**In Vitro Modeling of Blood-Brain Barrier with Human iPSC-Derived Endothelial Cells, Pericytes, Neurons, and Astrocytes via Notch Signaling**

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**SUMMARY**

The blood-brain barrier (BBB) is composed of four cell populations, brain endothelial cells (BECs), pericytes, neurons, and astrocytes. Its role is to precisely regulate the microenvironment of the brain through selective substance crossing. Here we generated an in vitro model of the BBB by differentiating human induced pluripotent stem cells (hiPSCs) into all four populations. When the four hiPSC-derived populations were co-cultured, endothelial cells (ECs) were endowed with features consistent with BECs, including a high expression of BBB-specific transporters, good barrier function, and the efflux of drugs. This method allowed us to investigate the permeability tests and assessed ten clinical drugs by nanoLC-MS/MS, finding a good correlation with the BBB permeability reported in previous cases. This technology should be useful for research on human BBB physiology, pathology, and drug development.

**INTRODUCTION**

The blood-brain barrier (BBB) is composed of specialized brain endothelial cells (BECs) that are surrounded by pericytes, astrocytes, and neurons. These neurovascular units form intracellular tight junctions between BECs, which limit the passive diffusion of molecules into the CNS. BECs are enriched with nutrient transporters, such as glucose transporters, amino acid transporters, and fatty acid transporters, for efficient uptake into the brain from the blood. On the other hand, the enrichment of polarized efflux transporters, such as P-glycoprotein (PGP), breast cancer resistance protein (BCRP), and multidrug resistance-associated proteins (MRPs) in BECs protects the brain from toxic factors and pathogens. Because of drug efflux by the BBB, the delivery of therapeutic drugs into the brain to treat CNS diseases has been a major challenge. The BBB is also associated with brain disease, as its impairment correlates with neurodegenerative diseases including Alzheimer's disease and Parkinson's disease (Desai et al., 2007; Saito and Ihara, 2014). These reasons have spurred researchers to establish a BBB model for analyzing the dysfunction of neurovascular units and drug permeability in vitro.

Current in vitro BBB models use brain microvessels and astrocytes isolated from non-human species such as pig, rat, or mouse (Deli et al., 2005). However, the characteristics and functions of the BBB from these species differ from that in humans (Aday et al., 2016; Hoshi et al., 2013; Syvänä et al., 2009; Warren et al., 2009). Therefore, a human-derived BBB model is needed for pre-clinical drug screening or research about human BBB physiology and pathology. Although human primary brain microvessels isolated from the brain specimens of tumors or epilepsy patients can be used, they have low availability and reproducibility (Cecchelli et al., 2007). Immortalized human brain microvessels have also been considered, but the resulting BBB models do not form strong barrier properties due to discontinuous tight junctions (Wekслer et al., 2005). Endothelial cells (ECs) derived from human induced pluripotent stem cells (hiPSCs) have been used to prepare human in vitro BBB models, but these cells must be co-cultured with primary rat astrocytes or C6 rat glioma cells for maturation of the BBB (Lippmann et al., 2012; Minami et al., 2015). In the present work, we sought to induce all four BBB components, ECs, pericytes, neurons, and astrocytes, from hiPSCs, which are an unlimited human source and recapitulate development, in order to create a reproducible and robust human BBB model.

In our protocol, we developed a hiPSC differentiation system to produce ECs that depends on cyclic AMP (cAMP). cAMP is crucial for enhancing the EC differentiation and arterial specification of mouse embryonic stem cells (Yamamizu et al., 2009, 2010, 2012a, 2012b). To generate BECs, we co-cultured the ECs with pericytes, neurons, and astrocytes, which were also derived from hiPSCs. Culturally induced BECs (ciBECs) showed properties consistent of the BBB, including an enrichment of BBB-specific transporters, good barrier function, and the efflux of drugs. This method allowed us to investigate the
Figure 1. Differentiation of iPSCs into ECs, Pericytes, Neurons, and Astrocytes

(A) Schematic representation of the EC and pericyte induction protocol.

(B) FACS analysis for mesoderm markers, KDR and PDGFRα. Positive cells appeared at 4 and 5 days after differentiation. The percentage of KDR-positive cells in total cells are indicated.

(legend continued on next page)
mechanisms of BBB formation, which led to our discovery that ciBEC specification occurs through Notch signaling via Dll1 in neurons. Our model should be useful for the study of BBB development and homeostasis, and also for the discovery of CNS drugs that bypass the BBB.

