Directed differentiation of human pluripotent stem cells to blood-brain barrier endothelial cells

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The blood-brain barrier (BBB) is composed of specialized endothelial cells that are critical to neurological health. A key tool for understanding human BBB development and its role in neurological disease is a reliable and scalable source of functional brain microvascular endothelial cells (BMECs). Human pluripotent stem cells (hPSCs) can theoretically generate unlimited quantities of any cell lineage in vitro, including BMECs, for disease modeling, drug screening, and cell-based therapies. We demonstrate a facile, chemically defined method to differentiate hPSCs to BMECs in a developmentally relevant progression via small-molecule activation of key signaling pathways. hPSCs are first induced to mesoderm commitment by activating canonical Wnt signaling. Next, these mesoderm precursors progress to endothelial progenitors, and treatment with retinoic acid leads to acquisition of BBB-specific markers and phenotypes. hPSC-derived BMECs generated via this protocol exhibit endothelial properties, including tube formation and low-density lipoprotein uptake, as well as efflux transporter activities characteristic of BMECs. Notably, these cells exhibit high transendothelial electrical resistance above 3000 ohm-cm². These hPSC-derived BMECs serve as a robust human in vitro BBB model that can be used to study brain disease and inform therapeutic development.

INTRODUCTION
An intact blood-brain barrier (BBB) serves as a key interface between the blood circulation and the central nervous system (CNS). The primary anatomical component of the BBB is provided by brain microvascular endothelial cells (BMECs) (1, 2) that work in concert with supporting cells, such as astrocytes, pericytes, and neurons, to form the neurovascular unit (1, 3, 4). BMECs are connected by tight junctions and display low levels of vesicular traffic, leading to extremely low vascular permeability. BMECs also express molecular influx and efflux transporters, which regulate the delivery of nutrients from the blood to the brain and removal of compounds from the brain, respectively. A functional BBB prevents most of the small-molecule drugs and nearly all large-molecule biologics from entering the brain (5). Thus, the BBB is a highly efficient barrier that protects the brain and limits CNS drug delivery (6). Moreover, BBB dysfunction has been associated with many CNS disorders, including stroke (7–9), Alzheimer’s disease (10, 11), multiple sclerosis (12), Parkinson’s disease (13), traumatic brain injury (14, 15), and HIV (16–18).

Although the BBB has been extensively studied in animal models (19–21), in vitro models based on primary human BMECs (22, 23), and immortalized human brain EC lines (2, 24, 25), these models lack key attributes of the human BBB. Animal models cannot fully represent the human BBB because of species differences, particularly in transporter expression and function (26). Human primary BMECs are difficult to obtain in sufficient quantities for drug screening and disease models and cannot be readily expanded in high-fidelity cultures. Immortalized cell lines exhibit a loss of BMEC-specific properties, including loss of tight junctions yielding subphysiologic transendothelial electrical resistance (TEER) (27). These limitations have prevented our full understanding of human BBB development, function, and disease (28).

Human pluripotent stem cells (hPSCs) have the potential to generate large quantities of specialized human cells for studying development and modeling disease (29–31). Previously, we reported the generation of pure populations of hPSC-derived BMECs via co-differentiation of hPSCs to neural and endothelial progenitors, followed by selective purification of the BMECs (32). In addition, we demonstrated that retinoic acid (RA) addition during BMEC differentiation enhanced barrier properties to physiologic levels (33). Presumably, the neural progenitors in this co-differentiation platform induce the endothelial progenitors to acquire BMEC-specific traits, which are then enhanced by RA treatment. However, the undefined nature of this co-differentiation platform complicates the investigation of mechanisms that specify BMEC fates in the hPSC-derived ECs. In addition, this undefined protocol can result in line-to-line and batch-to-batch variability in BMEC yield and phenotypes (32, 34–36), despite recent efforts that have explored the use of defined medium to accelerate portions of the differentiation process (36). Other studies have also shown that human BMEC-like cells can be generated from alternative stem and progenitor cell sources, including hematopoietic stem cells (37), endothelial progenitors (38), and hPSC-derived ECs cocultured with hPSC-derived pericytes, astrocytes, and neurons (39) or C6 glioma cells (40). Nevertheless, none of these previous studies report a chemically defined, robust process for generating human BMECs exhibiting physiologic BBB phenotypes.

