

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 nutrient transporters (*CAT3*, *MFS2A*) and efflux transporters (*ABCA1*, *BCRP*, *PGP*, *MRP5*), and strong barrier function based on tight junctions. Neuron-derived Dll1, which activates Notch signaling in ECs, was essential for the BEC specification. We performed in vitro BBB 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; Syvänen 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 reproductivity (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 (Weksler 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

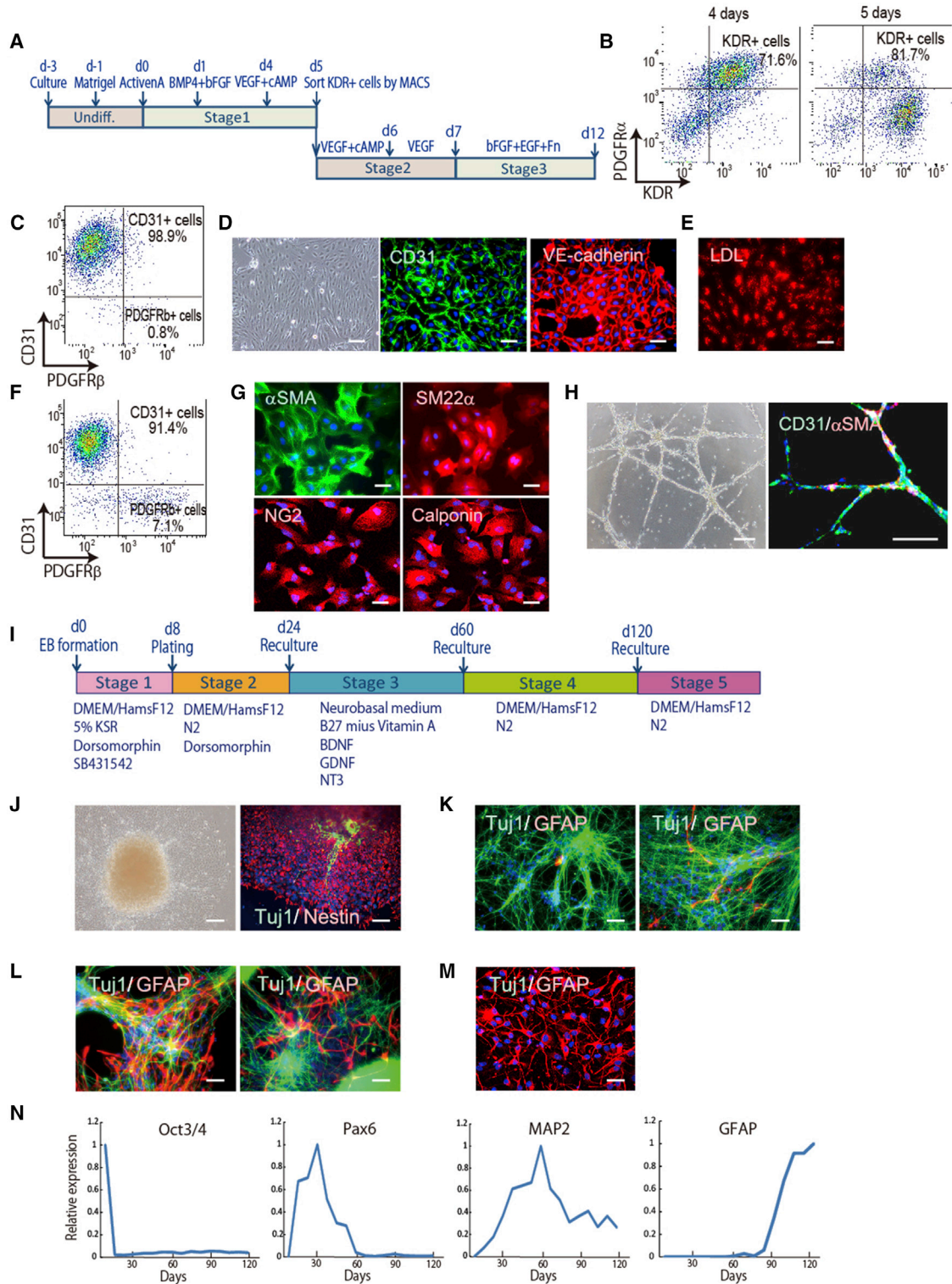


Figure 1. Differentiation of iPSCs into ECs, Pericytes, Neurons, and Astrocytes

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

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

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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) (Figure 1A). VEGF receptor type 2 (KDR)-positive and platelet-derived growth factor receptor α (PDGFR α)-positive cells appeared in approximately 70% of the cells at 4 days after differentiation. KDR single-positive cells and KDR/PDGFR α double-positive cells appeared after treatment with cAMP and VEGF, and KDR-positive cells were purified by auto MACS at day 5 after the differentiation and then recultured (Figure 1B). At day 9 after differentiation, the percentage of CD31/VE-CADHERIN double-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 about 91% and PDGFR β -positive pericytes about 7% (Figure 1F). The PDGFR β^+ /CD31 $^-$ pericytes were purified by fluorescence-activated cell sorting (FACS) and positive for α -smooth muscle actin (α SMA), SM22 α , NG2, and CALPONIN (Figure 1G). We examined