RESULTS

Differentiation of hiPSCs into ECs, Pericytes, Neurons, and Astrocytes

We first addressed the creation of an efficient EC and pericyte differentiation system derived from hiPSCs. We previously reported that the cAMP/protein kinase A/CREB pathway triggers the initiation of endothelial and hematopoietic cell differentiation via Etv2 induction (Yamamizu et al., 2009, 2012a, 2012b). Therefore, we treated cells from primitive mesoderm stage with cAMP and vascular endothelial growth factor (VEGF) based on our cardiomyocyte differentiation method (Masumoto et al., 2014).

(V) FACS analysis for an EC marker, CD31, and pericyte marker, PDGFRβ. Positive cell appearance at 9 days (C) and 12 days (F) after differentiation. Percentages of CD31-positive cells and PDGFRβ-positive cells are indicated.

We established a differentiation method by modifying previous procedures (Kondo et al., 2013) (Figure 1A). After plating embryoid bodies, almost all differentiated cells were positive for RESTIN, a neural progenitor marker, and some cell populations started to differentiate into class III β-tubulin (TUJ1)-positive neurons at stage 2 (Figure 1J). TUJ1-positive neurons were dramatically induced by changing the medium at stage 3 (Figure 1K). Glial fibrillary acidic protein (GFAP)-positive astrocytes were increased after neural maturation at stage 4, which was followed by brain development in vivo (Figure 1L). An astrocyte-enrichment culture showed a high population of GFAP-positive astrocytes at stage 5 (Figure 1M). qPCR clearly showed the induction of the neural progenitor marker Pax6 at stage 2, the mature neuron marker MAP2 at stage 3, and the astrocyte marker GFAP at stages 4 and 5 (Figure 1N). Taken together, our two systems efficiently differentiated hiPSCs into the four BBB components.

Generation of ciBECs with Four Cell Populations Derived from hiPSCs

The BBB is composed of specialized BECs surrounded by pericytes, astrocytes, and neurons. Thus, we hypothesized that BECs are generated by cell-cell interactions with the other three lineages and created a co-culture system with the four cell populations derived from hiPSCs described above. Astrocytes and neurons from 90 to 120 days after differentiation were recultured on differentiated cells with the EC and pericyte differentiation system at day 7 after differentiation (Figure 2A). Immunostaining showed that TUJ1-positive neurons and GFAP-positive astrocytes were nearly 99%, and these cells showed spindle-like structures (Figures 1C and 1D). All ECs took up low-density lipoprotein (LDL) into the cytoplasm, indicating functional ECs (Figure 1E). At day 12 after differentiation, the percentage of CD31-positive ECs was nearly 99%, and these cells showed spindle-like structures (Figures 1C and 1D). All ECs took up low-density lipoprotein (LDL) into the cytoplasm, indicating functional ECs (Figure 1E). At day 12 after differentiation, the percentage of CD31-positive ECs was nearly 99%, and these cells showed spindle-like structures (Figures 1C and 1D). All ECs took up low-density lipoprotein (LDL) into the cytoplasm, indicating functional ECs (Figure 1E). At day 12 after differentiation, the percentage of CD31-positive ECs was nearly 99%, and these cells showed spindle-like structures (Figures 1C and 1D). All ECs took up low-density lipoprotein (LDL) into the cytoplasm, indicating functional ECs (Figure 1E). At day 12 after differentiation, the percentage of CD31-positive ECs was nearly 99%, and these cells showed spindle-like structures (Figures 1C and 1D). All ECs took up low-density lipoprotein (LDL) into the cytoplasm, indicating functional ECs (Figure 1E).
were approximately 40% and 45% of the total population from 90 to 120 days after differentiation (Figure S1A). The endfeet of astrocytes elongated to the ECs, while neurons also interacted with ECs during the co-culture (Figures 2B and 2C). After co-culture with the four lineages derived from hiPSCs for 5 days, we purified ciBECs by FACS and analyzed their properties. Notably, 21 of the 22 BBB transporters and receptors analyzed in this study tended to have higher expressions in ciBECs compared with normal ECs, which were not co-cultured with astrocytes and neurons. Of the BBB-specific transporters analyzed, six, including cationic amino acid transporter 3 (CAT3), MFSD2A, which is a transporter for the essential ω-3 fatty acid docosahexaenoic acid (DHA), and the polarized efflux transporters, ATP-binding cassette transporters ABCA1, BCRP, PGP, and MRP5, were significantly increased in ciBECs (Figure 2D). We compared the properties of the ciBECs with those of the immortalized brain microvascular endothelial cell line, hCMC/D3, and human umbilical vein endothelial cells (HUVEC). The expressions of CAT1, PGP, and Transferrin receptor in ciBECs and hCMC/D3 are similar. However, the expressions of efflux transporters such as ABCA1, BCRP, and MRP5 are higher in ciBECs than in hCMC/D3 and HUVEC (Figure 2D). Immunostaining further showed that BCRP and PGP were highly expressed in ciBECs compared with ECs (Figure 2E). We next examined how these expressions changed with the culture. The co-culture of neurons (stage 3 in Figure 1I) with ECs and pericytes partially increased BBB-specific transporters and receptors. In contrast, co-culture of astrocytes (stage 5 in Figure 1I) with ECs and pericytes did not lead to an increase. Importantly, the co-culture of both neurons and astrocytes with ECs and pericytes was most efficient at inducing BBB-specific transporters and receptors. These results indicated that cell-cell communication between ECs and neurons and astrocytes is crucial in acquiring ciBEC properties.