During embryonic development, mesoderm-derived ECs form a vascular plexus covering the developing neural tube (41, 42). As nascent blood vessels enter the developing CNS, canonical Wnt signaling is necessary to induce BMEC barrier properties (43–45). RA has also been shown to regulate BMEC specification. During BBB development, radial glial cells supply the CNS with RA (46), and this RA signaling induces barrier formation and BBB-specific gene expression (33, 46, 47). In addition to Wnt regulation of BBB induction in vivo, previous studies have demonstrated that activation of canonical Wnt signaling can also direct hPSCs to mesodermal lineages in vitro (31, 48–50). Thus, we hypothesized that appropriate differentiation stage-specific modulation of canonical Wnt signaling would induce mesodermal and endothelial commitment in hPSCs, and combine...
with subsequent RA signaling to drive the acquisition of BMEC markers and phenotypes.

Here, we report a chemically defined method to differentiate hPSCs to BMECs via sequential Wnt and RA pathway activation. During this differentiation process, hPSCs progress through stages resembling primitive streak and intermediate mesoderm to vascular endothelial growth factor receptor 2-positive (VEGFR2⁺) endothelial progenitors that become virtually pure populations of CD31⁺ ECs displaying key BMEC phenotypes including tight junctions, low passive permeability, and polarized efflux transporters. The resultant, developmentally relevant BMEC differentiation strategy is defined, robust, and facile.

**RESULTS**

**hPSCs progress from intermediate mesoderm to BMECs**

Given the roles of canonical Wnt signaling in both mesoderm specification and BBB development, we first treated hPSCs with CHIR99021, a glycogen synthase kinase 3β (GSK-3β) inhibitor and canonical Wnt pathway agonist, to direct hPSCs to mesoderm-derived endothelial progenitors. Before treatment, IMR90-4 induced pluripotent stem cells (iPSCs) were seeded on a Matrigel-coated six-well plate at a density of 35 × 10³ cells/cm² and expanded for 3 days in mTeSR1 (Fig. 1A). Previously, we showed that 6 μM CHIR99021 treatment induced hPSC differentiation to a primitive streak-like stage.
in a serum- and albumin-free medium (51). Hence, at day 0, 6 μM CHIR99021 was added to DeSR1 [unconditioned medium (UM)] lacking KnockOut Serum Replacement: DMEM/F12, 1% minimum essential medium (MEM)–nonessential amino acids (NEAA), 0.5% GlutaMAX, and 0.1 mM β-mercaptoethanol (32)] to initiate differentiation. After 24 hours, the medium was removed and cells were transitioned to DeSR2 (DeSR1 plus B27 supplement) for another 5 days with daily medium changes. At day 0, pluripotency was verified by OCT4, NANOG, and TRA-1-60 immunofluorescence (Fig. 1, B to D). After 24 hours of CHIR99021 treatment, almost 100% of the cells expressed the primitive streak marker brachyury, as assessed by immunolabeling (Fig. 1E) and flow cytometry (Fig. 1F). In concert with brachyury expression, primitive streak genes (MIXL1 (52) peaked at day 2 and then markedly decreased (fig. S1). At day 4, more than 90% of the cells expressed the intermediate mesoderm marker PAX2 (Fig. 1, G and H), and PAX2 expression peaked at day 6 (Fig. S1). Nearly 100% of the cells expressed the endothelial progenitor marker VEGFR2 at day 5 (Fig. 1, I and J), whereas the expression level of the endothelial progenitor marker CD34 gradually increased and then diminished after day 6 (Fig. S1).

At day 6, cells were switched to hECSR1 medium [human endothelial serum-free medium (hESFM) supplemented with basic fibroblast growth factor (bFGF, 20 ng/ml), 10 μM RA, and B27] to induce RA signaling in the hPSC-derived endothelial progenitors in an attempt to drive the specification to BMECs. Cells were maintained in this medium for 2 days. At day 8, cells were replated onto a Matrigel-coated substrate in hECSR1, and at day 9, the medium was switched to hECSR2 [hECSR1 lacking RA and bFGF]. The expression of CDH5 [vascular endothelial cadherin (VE-cadherin)] was substantially induced after RA treatment (Fig. S1). The expression of the tight junction–related genes TJP1, CLDN5, and OCLN and the efflux transporter ABCB1 also increased during differentiation (Fig. S1). The resultant day 10 BMEC-like cells were a pure population expressing endothelial markers (CD31 and VE-cadherin), BBB glucose transporter (GLUT-1), tight junction proteins (ZO-1, claudin-5, and occludin), and efflux transporters (BCRP, MRP1, and Pgp) (Fig. 2, A to I). Thus, sequential treatment of hPSCs with CHIR99021 and RA directed hPSCs through endothelial progenitors to ECs that expressed BMEC markers. We next tested whether the differentiation protocol illustrated in Fig. 1A generated cells expressing BMEC markers in additional hPSC lines, including h9 human embryonic stem cells (hESCs) and 9-9-11 iPSCs. These lines also produced cells expressing endothelial and BMEC markers, including CD31, GLUT-1, ZO-1, claudin-5, occludin, MRP1, BCRP1, and Pgp, at day 10 (Fig. S2).