the function of these ECs by using the tube formation assay. The ECs generated vascular-like structures on Matrigel. Gel immunostaining showed that these structures consisted of CD31-positive ECs attached to α SMA-positive pericytes (Figure 1H).

We next induced astrocytes and neurons from hiPSCs. We established a differentiation method by modifying previous procedures (Kondo et al., 2013) (Figure 1I). After plating embryoid bodies, almost all differentiated cells were positive for NESTIN, 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

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

(D) A phase-contrast image of differentiated ECs and immunostaining for CD31 and VE-CADHERIN at 9 days after differentiation. Scale bar, 200 μ m.

(E) LDL uptake assay for ECs at 9 days after differentiation. Scale bar, 200 μ m.

(G) Immunostaining of purified pericytes at 12 days after differentiation for α SMA, SM22 α , NG2, and CALPONIN. Scale bar, 200 μ m.

(H) Tube formation assay for ECs at 12 days after differentiation. A phase-contrast image and immunostaining for CD31 and α SMA. Scale bar, 200 μ m.

(I) Schematic representation of the neuron and astrocyte induction protocol.

(J) Phase-contrast image at 11 days after differentiation and immunostaining for NESTIN and TUJ1 at 16 days after differentiation. Scale bar, 200 μ m.

(K–M) Double immunostaining for TUJ1 and GFAP at 32 days (K, left panel), 48 days (K, right panel), 86 days (L, left panel), 100 days (L, right panel), and 145 days (M) after differentiation. Scale bars, 200 μ m (K, L) and 100 μ m (M).

(N) qPCR for the mRNA expressions of *Oct3/4*, *Pax6*, *MAP2*, and *GFAP* during neuron and astrocyte differentiation ($n = 1$). mRNA expression on undifferentiated hiPSCs was set as 1.0.