We induced ciBECs with two hiPSC lines, 201B6 and 836B3. Furthermore, we performed the chimera differentiation assay, in which 836B3 iPSC-derived ECs and pericytes were co-cultured with 201B6 iPSC-derived neurons and astrocytes. This method also was able to generate ciBECs (Figure S3). These results indicated that our method is robust for generating ciBECs.

Figure 2. Generation of ciBECs Using Four Cell Populations Derived from iPSCs

(A) Schematic of the co-culture system with four lineages derived from iPSCs for ciBEC generation.

(B) A phase-contrast image at 2 days after co-culture. Asterisks, ECs; arrows, endfeet of astrocytes attached to ECs. Scale bar, 200 μm.

(C) Double immunostaining for CD31 and GFAP (left panel) or TUJ1 (right panel) at 5 days after co-culture. Scale bars, 200 μm.

(D) qPCR for the mRNA expressions of BBB-specific transporters and receptors in purified CD31-positive ECs (n = 6 independent experiments), ciBECs (n = 7 independent experiments), and immortalized cell lines, hCMC/D3 (n = 3 independent experiments) and HUVEC (n = 3 independent experiments) (*p < 0.05 versus ECs). mRNA expression on ECs was set as 1.0.

(E) Double immunostaining for CD31 and BCRP (upper panels) or PGP (bottom panels). Scale bars, 200 μm.
Induction of ciBECs via Notch Activation by Dll1 in Neurons

Our ciBEC generation method is amenable to investigating the underlying mechanisms of ciBEC specification. We first attempted to investigate the mechanisms of ciBEC specification by a pharmacological approach. We treated the cells with six inhibitors that are associated with various signaling pathways, such as Notch signaling and Wnt signaling, from the beginning of the co-culture of ECs, pericytes, astrocytes, and neurons. Interestingly, treatment with DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), a γ-secretase inhibitor which prevents Notch signaling, inhibited the upregulation of BBB-specific transporters in ECs co-cultured with astrocytes and neurons (Figure 3A). Similarly, LY294002, a phosphoinositide 3-kinase inhibitor, specifically inhibited the upregulation of BCRP in the co-culture system. Wnt inhibitors, including XAV939 and IWP4, did not have significant effects on the expression of BBB transporters (Figure 3A). We next examined Notch activation in ciBECs by immunostaining for Notch intracellular domain (NICD). NICD was highly expressed in the nuclei of ciBECs compared with ECs, and this localization was prevented by DAPT treatment (Figure 3B). These results suggested that Notch signaling is involved in ciBEC generation.

To investigate which cells activate Notch signaling in ciBECs, we examined the expression levels of Notch receptors and ligands in ECs, pericytes, astrocytes, and neurons. We first purified CD31+/PDGFRβ− ECs and PDGFRβ+/CD31− pericytes at 12 days after differentiation and collected CD44+/PSA−NCAM− astrocytes and PSA−NCAM+/CD44+ neurons from 90 to 120 days after differentiation. We then treated these cells with six inhibitors and performed qPCR for the mRNA expressions of the BBB-specific transporters CAT3, ABCA1, MFSD2A, BCRP, PGP, and MRP5 (n = 3 independent experiments; *p < 0.05, **p < 0.01 versus ECs, #p < 0.05, ##p < 0.01 versus ciBECs). mRNA expression in ECs was set as 1.0.

Figure 3. Induction of ciBECs via Notch Activation
(A) Pharmacological approach using six inhibitors to study the mechanism of BBB specification. qPCR for the mRNA expressions of the BBB-specific transporters CAT3, ABCA1, MFSD2A, BCRP, PGP, and MRP5 (n = 3 independent experiments; *p < 0.05, **p < 0.01 versus ECs, #p < 0.05, ##p < 0.01 versus ciBECs). mRNA expression in ECs was set as 1.0.
(B) Double immunostaining for CD31 and NICD at 5 days after co-culture. Scale bars, 50 μm.
Figure 4. Dll1 in Neurons Activates Notch Signaling to Generate ciBECs