Next, RNA sequencing was used to compare global gene expression profiles in the hPSC-derived BMECs differentiated, as shown in Fig. 1A, with BMECs generated from our previously reported co-differentiation system [UM (32)] and primary human BMECs. As expected, hPSC-derived BMECs from three independent differentiation clusters closely and were similar to those generated from the undefined UM platform. Moreover, the hPSC-derived BMECs clustered with primary human BMECs and were distinct from undifferentiated hPSCs and hPSC-derived ectoderm, endoderm, and mesoderm (Fig. 2J). The Pearson correlation coefficient between defined BMECs and primary human BMECs was 0.77 (P < 0.001), which indicates a strong positive association between these two groups. We next analyzed the expression of a subset of genes that regulate key BBB attributes, including tight junctions and molecular transporters. The gene set comprises 20 tight junction–related genes (I, 53–56) and an unbiased list of all 25 CLDN genes, all 407 solute carrier (SLC) transporters, and all 53 adenosine 5′-triphosphate (ATP)–binding cassette (ABC) transporters regardless of previous knowledge of BBB association (table S1). Primary human BMECs expressed 234 of these genes. BMECs differentiated from hPSCs via the defined method expressed many of these same genes (206 of 234 (88%)), as did BMECs differentiated via the UM method (208 of 234 (89%); Fig. 2K), indicating a close similarity between hPSC-derived BMECs and primary human BMECs with respect to transcripts having likely relevance to BBB function.

Initially, differentiation was performed on Matrigel, which has been shown to support BMEC generation from hPSCs (32). However, to remove the lot-to-lot variability inherent to Matrigel and to fully define the differentiation platform, we explored differentiation on Synthema and recombinant human vitronectin coatings. Undifferentiated IMR90-4 iPSCs were expanded on either Synthema- or vitronectin-coated surfaces for 3 days and then subjected to the differentiation process shown in Fig. 1A. Cells were replated onto a human placenta–derived collagen IV/human plasma–derived fibronectin-coated surface at day 8. Immunofluorescence at day 10 demonstrated the expression of key BMEC proteins in cells differentiated on defined matrices (fig. S3, A and B).

hPSC-derived BMECs exhibit BBB phenotypes

In addition to the examination of BMEC gene and protein expression, we also evaluated endothelial and BMEC phenotypes. After 8 days of differentiation, cells were replated onto a Matrigel-coated surface at 1 million cells/cm² and maintained in hECSR1 medium. At day 9, culture medium was switched to hECSR2. Day 10 hPSC-derived BMECs exhibited EC properties, including the expression of von Willebrand factor (vWF) (Fig. 3A), formation of tube-like structures on Matrigel in the presence of VEGF (Fig. 3B), uptake of acetylated low-density lipoprotein (LDL) (Fig. 3C), and up-regulation of intercellular adhesion molecule 1 (ICAM-1) expression after treatment with tumor necrosis factor–α (TNF-α) (Fig. 3, D to F). BMEC efflux transporter activities were also measured at day 10. Efflux transporter accumulation assays were performed by quantifying intracellular accumulation of fluorescent substrates, including the Pgp substrate rhodamine 123, the MRPl family substrate 2′,7′-dichlorofluorescein diacetate (DCFDA), and the BCRP family substrate Hoechst. In the presence of the transporter–specific inhibitors cyclosporine A (CsA; Pgp), MK571 (MRP), and Ko143 (BCRP), the intracellular accumulation of fluorescent substrates increased between 150 and 220%, indicating the activity of each class of transporters in hPSC-derived BMECs (Fig. 3, G to I). In addition, BMECs differentiated on defined vitronectin or Synthema substrates also exhibited Pgp, MRP, and BCRP transporter activities similar to those differentiated on Matrigel (fig. S3C). Next, polarization of Pgp activity was demonstrated by measuring rhodamine 123 flux across the BMEC monolayer in the presence and absence of the Pgp-specific inhibitor CsA and in both the apical to basolateral (A-B) and basolateral to apical (B-A) directions. As shown in Fig. 3J, CsA treatment increased rhodamine 123 transport across the BMEC monolayer by 160% in the A-B direction. In contrast, CsA inhibition resulted in a 23% decrease in rhodamine 123 crossing the barrier in the B-A direction (indicated in Fig. 1A), indicating Pgp efflux function polarized in the B-A direction. Finally, BMECs differentiated via the defined protocol exhibited Pgp accumulation and transport similar to those differentiated via our previously reported undefined co-differentiation protocol (fig. S4, UM protocol).
Finally, previous studies have shown that coculturing BMECs, including those that are iPSC-derived, with neural progenitor cells, astrocytes, and pericytes can enhance BBB properties such as TEER (33, 57–60).