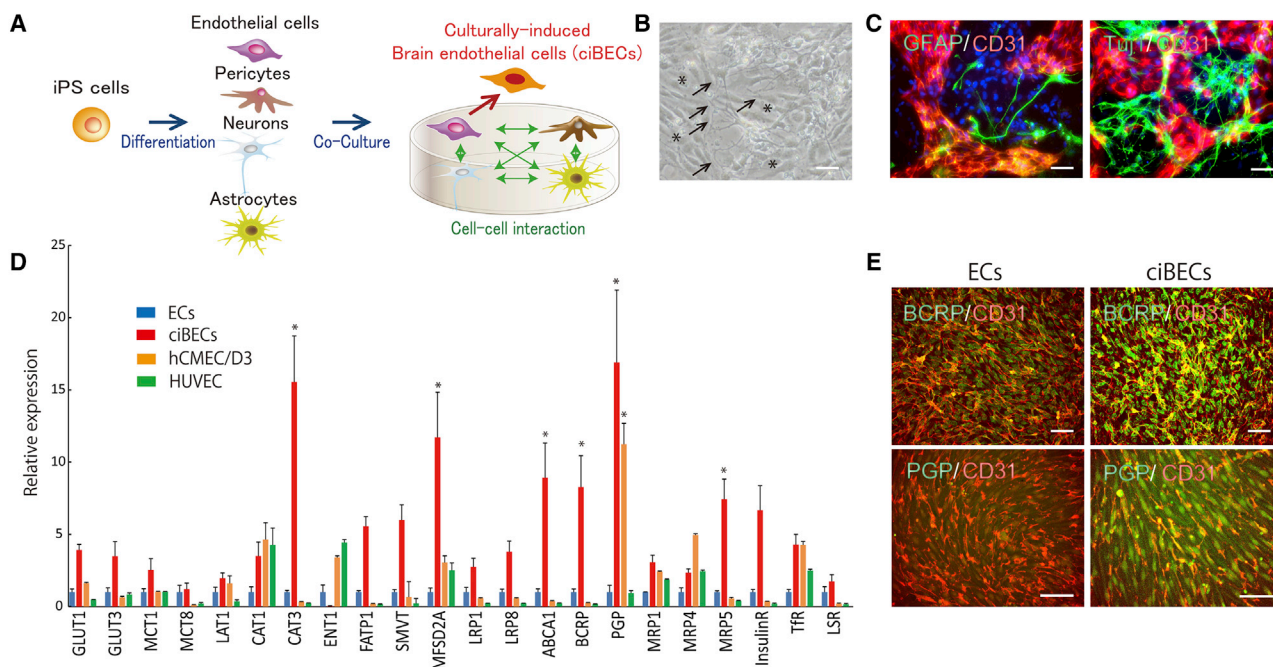


Figure 2. Generation of ciBECs Using Four Cell Populations Derived from iPSCs

(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 μ m.

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

(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, hCMEC/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 μ m.

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, hCMEC/D3, and human umbilical vein endothelial cells (HUVEC). The expressions of CAT1, PGP, and Transferrin receptor in ciBECs and hCMEC/D3 are similar. However, the expressions of efflux transporters

such as ABCA1, BCRP, and MRP5 are higher in ciBECs than in hCMEC/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 (Figure S2). 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.