(A) qPCR for the mRNA expressions of CD31, SM22α, MAP2, and GFAP in purified ECs, pericytes, neurons, and astrocytes by FACS (n = 3 independent experiments). mRNA expression on ECs was set as 1.0.
differentiation by FACS (Figures 1B and S1C). We checked lineage-specific gene expressions by qPCR. CD31, SM22a, MAP2, and GFAP were highly expressed in ECs, pericytes, neurons, and astrocytes, respectively (Figure 4A). Next, we investigated mRNA expressions of Notch receptors and ligands in the four lineages. Notch1 and 4 were highly expressed in ECs and Notch2 and 3 were enriched in astrocytes. Notch ligands, Dll4 and Jagged2, were highly expressed in ECs and Jagged1 was enriched in pericytes. We focused on Dll1, which was highly expressed in neurons, to analyze the generation of ciBECs (Figure 4B).

To define the biological effect of the high Dll1 expression, we performed a loss-of-function study using Dll1 small interfering RNA (siRNA) (siDll1). Approximately 81% inhibition of Dll1 mRNA expression in neurons and astrocytes at 120 days after differentiation was induced with the transfection of siDll1#2 (Figure S4A). We co-cultured the siRNA-treated neurons and astrocytes (siDll1 or control siRNA [siCon]) on ECs and pericytes for 5 days. siCon-treated neurons and astrocytes significantly induced the six BBB-specific transporters compared with ECs. siDll1 treatment tended to inhibit the six BBB-specific transporters and resulted in significant inhibition of ABCA1, MFS2DA, BCRP, and MRPs compared with siCon treatment (Figure 4C). Taken together, these results indicated that Dll1 in neurons is crucial for the generation of ciBECs by activating Notch signaling.

BBB Model Using ciBECs Derived from hiPSCs
To create a BBB model with hiPSC-derived ciBECs, we seeded ciBECs purified from the co-culture with pericytes, neurons, and astrocytes by FACS onto Transwell filters coated with fibronectin and grew them to confluence for 7 days (Figure 5A). Immunostaining of ZO-1 and CLAUDIN5 showed the maintenance of continuous cell-cell contacts between ciBECs on the Transwell filter (Figure 5B). Western blotting with membrane protein showed that CLAUDIN5 was highly expressed in ciBECs compared with ECs (Figure S4B). A hallmark of the BBB is high transendothelial electrical resistance (TEER) due to tight junctions between ciBECs. As expected, ciBECs showed a significant increase of TEER compared with ECs. When we co-cultured the cells with astrocytes from days 125–180 after differentiation on the bottom side of the Transwell, the TEER of ciBECs, but not of ECs, was dramatically enhanced (Figure 5C), indicating ciBECs had a brain-like phenotype. To confirm the existence of tight junctions between ciBECs, we analyzed the cells by transmission electron microscopy. ciBECs co-cultured with astrocytes clearly formed tight junctions (Figure 5D). To further validate the barrier function in our iPSC-derived BBB model, we investigated the permeability of fluorescein-Na (FN, 376 Da) and fluorescein isothiocyanate (FITC)-dextran of different masses (4, 10, 20, 40, and 70 kDa), which cross the BBB non-specifically by paracellular diffusion. ciBECs showed significantly decreased permeability of FN (39.3% ± 3.8%) and 4-kDa FITC-dextran (26.1% ± 5.6%) compared with ECs, hCMEC/D3, and HUVEC (Figures 5E and S6). Heavier FITC-dextran also tended to have less permeability across ciBECs compared with ECs (Figure 5E). Taken together, hiPSC-derived ciBECs have a strong barrier function, including high transendothelial resistance and low rates of paracellular leakage.

Screening System of Drug Permeability with BBB Model
We found that the induced ciBECs highly expressed polarized efflux transporters, such as PGP, BCRP, and MRPs. To define the biological function of these high expressions, we first examined PGP function using rhodamine-123, a cell-permeable PGP substrate. ECs and HUVEC showed about 1.2-fold increase and hCMEC/D3 about 2-fold increase in rhodamine-123 accumulation in the presence of verapamil, a PGP inhibitor. Notably, cell accumulation of rhodamine-123 in ciBECs was significantly increased approximately 2.5-fold with verapamil, demonstrating the functional PGP efflux activity of ciBECs (Figures 6A and S5A–S5C).