Day 8 iPSC-derived BMECs seeded on Transwells were either maintained as a monoculture or cocultured with primary human pericytes for the first 24 hours, followed by coculture with hPSC EZ sphere-derived astrocytes and neurons (1:3) (61) for additional 3 days. Maximum TEER for the monoculture system was ~3000 ohm-cm², and

Fig. 2. hPSC-derived BMECs express key BMEC proteins and have gene expression profiles similar to those of primary human BMECs. (A to I) At day 10, BMECs differentiated as shown in Fig. 1A were characterized by immunocytofluorescence (A) and flow cytometry (B to I) for key endothelial and BMEC markers. Scale bars, 100 μm. (J) Hierarchical clustering of whole transcripts counted by RNA sequencing was plotted using GENE-E. Hierarchical clustering analysis was performed on log-transformed gene counts of RNA sequencing expression data of undifferentiated hPSCs (76–78); hPSC-derived endoderm (Endo) (76), ectoderm (Ecto) (77), and mesoderm (Mes) (78); BMECs differentiated under defined conditions as illustrated in Fig. 1A (D-BMEC1, D-BMEC2, and D-BMEC3: IMR90–4–derived BMECs at day 10 generated from three independent differentiations); BMECs differentiated in UM (UM-BMECs); and human primary BMECs (hBMECs). Distances were computed using one minus Pearson correlation with average linkage. (K) A set of 506 tight junction and transporter genes (table S1) was used to investigate gene expression similarity between primary human BMECs, hPSC-derived BMECs differentiated under defined conditions, and hPSC-derived BMECs differentiated in UM (32). The gene set included 20 tight junction–related genes (1, 53–56), all 25 CLDN genes, all 407 solute carrier (SLC) transporters, and all 53 ATP-binding cassette (ABC) transporters. CLDN, SLC, and ABC genes were included without previous knowledge of BBB association. A threshold of >1 fragment per kilobase million (FPKM) was used to define expressed versus nonexpressed transcripts.
coculture further elevated TEER by 30% at day 2 and helped maintain an elevated TEER throughout the duration of the experiment compared to the monoculture control (Fig. 3K).

**Cell density is crucial for BMEC differentiation**

Cell density has been shown to be crucial for efficient hPSC differentiation to a variety of lineages, including BMECs (35, 62–64). Thus, in addition to the optimal initial day −3 cell seeding density used above (35 × 10^3 cells/cm^2), we tested a range of seeding densities, from 8.8 × 10^3 to 140 × 10^3 cells/cm^2, to explore how density affects BMEC yield and phenotype. As shown in Fig. 4A, TEER was a strong function of seeding density, with only 35 × 10^3 cells/cm^2 yielding BMECs with substantial barrier function at day 2 after transfer onto Transwells. The BMEC TEER peaked 2 days after replating and plateaued above...
2000 ohm·cm² through day 7 (Fig. 4B). At non-optimum seeding densities, TEER gradually increased through 6 days after replating, reaching approximately 1000 ohm·cm². We next assessed the expression of endothelial markers to investigate the endothelial specification process. Cells differentiated at all densities tested yielded EC populations with nearly 100% VEGFR2⁺ cells at day 5 and more than 90% CD31⁺ cells at day 10 (fig. S5). This suggested that deficits in barrier function may be a result of poor BMEC specification. Thus, we assessed BMEC
marker expression in populations differentiated at different seeding densities. Nearly 100% of cells expressed Pgp after the differentiation process at either day 8 or day 10 (fig. S6). However, only cells differentiated at the optimal seeding density of $35 \times 10^3$ cells/cm² yielded a pure claudin-5–expressing population with maximal claudin-5 expression (fig. 4, C to E). In addition, a subpopulation of cells differentiated from non-optimal starting densities also lacked junctional claudin-5 (fig. 4F, white arrows) or exhibited discontinuous claudin-5 localization at cell junctions (fig. 4F, red arrows). Similarly, only cells differentiated from seeding densities of at least $35 \times 10^3$ cells/cm² yielded a nearly pure population of occludin-expressing cells (fig. 4, G to I).