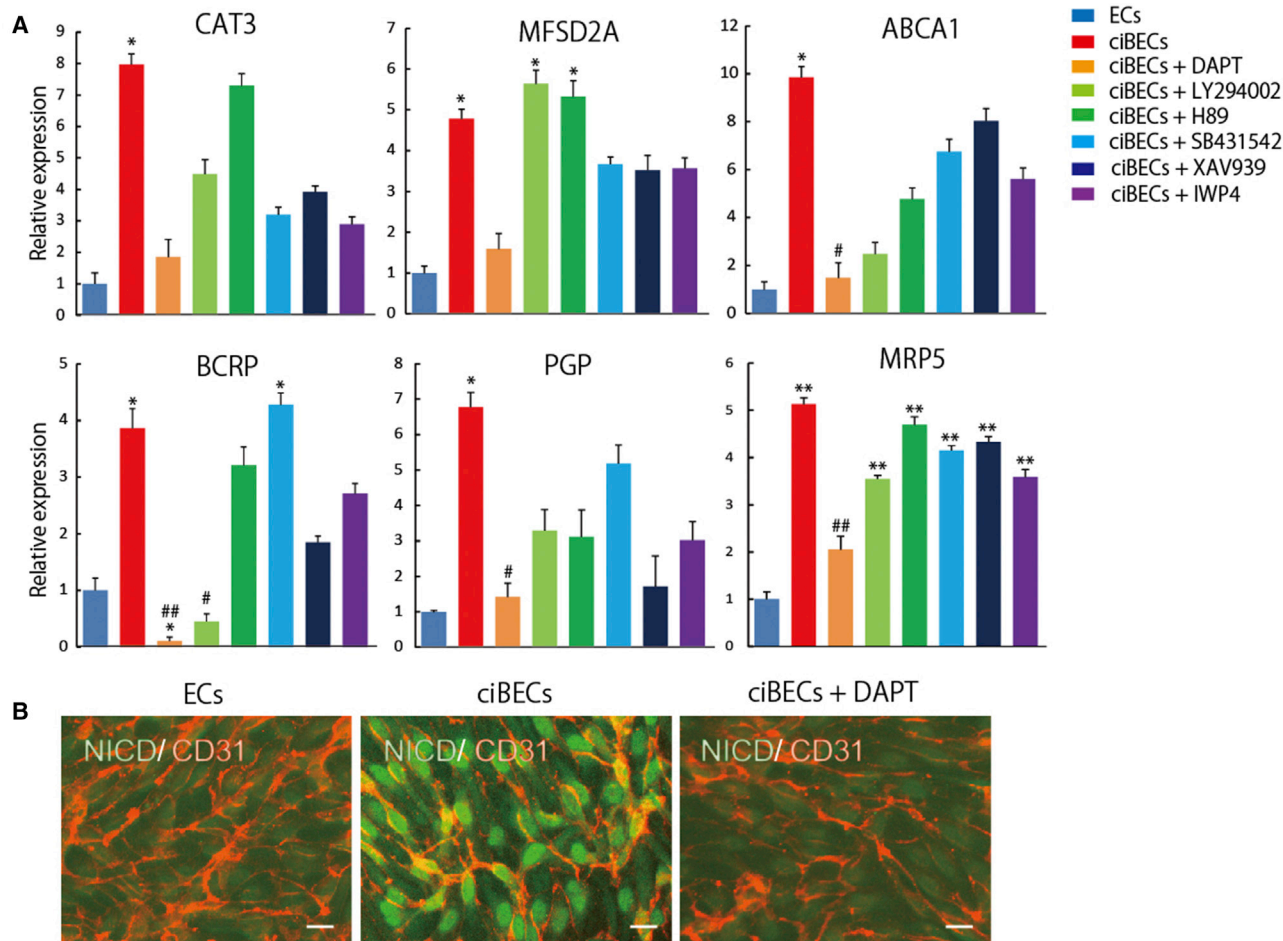


Figure 3. Induction of ciBECs via Notch Activation

(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 .

Induction of ciBECs via Notch Activation by DII1 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, specif-

ically 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⁺/PDGFR β ⁻ ECs and PDGFR β ⁺/CD31⁻ pericytes at 12 days after differentiation and collected CD44⁺/PSA⁻NCAM⁻ astrocytes and PSA⁻NCAM⁺/CD44⁻ neurons from 90 to 120 days after