Finally, we screened the BBB permeability for small molecules of clinical drugs by nano liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) using our hiPSC-derived BBB model. We selected ten drugs with known permeability properties for the evaluation. Notably, propanolol, caffeine, antipyrim, carbamazepine, and trazodone, which can all cross the BBB into the CNS by lipid-mediated free diffusion (indicated as CNS-positive drugs in Figure 6C), displayed high permeability coefficient values (Pe) (144.8 ± 26.3 to 879.4 ± 161.1 × 10⁻⁶ cm/s). In contrast, sulpiride, epinastine, cimetidine, quinidine, and prazosine, which have low BBB permeability as efflux transporter substrates (indicated as CNS-negative drugs in Figure 6C), showed low Pe (5.9 ± 2.6 to 53.2 ± 22.8 × 10⁻⁶ cm/s) (Figures 6B and 6C). These results indicated
that our human iPSC-derived BBB model could be used to predict the permeability of developmental drugs.

We also investigated DHA permeability for analyzing the function of the nutrient transporter in our BBB model. An analysis of DHA kinetics with nanoLC-MS/MS showed that ciBECs significantly increased the permeability of DHA compared with ECs (Figure S5D). This result suggested that nutrient transporters such as MFSD2A in hiPSC-derived ciBECs are functional in the BBB model.

**DISCUSSION**

Understanding how the BBB operates is crucial for drug development, as it impedes chemical molecules from reaching the CNS. Current non-primate BBB models fail to adequately recapitulate human BBB function because of species-specific differences (Aday et al., 2016; Hoshi et al., 2013; Syvänen et al., 2009; Warren et al., 2009). We therefore considered human iPSCs as a resource for creating a reproducible and robust human BBB model. Here, we generated hiPSC-derived BBB that expressed specific transporters and receptors and showed low permeability properties, suggesting that it could be used as a platform for initial drug screening before in vivo clinical tests.

Importantly, using simple co-culturing systems with hiPSC-derived ECs, pericytes, neurons, and astrocytes, we revealed a molecular mechanism for ciBEC specification. In particular, this technology allows us to investigate cell-cell interactions, which led to the discovery that Dll1 expression by neurons is crucial for activating the Notch signaling that leads to ciBEC specificity. Notch is a single-pass transmembrane receptor known for its function in controlling cell-fate decisions and creating boundaries through cell-cell communication. Notch signaling in ECs is known to specify arterial ECs (Yamamizu et al., 2010). Dll1, a Notch ligand in neurons, functions in neural differentiation and maintenance (Grandbarbe et al., 2003; Kageyama et al., 2008). Furthermore, it communicates with radial glial cells for the differentiation to astrocytes (Namihira et al., 2009). Our results showed that Dll1 was highly expressed in mature neurons (Figure S6A), and we demonstrated that it
interacted with ECs for ciBEC specification (Figure 4C). Some reports have shown that the Wnt canonical pathway is crucial for proper neurovascular development. For example, the simultaneous ablation of Wnt7a/7b is embryonic lethal for neurovascular phenotypes (Daneman et al., 2009; Stenman et al., 2008). Our results showed that Wnt7a was expressed in neurons and Wnt7b was expressed in pericytes and astrocytes (Figure S6B), but treatment with Wnt inhibitors in our co-culture system did not cause significant differences in the expression of BBB-specific transporters (Figure 3A). Moreover, we found that Wnt7a was highly expressed in early-stage neural progenitors (Figure S6C), indicating that Wnt7a may be necessary for early vascularization in the brain. Overall, our technology could help elucidate the underlying molecular machinery for finely regulated BBB formation, which should offer new therapeutic strategies to repair the BBB following stroke or other neurological impairments.

The brain vasculature sprouts into the neuronal tube and elongates toward the ventricular zone to form vascular networks that are surrounded by neuroepithelial cells, radial glia, neuroblasts, and neurons at around embryonic day 9.5 (E9.5) in mouse embryo (Engelhardt, 2003). The vascular permeability decreases at E16 as it interacts with BECs, neurons, and radial glial cells (Risau et al., 1986). Our ciBEC specification process seems to involve Notch signaling in response to neuron-derived Dll1 (Figure 4C), which may recapitulate the in vivo microenvironment for BBB development. We found that mature barrier characteristics of the BBB were induced by crosstalk between iPSC-derived astrocytes (Figure 5C). Several astrocytic factors for BBB maturation, including transforming growth factor, glial cell-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), interleukin-6, angiopoietin-1 (Ang1), and hydrocortisone, are capable of glial-mediated barrier induction (Abbott, 2002; Lee et al., 2003). Indeed, hiPSC-derived astrocytes highly expressed GDNF, bFGF, EGF (epidermal growth factor), and Ang1 (Figure S6D). Further analyses are needed to identify the BBB maturation factors from hiPSC-derived astrocytes. In addition, previous anatomical examination of the brain microvasculature showed that the endfeet of astrocytes form a lacework of fine lamellae to closely support the outer surfaces of the endothelium (Kacem et al., 1998). Although we successfully used Transwell dishes, which separate the apical and basolateral sides, to analyze drug kinetics, the construction of a three-dimensional BBB model from all four cell populations will expedite mature BBB formation and BBB physiology analysis.
The widespread hope for a new era of prevention and treatment for chronic neurological disorders, such as stroke, Alzheimer’s disease, and Parkinson’s disease, will likely depend on the discovery of new CNS drugs. However, currently over 99% of candidate CNS drugs fail in clinical tests because of side effects and/or an inability to cross the BBB (Alavieh et al., 2005). Our human iPSC-derived BBB shows promise as a model for CNS drug development at the pre-clinical stage. BBB models constructed from CNS disease patient-specific iPSCs should also provide new insights into how vascular dysfunction contributes to CNS diseases.