Immunofluorescence analysis of occludin also showed that a large fraction of cells differentiated from initial densities less than $35 \times 10^3$ cells/cm² lacked occludin expression (fig. 4), white arrows). Unlike claudin-5 and occludin, seeding density did not have a significant effect on ZO-1 expression, but cells differentiated at initial densities of $8.8 \times 10^3$ cells/cm² showed discontinuous ZO-1 localization (fig. S7, red arrows). Immunofluorescence for additional BMEC markers also indicated poor localization of CD31, MRPI, and BCRP in cells differentiated at non-optimum initial cell densities (fig. S7, compare 35k to other densities). H9 hESC and 19-9-11 iPSCs yielded BMECs exhibiting TEER at or above 2000 ohm·cm² at an optimal initial seeding density of $35 \times 10^3$ cells/cm² (fig. S8, A and B). We also compared the differentiation reproducibility with that of the previously reported UM protocol (33). Although both methods produce BMECs capable of substantial barrier formation from multiple hPSC lines, BMECs differentiated from H9 hESCs and 19-9-11 iPSCs using the defined method exhibited higher TEER and lower batch-to-batch variation (fig. S8A). Finally, $35 \times 10^3$ cells/cm² was also found to be the optimal seeding density for differentiation on Synthemax and vitronectin substrates, with vitronectin substrates performing more closely to Matrigel than Synthemax substrates in TEER assays (fig. S9).

**RA enhances BMEC phenotypes**

Previously, we have shown that RA induces BBB properties in hPSC-derived BMECs (33). Other studies have also demonstrated that RA signaling regulates BBB formation and induces BBB phenotypes (46, 47). To determine the role of RA in specifying BMEC differentiation and enhancing the BMEC phenotypes described in Figs. 2 and 3, we compared differentiation in the presence and absence of RA using the protocol illustrated in Fig. 1A. From day 6 to day 8, cells were maintained either in hECSR1 or in hECSR1 lacking RA. Quantitative polymerase chain reaction (qPCR) showed that the expression of the tight junction–related genes TJPI, CLDN5, and OCLN and the efflux transporters ABCG2, ABCC1, and ABCB1 was greater (3- to 20-fold) in cells exposed to RA (fig. 5A). Nearly 100% of cells expressed CD31 at day 6, and this expression was preserved in the presence of RA induction at day 8 (fig. 5B). Immunofluorescence for CD31 and other BMEC markers for cells differentiated in the absence of RA, including VE-cadherin, GLUT-1, and MRPI, is shown in fig. S10A. Nearly 100% of cells differentiated in the absence and presence of RA expressed Pgp, but RA-treated cells expressed more Pgp than nontreated cells (fig. 5C). To evaluate the barrier formation potential of the differentiated BMECs and to assess the effects of RA treatment, we replated day 8 BMECs onto Transwells and measured TEER at day 10. As shown in fig. 5D, cells differentiated in the presence of RA exhibited physiologically relevant TEER (~4000 ohm·cm²), whereas those differentiated in the absence of RA exhibited significantly reduced barrier properties. Similarly, BMECs differentiated on vitronectin or Synthemax required RA supplementation to achieve substantial barrier properties (fig. S11). We then investigated the expression and localization of tight junction proteins. Both occludin and ZO-1 were expressed in nearly all cells at day 10 regardless of RA treatment; however, RA treatment significantly increased the expression levels of occludin and ZO-1 (fig. 5, E and F). Although occludin and ZO-1 expression was lower in the absence of RA, immunofluorescence results indicate that nearly all the cells differentiated in the absence of RA still expressed occludin and ZO-1; however, the junctional distribution was nonuniform (fig. 5G, indicated with red arrows and compare to fig. 2A). In contrast to the results with occludin and ZO-1, in the absence of RA, only around 60% of the ECs expressed claudin-5 compared to 100% of RA-treated cells expressing claudin-5 (fig. 5, H and I). In addition, claudin-5 expression was also substantially greater in RA-treated cells (fig. 5I). Immunofluorescence indicated that in the absence of RA, many of the cells did not express claudin-5 (fig. 5, white arrows), and those that did exhibited nonuniform junctional distribution of claudin-5 similar to that observed with occludin and ZO-1 (fig. 5J, red arrows). Together, these results suggest that RA is not necessary for hPSC differentiation to ECs but enhances key BMEC phenotypes in the hPSC-derived ECs, including the expression and localization of tight junction proteins that promote barrier function as measured by TEER.

**DISCUSSION**

Here, we demonstrate a robust and efficient process to differentiate hPSCs to BMECs in a defined manner. The cells progress as a homogeneous population from a pluripotent state to primitive streak–like and intermediate mesoderm–like stages, to endothelial progenitors, and eventually to ECs that express BMEC markers and exhibit BBB barrier and efflux transporter properties. This differentiation method uses a completely defined platform, including culture medium and substrates. Defined reagents exhibit less lot-to-lot variability, leading to more robust and efficient differentiation and allowing differentiation results to be more reliable and reproducible. We have tested three different hPSC lines with this differentiation protocol, and all of these lines were able to differentiate into pure populations of BMEC, with definitive BMEC properties at the same optimum cell density.

