of the ECs, zebrafish embryos (including non-implanted controls) were maintained at 33°C; this temperature was selected as a compromise between the optimal temperature for fish and mammalian cells. For each cell line
or condition, data representative of at least 2 independent experiments with at least 100 embryos per group were included. Experiments were discarded when the survival rate of the control group was less than 80%.

**Zebrafish Immunofluorescent Staining**

Embryos were dehydrated by sequential embedding for 5 minutes in 100% MetOH-75% MetOH-50% MetOH and 25% MethOH in PBS-Tween-20 (PBT-T). Embryos were additionally washed four times in PBS-T. Proteinase K (10ug/ml) was used to permeabilize embryos for 30 min at RT. Embryos were washed once with PBS-T and blocked with 1% BSA in PBS-T for 2h at RT. Incubation with primary antibodies was performed at +4°C overnight. The following primary antibodies were used: anti-Ki67 (Millipore, Ab9260, 1:200), anti-Caspase-3 (Cell Signaling, 96579, 1:200) and anti-PECAM1 (Scbt, sc-1506-R, (M-20), 1:200). Embryos were rinsed three times in PBS-T, then washed four times for 10 min in PBS-T. Embryos were additionally blocked with 1% BSA in PBS-T for 1h at RT prior to incubation with secondary antibodies for 2h at RT. Embryos were rapidly rinsed three times in PBS-T, followed by an additional wash in PBS-T for 10 min each. Embryos were stored at +4°C prior to imaging.

**Zebrafish Microscopy and Analysis**

Fixed embryos were imaged in PBS. Fluorescent image acquisition was performed using a Leica MZ16FA stereo-microscope, or a Leica SP5 STED confocal microscope. Confocal stacks were processed for maximum intensity projections with Leica software or Adobe Photoshop CS4 software. Images were adjusted for brightness and contrast using Adobe Photoshop CS4. Overlays were created using Adobe Photoshop CS4.

**Statistical analysis**
The unpaired Student's t-test with Welch's correction was used to determine whether results are statistically significant. Results are presented as mean±SD (standard deviation of mean).
References


Supplemental Material

**Supplemental Table I.** Primer sequences used for the gene expression analysis.

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Supplemental Figure I

A

Supplemental Figure I. Schematic workflow of ECs differentiation. (A) Workflow schematic overview of the mesoderm induction and vascular specification from hPSCs. (B) Bright field images of undifferentiated hPSCs in mTeSR, endothelial cells islands at day 10 of differentiation and isolated CD31+ ECs.
Supplemental Figure II. (A) Flow cytometry analysis of the time course differentiation experiment (BOECs-iPSC) for the surface expression of VE-Cadherin (VEC), early hematopoietic (CD43), endothelia and MSCs markers (CD73, CD105, KDR, PDGFRb). (B) Quantification of the flow cytometry data for % of VE-Cadherin positive (VEC+) and VEC+CD73+ endothelial cells. Representative data of one experiment (three independent wells per time point) of two independent biological replicates.
Supplemental Figure III. Comparison of BVC versus BVAC conditions for endothelial differentiation. Flow cytometry analysis of differentiation efficiency across multiple hiPSC lines based on % of CD31+CD34+ at day 10 of differentiation induction.
Supplemental Figure IV. Flow cytometry analysis of whole blood, and CD31+ ECs and CD31- pericytes/MSCs derived from BOECs-iPSC for the surface expression of hematopoietic (CD45, CD43 and CD14), endothelial and leukocyte (CD31), and endothelial, monocyte and MSCs (CD105, CD73) markers.
Supplemental Figure V

A

Representative immunofluorescent images of the PKH67 (white) labeled endothelial cells derived from hPSCs upon seeding on Matrigel basement membrane matrix. The images were taken with the BD Pathway imaging system 4x objective in the 2x2 montage mode at different time points (6h, 24h and 48h).

B

Quantification of total sprout length and total area (Wimasis).