**EXPERIMENTAL PROCEDURES**

**Differentiation of hiPSCs into ECs and Pericytes**

We used the human iPSC lines 201B6 and 836B3 (Takahashi et al., 2007). The methods for culturing and passaging human iPSCs were reported previously (Masumoto et al., 2014). hCMEC/D3 (Millipore) was cultured with EGM2 (Lonza) and 5% fetal bovine serum (FBS) (Life Technologies). HUVEC (Lonza) was cultured with EGM2 (Lonza).

For EC and pericyte differentiation, iPSCs were detached with Versene (0.48 mM EDTA solution; Life Technologies) and plated onto Matrigel (growth factor reduced, 1:60 dilution; Life Technologies)-coated plates at a density of approximately 70,000 cells/cm² in mouse embryonic fibroblast conditioned medium (MEF-CM; DMEM [Nacalai Tesque] containing 10% fetal calf serum, 2 mM L-glutamine, and 1% non-essential amino acids (NEAA) (Life Technologies) with 4 ng/mL bFGF (Wako Pure Chemicals Industries) for 2 days before induction. Cells were covered with Matrigel (1:60 dilution) 1 day before induction. MEF-CM was replaced with RPMI + B27 medium (RPMI-1640, 2 mM L-glutamine, B27 supplement without insulin) supplemented with 100 ng/mL of activin A (R&D Systems) for 1 day, followed by 10 ng/mL bone morphogenetic protein 4 (R&d), 10 ng/mL bFGF, and Matrigel (1:60 dilution) for 3 days without culture medium change. After differentiation at 4 days, the culture medium was replaced with culture medium with RPMI + B27 medium with 100 ng/mL VEGF165 (Wako) and 1 mM 8-bromoadenosine-3',5'-cyclic monophosphate sodium salt (8-bromo-cAMP) (Nacalai Tesque) for 1 day. Primitive mesoderm cells were purified with autoMACS (Miltenyi Biotec) using anti-KDR antibody and anti-APC MicroBeads (Miltenyi Biotec) and recultured at a density of 60,000 cells/cm² with RPMI + B27 medium, 100 ng/mL VEGF165, and 1 mM 8-bromo-cAMP. One day after MACS sorting, the culture medium was replaced with RPMI + B27 medium and 100 ng/mL VEGF165. Then ECs were cultured with human endothelial serum-free medium (SFM) (Life Technologies) supplemented with 20 ng/mL bFGF (Wako), 10 ng/mL EGF (Life Technologies), and 10 µg/mL human plasma fibronectin (Life Technologies), and refreshed every 3 days.

**Differentiation of hiPSCs into Astrocytes and Neurons**

iPSCs were dissociated to single cells and quickly reaggregated in U-bottomed 96-well plates (Greiner Bio-One) pre-coated with 2% Pluronic F-127 (Sigma-Aldrich) for embryoid body (EB) formation. EBs were cultured in DMEM/Ham’s F12 (Life Technologies) supplemented with 5% knockout serum replacement, 2 mM L-glutamine, 1% NEAA, 0.1 µM 2-mercaptoethanol (Life Technologies), 2 µM dorsomorphin, and 10 µM SB431542 (Wako) at stage 1 of the neural induction (days 0–8). EBs were transferred onto Matrigel (1:60 dilution)-coated 6-well plates and cultured in DMEM/Ham’s F12 supplemented with N2 supplement (Life Technologies) and 2 µM dorsomorphin (Wako) at stage 2 (days 8–24). At 24 days after differentiation, neural precursor cells were cultured in Neurobasal Medium (Life Technologies) supplemented with B27 without vitamin A, Glutamax, 10 ng/mL brain-derived neurotrophic factor, 10 ng/mL GDNF, and 10 ng/mL NT3 on Matrigel-coated 10 cm dishes and refreshed every 3 days at stage 3 (days 24–60). Cells at 60 days after differentiation were transferred onto Matrigel (1:60 dilution)-coated 10-cm dishes and cultured in DMEM/Ham’s F12 supplemented with N2 supplement (Life Technologies) and refreshed every 3 days at stage 4 (days 60–120). Cells at 120 days after differentiation were passaged on non-coated polystyrene 10-cm dishes for astrocyte culture, because neurons weakly attach to non-coated polystyrene 10-cm dishes whereas astrocytes attach strongly and proliferate. By repeated passage in the same manner at days 140, 160, and 180, astrocytes increased their own abundance ratio and showed positive GFAP immunostaining (more than 80%) at stage 5 (days 120–180).