In vivo, ECs that form the BBB originate from mesoderm progenitors located outside the CNS (65). In contrast to previous BMEC differentiation protocols (32, 37) that rely on coculture of endothelial progenitors with pericytes, astrocytes, or differentiating neural cells, this differentiation strategy instead relies on sequential Wnt and RA signaling activation to first specify ECs and then enhance BMEC properties, respectively. First, activation of canonical Wnt signaling by CHIR99021 addition directs hPSCs to brachyury-positive primitive streak–like cells that then differentiate to PAX2-positive intermediate mesoderm and EC progenitors when differentiated at the appropriate density in DeSR2 medium. Next, RA treatment for 2 days drives these endothelial progenitor cells to express key BMEC markers and exhibit BMEC-specific properties, including high TEER and efflux transporter activity. Our experiments showed that although RA was not necessary to obtain ECs, RA treatment significantly increased BBB properties such as TEER. The TEER enhancement correlated with increased expression and improved localization of the tight junction proteins occludin and claudin-5. These findings are similar to those results observed after RA treatment of hPSC-derived BMECs generated by co-differentiation with neural cells using our previously reported undefined protocol (33), in
addition to those studies that have explored the barrier-enhancing effects of astrocyte or neuron coculture with hPSC-derived BMECs (33, 60). However, even in the absence of RA treatment, the previously reported undefined protocol still yielded pure populations of claudin-5–expressing BMECs, whereas RA was critical in the defined protocol for generating pure claudin-5–expressing populations. Thus, the effects of defined differentiation do not exactly mimic those provided by cocultured neural cells, yet the resultant BMECs were quite similar on a whole-transcriptome level to BMECs generated using the undefined coculture approach and to primary human BMECs.

Previously, we have shown that cell seeding density can affect BMEC differentiation from hPSCs using the neural co-differentiation protocol (35). Other studies have also demonstrated that cell seeding density is crucial for efficient hPSC differentiation to various lineages (66–68). An initial cell seeding density of \(35 \times 10^3 \text{ cells/cm}^2\) at day \(-3\) is necessary to yield homogeneous populations of BMECs with high expression and proper localization of key BBB proteins, in turn leading to optimal barrier properties. In addition, this optimum seeding density translated to multiple hPSC lines and to differentiation on defined matrices. Cells differentiated at non-optimal seeding densities expressed BMEC markers but exhibited a reduced TEER, likely resulting from diminished claudin-5 and occludin expression and improper junctional localization. Thus, RA signaling and cell density similarly regulate the capability for the endothelial progenitors to acquire BMEC properties. Although we determined that seeding density is a crucial regulator of hPSC differentiation to BMECs, we do not yet know the mechanism by which density affects differentiation efficiency.
Coculturing hPSC-derived BMECs with pericytes, astrocytes, and neurons further elevated TEER, consistent with previous studies that showed that coculturing BMECs with these neural cells can enhance BBB properties (57, 58, 69–71). These data suggest that it will be possible to integrate these defined hPSC-derived BMECs with other cells of the neurovascular unit to create an isogenic patient-derived model that can be used to study the role of neurovascular unit in human neurological diseases (60). In addition, this defined BMEC differentiation method has the potential to be a powerful and robust tool for preclinical studies of pharmaceutical transport through the BBB.