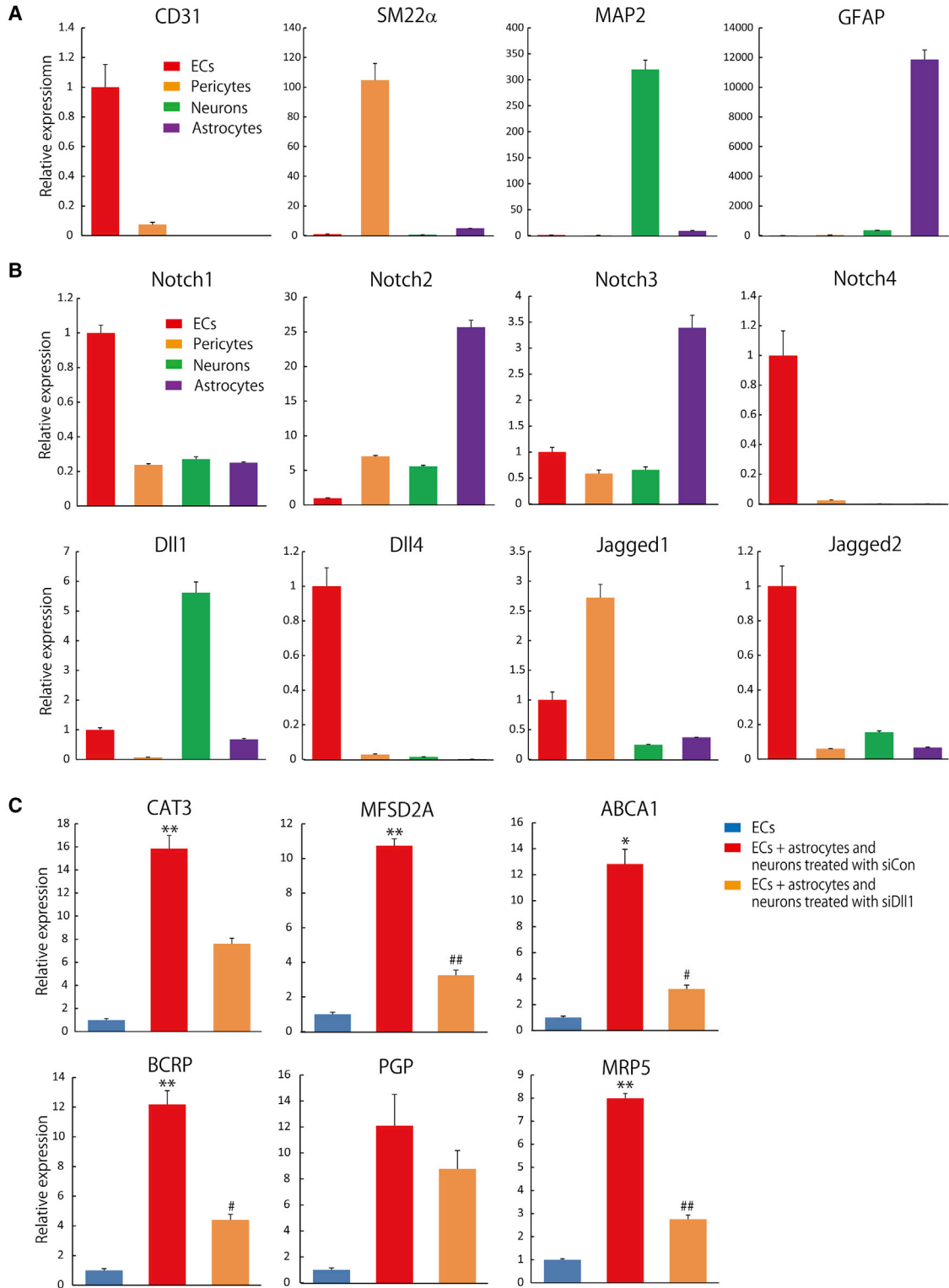


Figure 4. Dll1 in Neurons Activates Notch Signaling to Generate ciBECs

(A) qPCR for the mRNA expressions of *CD31*, *SM22 α* , *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.

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differentiation by FACS (Figures 1B and S1C). We checked lineage-specific gene expressions by qPCR. *CD31*, *SM22 α* , *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) (si*Dll1*). Approximately 81% inhibition of *Dll1* mRNA expression in neurons and astrocytes at 120 days after differentiation was induced with the transfection of si*Dll1*#2 (Figure S4A). We co-cultured the siRNA-treated neurons and astrocytes (si*Dll1* 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. si*Dll1* treatment tended to inhibit the six BBB-specific transporters and resulted in significant inhibition of *ABCA1*, *MFSD2A*, *BCRP*, and *MRP5* 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 ($39.3\% \pm 3.8\%$) and 4-kDa FITC-dextran ($26.1\% \pm 5.6\%$) 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, propranolol, caffeine, antipyrin, 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 (P_e) (144.8 ± 26.3 to $879.4 \pm 161.1 \times 10^{-6}$ cm/s). In contrast, sulphiride, epinastine, cimetidine, quinidine, and prazosine, which have low BBB permeability as efflux transporter substrates (indicated as CNS-negative drugs in Figure 6C), showed low P_e (5.9 ± 2.6 to $53.2 \pm 22.8 \times 10^{-6}$ cm/s) (Figures 6B and 6C). These results indicated

(B) qPCR for the mRNA expressions of Notch receptors *Notch1*, *Notch2*, *Notch3*, and *Notch4* and Notch ligands *Dll1*, *Dll4*, *Jagged1*, and *Jagged2* ($n = 3$ independent experiments). mRNA expression in ECs was set as 1.0.

(C) Loss-of-function using *Dll1* siRNA. 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 ECs + astrocytes and neurons treated with siCon). mRNA expression in ECs was set as 1.0.