**Induction of ciBECs**

BECs were induced by having ECs interact with pericytes, astrocytes, and neurons. To promote this interaction, we prepared a co-culture system with hiPSC-derived ECs, pericytes, astrocytes, and neurons. Astrocytes and neurons (total 3.5 × 10⁶ cells) from day 90 to day 120 after differentiation were co-cultured on differentiated ECs and pericytes (about 3.5 × 10⁶ cells) at day 7 in SFM (Life Technologies) supplemented with 20 ng/mL bFGF (Wako), 10 ng/mL EGF (Life Technologies), and 10 µg/mL human plasma fibronectin (Life Technologies) for 5 days. The population of the co-culture at day 7 included approximately 49.5% CD31-positive ECs, 0.5% PDGFRα-positive pericytes, 20% TUJ1-positive neurons, and 22.5% GFAP-positive astrocytes. We refreshed the medium every 3 days. The population of the co-culture at day 12 included approximately 10% CD31-positive ECs, 5% PDGFRα-positive pericytes, 20% TUJ1-positive neurons, and 45% GFAP-positive astrocytes (Figures 1 and S1). ciBECs and ECs were purified with FACS Ariall (Becton Dickinson) using anti-CD31 (PECAM1) conjugated with APC at 12 days after differentiation. The ciBECs were used to form the BBB model.

**Immunostaining and FACS Analysis**

Immunostaining of the cultured cells was carried out as described previously (Yamamizu et al., 2012a, 2010). In brief, 4% paraformaldehyde-fixed cells were blocked by 1% skim milk (BD Biosciences) and incubated overnight with primary antibodies at 4°C. For immunofluorescence staining, anti-mouse, rat, rabbit, or goat immunoglobulin G (IgG) antibodies conjugated with Alexa 488 or Alexa 546 (Life Technologies) were used as secondary antibodies. Nuclei were visualized with DAPI (Life Technologies). Stained cells were photographed with the inverted fluorescence microscope BZ-9000 (Keyence) or Eclipse TE2000-U (Nikon) and a digital camera system (AxioCam HRC, Carl Zeiss) with the use...
Acetylated LDL Uptake Assay and Tube Formation Assay
LDL uptake assays for ECs were performed as previously reported (Yamamizu et al., 2013). LDL uptake by cells was assessed by fluorescence microscopy after incubation with 10 μg/mL acetylated LDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL) (Biomedical Technologies) for 4h at 37°C.

The tube formation assay for ECs was carried out as described previously (Yamamizu et al., 2013). ECs and pericytes (total 7 × 10^4) were plated on 500 μm Matrigel Basement Membrane Matrix (BD Biosciences) and allowed to equilibrate at room temperature from the cell-culture incubator (37°C, 5% CO₂) for 20 min. TEER values were measured using an EVOM2 voltmeter with STX-2 electrodes (World Precision Instruments). For calculation of TEER (Ω [ohms] × cm²), electrical resistance across a fibronectin-coated insert without cells was subtracted from the readings obtained on inserts with cells and then multiplied by the surface area of the insert (0.33 cm²).

Transmission Electron Microscopy
ciBECs were cultured onto cell-culture inserts as described above. ciBECs were washed once with PBS and fixed in 2% glutaraldehyde (Microscopy Sciences) and 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C. All sections were treated with OsO₄ (1% for 1 min and 0.5% for 20 min at 4°C) in PBS, dehydrated in ethanol and propylene oxide, and embedded in Luveak 812 (Nacalai Tesque). Ultrathin sections were cut with an ultramicrotome (Leica) and observed with a transmission electron microscope (H-7650; Hitachi).