**MATERIALS AND METHODS**

**hPSC culture and differentiation**

Human iPSCs [iPS(IMR90)-4 (72), iPS-DF 19-9-11T (73), and hESCs (H9) (29)] were maintained on Matrigel (Corning)–coated surfaces in mTeSR1 (STEMCELL Technologies), as previously described (74). Before differentiation, hPSCs were singularized with Accutase (Innovative Cell Technologies) and plated onto Matrigel-coated plates at a density between 25 × 10^3 and 50 × 10^3 cells/cm² in mTeSR1 supplemented with 10 µM Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Selleckchem). hPSCs were expanded in mTeSR1 for 3 days. For differentiation on defined substrates, singularized hPSCs were plated onto the vitronectin-coated (Thermo Fisher Scientific) or Synthemax-coated (Corning) surface at a density between 25 × 10^3 and 50 × 10^3 cells/cm² in mTeSR1 supplemented with 10 µM ROCK inhibitor Y-27632 (Selleckchem). To initiate differentiation at day 0, cells were treated with 6 µM CHIR99021 (Selleckchem) in DeSR1: DMEM/Ham’s F12 (Thermo Fisher Scientific), 1× MEM-NEAA (Thermo Fisher Scientific), 0.5× GlutaMAX (Thermo Fisher Scientific), and 0.1 mM β-mercaptoethanol (Sigma). After 24 hours, the medium was changed to DeSR2: DeSR1 plus 1× B27 (Thermo Fisher Scientific) every day for another 5 days. At day 6, the medium was switched to hECSR1: hESFM (Thermo Fisher Scientific) supplemented with bFGF (20 ng/ml), 10 µM RA, and 1× B27. After 2 days of culture in hECSR1 medium, day 8 cells were dissociated with Accutase and plated at 1 × 10^6 cells/cm² in hECSR1 onto 48-well tissue culture plates or 1.12-cm² Transwell-Clear permeable inserts (0.4 µm pore size) coated with Matrigel (100 µg/ml). For cells differentiated on vitronectin or Synthema-coated substrates, cells were dissociated with Accutase and plated at 1 × 10^6 cells/cm² in hECSR1 onto 48-well tissue culture plates or 1.12-cm² Transwell-Clear permeable inserts (0.4 µm pore size) coated with human placenta–derived collagen IV (400 µg/ml/human placma–derived fibronectin (100 µg/ml). At day 9, the medium was changed to hECSR2 (hECSR1 without RA or bFGF) for longer-term maintenance. Y-27632 was added to increase the attachment (75) of cells differentiated in the absence of RA and was permitted to ensure confluent monolayer formation and confirm that the reduction in TEER was not only a result of incomplete monolayer formation.

**Immunohistochemistry**

Cells were rinsed with ice-cold phosphate-buffered saline (PBS) once and fixed in either ice-cold methanol or 4% parafomaldehyde (PFA) for 15 min. Cells were then blocked with 10% goat serum in PBS containing 0.3% Triton X-100 for 30 min (10% PBSGT). Primary antibodies were diluted in 10% PBSGT, and cells were incubated in the antibody solutions at 4°C overnight or at room temperature for 2 hours. After three PBS washes, cells were incubated with secondary antibodies in 10% PBSGT (goat anti-rabbit Alexa Fluor 594 and goat anti-mouse Alexa Fluor 488; 1:200) for 1 hour at room temperature. Cells were then washed with PBS three times, followed by treatment with DAPI Fluoromount-G (Southern Biotech), and were visualized. A detailed list of antibodies is shown in table S2.

**Flow cytometry**

Cells were dissociated with Accutase, fixed in 1% PFA for 15 min at room temperature, and then washed with 0.5% bovine serum albumin (BSA) (Bio-Rad) plus 0.1% Triton X-100 three times. Cells were stained with primary and secondary antibodies diluted in 0.5% BSA plus 0.1% Triton X-100, as previously described (31). Data were collected on a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using FlowJo. Corresponding isotype antibodies were used as controls (fluorescence-activated cell sorting) gating control. Details about antibody source and usage are provided in table S2.

**Quantitative real-time PCR**

Total RNA was extracted with the RNeasy Mini Kit (Qiagen) and treated with deoxyribonuclease (Qiagen). One microgram of total RNA was reverse-transcribed into complementary DNA using an oligo(dT) primer with SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was done in triplicate with iQ SYBR Green SuperMix (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous housekeeping control. Primer sequences are provided in table S3.

**LDL uptake assay**

Differentiated BMECs at day 10 were analyzed with the LDL Uptake Assay Kit (Abcam). Culture medium was aspirated and replaced with LDL-DyLight 550 working solution. Cells were then incubated for 3 hours at 37°C, followed by three washes with PBS, and then visualized via fluorescent microscopy with excitation and emission wavelengths of 540 and 570 nm, respectively. After visualization, cells were fixed with a cell-based fixative solution for 10 min. Cells were then washed with tris-buffered saline plus 0.1% Triton X-100 for 5 min, followed by 30-min blocking with Cell-Based Assay Blocking Solution. Cells were then stained with rabbit anti-LDL receptor primary antibody and DyLight 488–conjugated secondary antibody. Images were taken with a fluorescent microscope with excitation and emission wavelengths of 485 and 535 nm, respectively.