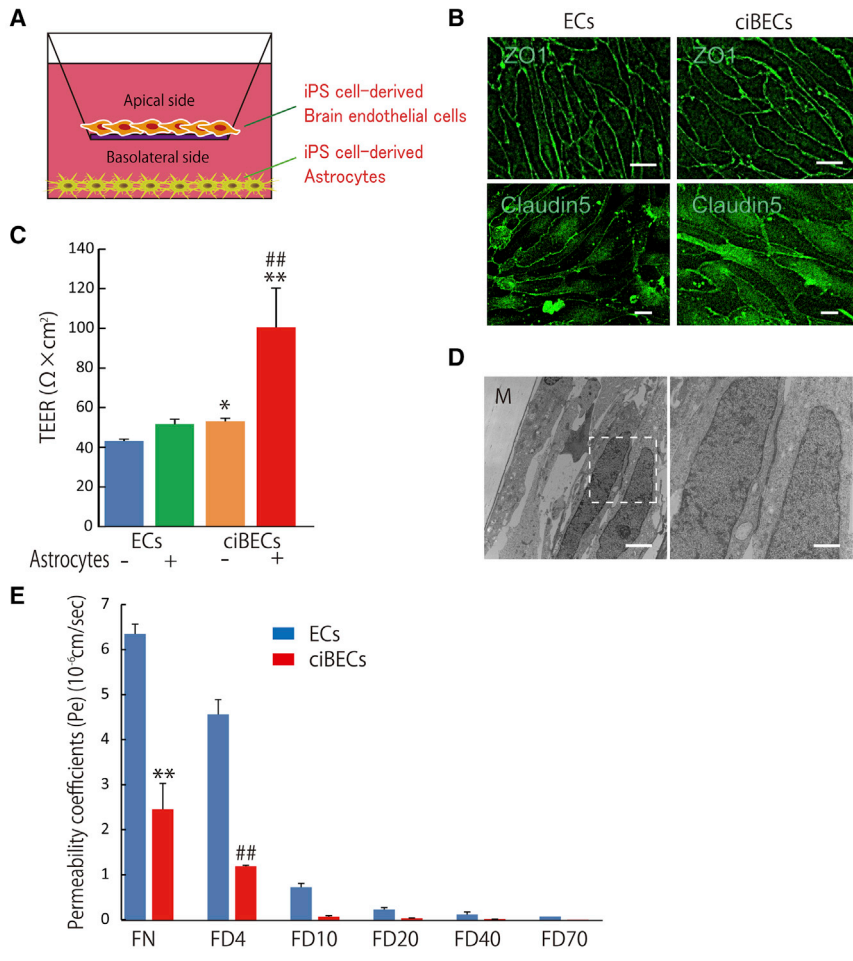


Figure 5. BBB Model Using ciBECs Derived from iPSCs

(A) Schematic of the two-compartment BBB model. iPSC-derived ciBECs were seeded onto a Transwell filter coated with fibronectin and co-cultured with iPSC-derived astrocytes to analyze BBB properties.

(B) Double immunostaining for CD31 and ZO-1 (upper panels; scale bar, 25 μm) or CLAUDIN5 (bottom panels; scale bar, 10 μm).

(C) Measurement of TEER at 7 days after plating onto the Transwell filter. iPSC-derived ciBECs responded to soluble factors from astrocytes (n = 4 independent experiments; *p < 0.05, **p < 0.01 versus ECs without astrocytes, ##p < 0.01 versus ciBECs without astrocytes).

(D) Transmission electron microscopy of iPSC-derived ciBECs after co-culture with iPSC-derived astrocytes for 24 hr. M, Transwell filter. Right panel shows higher magnification of the content in the dashed box. Scale bars, 2 μm (left panel) and 750 nm (right panel).

(E) Permeability assay using fluorescein-Na/FITC-labeled dextran (n = 3 independent experiments; **p < 0.01 versus ECs with fluorescein-Na, ##p < 0.01 versus ECs with 4-kDa FITC-labeled dextran).

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 (Namihiro et al., 2009). Our results showed that Dll1 was highly expressed in mature neurons (Figure S6A), and we demonstrated that it