Supplemental Figure V. Matrigel tube formation assay. (A) Representative immunofluorescent images of the PKH67 (white) labeled endothelial cells derived from hPSCs upon seeding on Matrigel basement membrane matrix. The images were taken with the BD Pathway imaging system 4x objective in the 2x2 montage mode at different time points (6h, 24h and 48h). (B) Quantification of total sprout length and total area (Wimasis).
Supplemental Figure VI. Characterization of pericytes/MSCs derived in serum-free media (BPEL) upon additional supplementation with TGFβ (1ng/ml). (A) Flow cytometry analysis of CD31- pericytes/MSCs derived from BOECs-iPSC for the surface expression of pericyte (PDGFRβ, NG2 and CD146) and MSCs (CD44, CD105, CD73) markers. (B) Immunofluorescent staining for the expression of smooth muscle cell markers: SMA (green), CNN1 (red), SM22 (white). Scale bar 100 µm.
Supplemental Figure VII

Supplemental Figure VII. In vitro vascular plexus formation upon co-culture of hiPSC-derived ECs and hiPSC-derived mesenchymal cells. (A) Representative images of the endothelial network counterstained with anti-CD31 (in white). Images were acquired with 4x objective 2x2 montage mode (BD Pathway855). (B) Quantification of the total area per object covered by endothelial sprouts (CellProfiler).
**Supplemental Figure VIII.** Increased endothelial cell proliferation in co-culture system upon supplementation with the ALK5 inhibitor SB. (A) Representative immunofluorescent images of the day 7 co-culture in standard growth medium and upon supplementation with DAPT (10µM) and/or SB431542 (10µM). Immunofluorescent staining of the co-culture at day 7 with endothelial specific marker CD31 (in green) and Ki67 (in white)(co-culture). Images were acquired with 10x objective in 3x3 montage mode (BD Pathway855). (B) Quantification of the Ki67 positive cells and Ki67+ ECs (CellProfiler).
**Supplemental Figure IX.** Modeling of endothelial-pericyte cell interactions in vitro.

(A) Representative immunofluorescent images of the day 7 co-culture in standard growth medium and upon supplementation with DAPT (10µM) and/or SB431542 (10µM). Immunofluorescent staining of the co-culture at day 7 or pericytes/MSCs only either with endothelial specific marker CD31 (in white) and SM22 (in white)(co-culture), or SM22 (in white) (for pericytes/MSCs). Overlay images are displayed with CD31 (in white) and SM22 (in red). Images were acquired with 10x objective in 3x3 montage mode (BD Pathway855).

(B) Quantification of the total area covered by SM22 positive cells (CellProfiler).

(C) Relative gene expression profiling of SM22 expression in the co-culture system at day 7. Representative data of one experiment (three independent wells) of total four biological replicates for CD31 quantification, and three biological replicates for SM22 quantification.
Supplemental Figure X

(A) Representative immunofluorescent images of the day 7 co-culture in standard growth medium or ECs conditioned medium for endothelial specific marker CD31 (in white) and SM22 (in white)(co-culture). Overlay images are displayed with CD31 (in white) and SM22 (in red). Images were acquired with 10x objective in 3x3 montage mode (BD Pathway855).

(B) SM22 Area Covered, px

Supplemental Figure X. The effect ECs conditioned medium for extra 4 days with or without extra supplementation with SB431542 (10μM). (A) Representative immunofluorescent images of the day 7 co-culture in standard growth medium or ECs conditioned medium for endothelial specific marker CD31 (in white) and SM22 (in white)(co-culture). Overlay images are displayed with CD31 (in white) and SM22 (in red). Images were acquired with 10x objective in 3x3 montage mode (BD Pathway855).
Supplemental Figure XI. Representative bright field and fluorescent images of (A) normal control zebrafish, (B) blastula stage injected BOECs-iPSC-ECs (in red) and (C) yolk sac injected BOECs-iPSC-ECs (in red) and zebrafish vasculature Tg(fli1:GFP) (in green). Representative images were taken at 5dpi.
Supplemental Figure XII. Representative images of yolk sac injected (48hpf) Fib-iPSC-ECs (in red) and zebrafish vasculature Tg(fli1:GFP) (in green) and proliferation marker Ki67 (in white). Scale bar 100µm.
Supplemental Figure XIII. Representative images of yolk sac injected (48hpf) Fib-iPSC-ECs (in red) and zebrafish vasculature Tg(fli1:GFP) (in green) and apoptosis marker Caspase-3 (in white). Scale bar 100µm.
Supplemental Figure XIV. Representative images of blastula stage (256/512 cell) injected BOECs-iPSC-ECs (in red) and zebrafish vasculature Tg(fli1:GFP) (in green) and human specific anti-PECAM1 (in white). Scale bar 20µm.