**Efflux transporter accumulation and transport assay**

Pgp, BCRP, and MRP functionality was assessed by intracellular accumulation of fluorescent transporter substrates and transport of fluorescent substrates across BMEC monolayers. Rhodamine 123 (10 µM; Sigma), Hoechst (20 µM; Thermo Fisher Scientific), and DCFDA (10 µM; Life Technologies) were used as the specific substrates for Pgp, BCRP1, and MRPI, respectively. BMECs at day 10 were pretreated for 1 hour with or without specific transporter inhibitors (10 µM CsA (Pgp inhibitor), 10 µM Ko143 (BCRP inhibitor), Sigma, and 1 µM MK571 (MRP inhibitor) (Sigma) in Hanks’ balanced salt solution (HBSS)). Cells were then treated with transporter substrates in HBSS and incubated for 1 hour at 37°C on an orbital shaker. Cells were washed with PBS three times and then lysed with radioimmunoprecipitation assay buffer (Pierce Biotechnology). Fluorescence intensity was measured on a plate reader (485-nm excitation and 530-nm emission for rhodamine 123 and DCFDA and 360-nm excitation and 497-nm emission for Hoechst). Fluorescence intensity was subsequently normalized to cell number determined using a hemocytometer.
Endothelial cell tube formation
Each well of a 24-well tissue culture plate was coated with 300 µl of Matrigel (10 mg/liter). BMECs at day 10 were dissociated with Accutase and plated in hECSM1 plus VEGF (50 ng/ml) without RA or bFGF at 2 × 10⁵ cells per well. Phase-contrast images were acquired after 24 hours.

RNA sequencing and data analysis
Total RNA of day 10 IMR90-4 iPSC–derived BMECs with either defined protocol or UM protocol and primary human brain microvascular ECs (ACBRI 376, Cell Systems) were prepared with the Direct-zol RNA MiniPrep Plus Kit (Zymo Research) according to the manufacturer’s instructions. Samples were sequenced on Illumina HiSeq 2500 at the University of Wisconsin–Madison Biotechnology Center. The resulting sequence reads were mapped to the human genome (hg19) using HISAT2-49, and the RefSeq transcript levels (FPKMs) were quantified using the Python script rpkmforgenes.py50. A hierarchical clustering of whole transcripts was performed using GENE-E on the log2-transformed gene counts. Distances were computed using one minus Pearson correlation with average linkage. FASTQ files of hPSCs (76–78) and hPSC-derived ectoderm (77), endoderm (76), and mesoderm (78) were downloaded from the Gene Expression Omnibus (GEO) or ArrayExpress (www.ebi.ac.uk/arrayexpress/) database. The expression of a subset of genes that regulate key BBB attributes, including tight junctions and molecular transporters, was analyzed. The gene set comprises 20 tight junction–related genes (I, 53–56) and an unbiased list of all 25 CLDN genes, all 407 solute carrier (SLC) transporters, and all 53 ATP-binding cassette (ABC) transporters regardless of previous knowledge of BBB association (table S1). Transcript levels (FPKMs) were set at a threshold of >1 FPKM, which indicates moderate expression (79). Primary human BMECs were used to screen out the BBB-related genes from the gene list with the threshold of >1 FPKM.

Coculture of hPSC-derived BMECs with pericytes, neurons, and astrocytes
hPSC-derived BMECs at day 8 were replated onto 12-well Transwell inserts at a density of 1 × 10⁶ cells/cm² and cocultured with primary human pericytes residing on the bottom of the 12-well plates (1 × 10⁶ cells per well) for 24 hours in hECSM1 medium. Following coculture with pericytes, BMECs were cocultured with EZ sphere–derived neurons and astrocytes (a total of 1 × 10⁶ cells per well; 1:3) in hECSM2 for the remainder of the experiment. TEER was measured as a function of time following initiation of coculture.

Statistics
Data are presented as means ± SEM. Statistical significance was determined by Student’s t test (two-tailed) between two groups. Three or more groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc tests. P < 0.05 was considered statistically significant.

REFERENCES AND NOTES

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/11/e1701679/DC1
fig. S1. Gene expression during hPSC differentiation to BMECs.
fig. S2. BMECs differentiated from hPSCs with either defined or undefined protocols exhibit similar Pgp activities.
fig. S5. BMECs differentiated at different seeding densities express VEGFR2 and CD31.
fig. S6. BMECs differentiated at different seeding densities express Pgp.
fig. S7. BMECs differentiated at different densities can exhibit aberrant protein localization.
fig. S8. TEER of BMECs differentiated from different hPSC lines.
fig. S9. TEER in BMECs differentiated from hPSCs at different seeding densities.
fig. S10. BMECs differentiated in the absence of RA exhibit low expression and mislocalization of Matrigel-, vitronectin-, and Synthemax-coated surfaces.
table S1. Expression of tight junction and transporter genes in ipsc-derived and primary human BMECs.
table S2. Antibodies used in this study.
table S3. qPCR primers used in this study.

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Directed differentiation of human pluripotent stem cells to blood-brain barrier endothelial cells
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