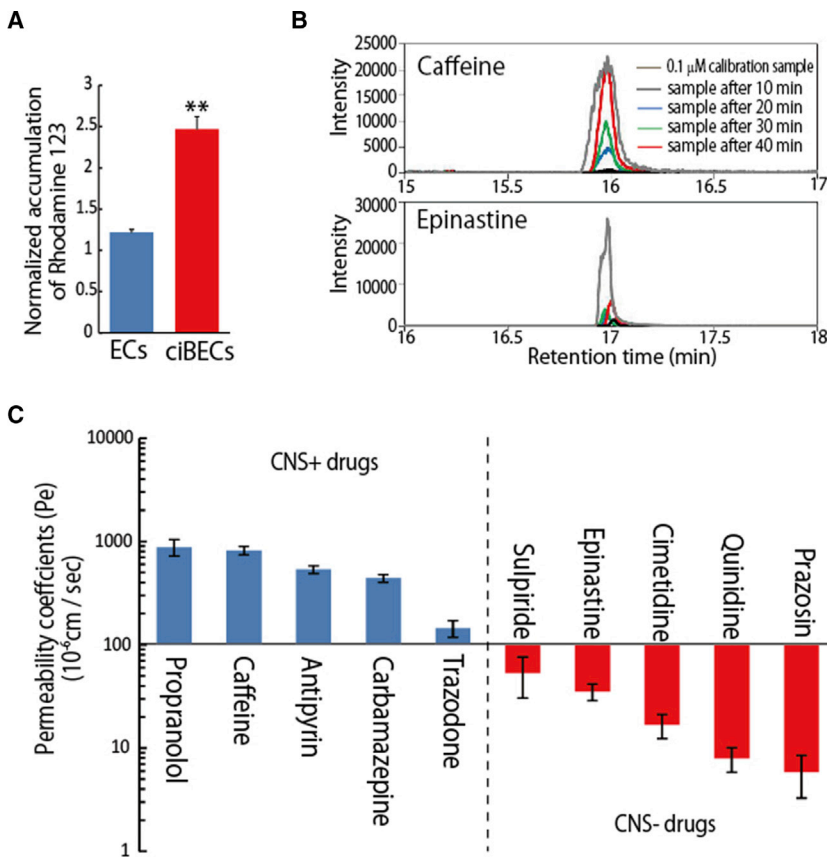


Figure 6. Screening System of Drug Permeability with BBB Model

(A) PGP functional efflux assay using rhodamine-123 at 7 days after plating onto a Transwell filter co-cultured with astrocytes. Cell accumulation ratio of rhodamine-123 with verapamil treatment and vehicle (0.5% DMSO) ($n = 3$ independent experiments; $**p < 0.01$ versus ECs).

(B) Representative results of nanoLC-MS/MS. Upper panel shows the intensity of caffeine; lower panel shows the intensity of epinastine. Gray data, 0.1 μ M calibration samples; black data, samples after 10 min; blue data, samples after 20 min; green data, samples after 30 min; red data, samples after 40 min.

(C) Drug permeability measured by nanoLC-MS/MS. We selected ten drugs with known permeability to test our BBB model ($n = 3$ independent experiments). CNS-positive drugs could cross the BBB into the CNS by lipid-mediated free diffusion. CNS-negative drugs had low BBB permeability for efflux transporter substrates.

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 (Alavijeh 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 RPMI + B27 medium with 100 ng/mL VEGF165 (Wako) and 1 mM 8-bromo-adenosine-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 hiPSCs-derived ECs, pericytes, astrocytes, and neurons. Astrocytes and neurons (total 3.5×10^6 cells) from day 90 to day 120 after differentiation were co-cultured on differentiated ECs and pericytes (about 3.5×10^6 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 about 49.5% CD31-positive ECs, 0.5% PDGFRβ-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% PDGFRβ-positive pericytes, 20% TUJ1-positive neurons, and 45% GFAP-positive astrocytes (Figures 1 and S1). ciBECs and ECs were purified with FACS AriaII (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



of AxioVision 4.7.1 Software (Carl Zeiss). The following primary antibodies were used for immunostaining: anti-mouse CD31 antibody (BBA7; R&D; 1:1,000), anti-rabbit CD31 antibody (ab134168; Abcam; 1:1,000), anti-VE-CADHERIN antibody conjugated with phycoerythrin (PE) (12-1449-80; BD Biosciences; 1:200), anti- α SMA antibody (A2547; Sigma-Aldrich; 1:1,000), anti-SM22 α antibody (ab10135; Abcam; 1:500), anti-CALPONIN (ab46794; Abcam; 1:500), anti-NG2 (14-6504-80; eBioscience; 1:500), anti-NESTIN antibody (MAB5326; Millipore; 1:500), anti-mouse TUJ1 antibody (MAB1637; Millipore; 1:1,000), anti-rabbit TUJ1 antibody (845501; Covance; 1:1,000), anti-GFAP antibody (IS524; Dako; 1:100), anti-BCRP antibody (MAB995; R&D; 1:200), anti-PGP antibody (MA1-26528; Thermo Fisher; 1:200), anti-NICD antibody (ab52301; Abcam; 1:300), anti-CLAUDIN5 antibody (352500; Life Technologies; 1:500), and anti-ZO1 antibody (339100; Novex; 1:500).