Permeability Assay Using Fluorescein-Na/FITC-Labeled Dextran
ciBECs were cultured on cell inserts with astrocytes on the bottom side of Transwell for 7 days. For the transport experiments, all media were removed from the upper chamber of the inserts and...
replaced with 50 µL of pre-warmed Hank’s balanced salt solution (HBSS) (Life Technologies) supplemented with 10 mM HEPES (Life Technologies) and 0.1% BSA (Sigma-Aldrich) by equilibration to 37°C for 15 min. At a time point 0 min, 50 µL of 2 µM fluorescein-Na or 2 mg/mL FITC-labeled dextran was added to the upper chamber of the inserts, which were then transferred to 24-well plates containing 1 mL of pre-warmed HBSS supplemented with 10 mM HEPES. Samples (100 µL) were collected at 15, 30, 45, and 60 min from the bottom chamber and transferred to a black-walled 96-well plate (Nunc). The fluorescence was measured with an Envision fluorescence plate reader (PerkinElmer). Concentrations were calculated using standard curves generated from the stock solutions of each compound. Permeability coefficients (Pe) that take into account the barrier to transport from both the endothelial monolayer and the cell-culture insert were calculated as described previously (Perrière et al., 2005; Nakagawa et al., 2009; Watson et al., 2013).

PGP Functional Efflux Assay
ciBECs were cultured onto cell-culture inserts as described above. The media were replaced with HBSS supplemented with 10 mM HEPES and 0.1% BSA containing either 100 µM verapamil or vehicle (0.5% DMSO), followed by incubation at 37°C for 30 min. Cells were dye-loaded by removing buffer from the upper chamber and replacing it with fresh buffer containing 200 ng/mL rhodamine-123 at 37°C for 30 min. The cells were washed three times in HBSS supplemented with 10 mM HEPES and 0.1% BSA. Fresh assay buffer was added onto the cell-culture inserts and incubated at 37°C for 1 hr to allow dye efflux. At the end of the incubation the inserts were transferred to a fresh plate, and the cells were washed three times in PBS and lysed for 20 min in cell lysis buffer. The fluorescence was measured with an Envision fluorescence plate reader (PerkinElmer). The cellular uptake of rhodamine-123 was calculated using standard curves generated from stock solutions of the dye.

Analysis of Drug Permeability Using NanoLC-MS/MS
Drugs were dissolved in DMSO to a concentration of 1 mM and further diluted in HBSS supplemented with 10 mM HEPES. ciBECs were cultured onto cell-culture inserts as described above. We refreshed 50 µL of pre-warmed HBSS supplemented with 10 mM HEPES and 0.1% BSA by equilibration to 37°C for 15 min. At the time point 0 min, 50 µL of 4 µM drug was added to the upper chamber of the inserts, which were then transferred to 24-well plates containing 1 mL of pre-warmed HBSS supplemented with 10 mM HEPES. Samples were collected from the lower chamber at 10, 20, 30, and 40 min, 15, 30, 45, and 60 min, or 30, 60, 90, and 120 min. Samples were desalted with reversed-phase StageTips (Rappaport et al., 2007) and suspended in loading buffer (0.5% trifluoroacetic acid and 4% acetonitrile) for subsequent nanoLC-MS/MS analysis.

NanoLC-MS/MS was performed using a TripleTOF 5600 system (AB Sciex) equipped with an HTC-PAL autosampler (CTC Analytics). Loaded samples were separated on a self-pulled analytical column (150 mm length, 100 µm internal diameter) using a Dionex Ultimate 3000 RSLC Nano System. The mobile phases consisted of (A) 0.5% acetic acid with 5% DMSO and (B) 0.5% acetic acid in 80% acetonitrile with 5% DMSO (Hahne et al., 2013). The linear gradient condition was 5%–99% B for 10 min, 99% B for 5 min, and 5% B for 30 min at a flow rate of 500 µL/min. A spray voltage of 2,300 V was applied. The MS scan range was 100–1,500 m/z every 0.25 s. Blank runs were inserted between different samples. All raw data files from the TripleTOF 5600 system were analyzed by PeakView software version 2.1 (AB Sciex). Extracted-ion chromatograms were observed with ±0.01 m/z width for each of caffeine (m/z = 195.0867), cimetidine (m/z = 253.123), propranolol (m/z = 260.1645), epinastine (m/z = 250.13387), prazosin (m/z = 384.16663), quinidine (m/z = 323.19105), sulpiride (m/z = 342.1482), trazodone (m/z = 372.15856), antipyrine (m/z = 189.10224), and carbamazepine (m/z = 237.10224).

Statistical Analysis
At least three independent experiments were performed. Statistical analysis of the data was performed using ANOVA. P < 0.05 was considered significant. Values are reported as mean ± SEM.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.01.023.

AUTHOR CONTRIBUTIONS
K.Y. conceived the project, performed all experiments, and wrote the manuscript; M.I. and M.N. performed nanoLC-MS/MS for drug kinetics; H.T., T.I., and M.M. helped with the iPSC maintenance and EC and pericyte differentiation; T.S. and Y.N. performed nanoLC-MS/MS for DHA; T.K. and H.I. helped with the neuron and astrocyte differentiation; J.K.Y. supervised the experiments.

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