For FACS analysis, differentiated cells were stained with combinations of APC-conjugated PDGFR α (FAB1264A; BD Biosciences) and PE-conjugated KDR (CD309; Miltenyi Biotec), APC-conjugated CD31 (17-0319-42; BD Biosciences) and PE-conjugated PDGFR β (558821; BD Biosciences), or APC-conjugated PSANCAM (130-093-273; Miltenyi Biotec) and FITC-conjugated CD44 (338804; BD Biosciences), then subjected to analysis using FACS AriaII (Becton Dickinson). Negative controls used were non-staining cells or cells stained with APC-conjugated anti-rabbit IgG (711-136-152; Jackson ImmunoResearch) and PE-conjugated anti-mouse IgG (115-116-072; Jackson ImmunoResearch).

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 μ g/mL acetylated LDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL) (Biomedical Technologies) for 4 hr 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) at 12 days after differentiation were cultured in a 24-well plate (Life Technologies) coated with 150 μ L of Matrigel Basement Membrane Matrix GFR (BD Biosciences).

RNA Isolation and qRT-PCR

Total RNA was isolated from differentiated cells using Qiazol (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed with the SuperScript III first-strand synthesis system (Life Technologies). qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and a StepOnePlus system (Applied Biosystems). The amount of target RNA was normalized relative to the amount of 18S rRNA mRNA. Primer sequences are shown in Table S1.

DIII Knockdown Using siRNA

siRNA targeting human DIII No. 1, 5'-ACG GAU CUC GAG AAC AGC UAC UCC U-3', DIII No. 2, 5'-CGG ACU CGG GCU GUU CAA CUU CAA A-3', DIII No. 3, 5'-CGG GAU GGC GUG AAC GAC UUC UCC U-3', and negative control DIII, 5'-CCA CAA

GGA UGG GAA CGU CUU AGG A-3' were purchased from Life Technologies (Stealth RNAi). We checked knockdown efficiencies of the siRNA and selected siRNA targeting human DIII No. 2 (Figure S2). Stealth RNAi for DIII No. 2 (20 nM) or control (20 nM) were transfected into astrocytes and neurons before 2 days of co-culture with ECs and pericytes using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions. After co-culture day 5, differentiated cells were examined by qPCR and immunohistochemistry.

Generation of BBB Model

Purified CD31-positive ciBECs and ECs were suspended with SFM medium supplemented with 20 ng/mL bFGF, 10 ng/mL EGF, and 10 μ g/mL human plasma fibronectin. A total of 12×10^4 ciBECs were plated on 500 μ g/mL human plasma fibronectin-coated cell-culture inserts in the 24-well Transwell at 200 μ L/well (Costar; polyester membrane, 0.4 mm pore size). Pre-seeded astrocytes from D125 to D180 on the bottom side of the culture dish were switched from DMEM/Ham's F12 supplemented with N2 supplement into SFM medium supplemented with 20 ng/mL bFGF, 10 ng/mL EGF, and 10 μ g/mL human plasma fibronectin, and the inserts with ciBECs were added. After 3 days the medium was changed to minimum essential medium (Life Technologies) with 10% FBS (Life Technologies) and 100 ng/mL VEGF (Wako) for 2 days and then changed again to SFM medium supplemented with 20 ng/mL bFGF, 10 ng/mL EGF, and 10 μ g/mL human plasma fibronectin for 2 days, and were used for further experiments such as drug kinetics.

Measurement of Transendothelial Electrical Resistance

Cells were 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] \times 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 μ L 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 μ L of 2 μ M 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 μ L) 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 μ M 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 μ L 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 μ L of 4 μ M 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 (Rappsilber 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 μ m 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 μ L/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 ($m/z = 325.19105$), sulphiride ($m/z = 342.1482$), 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 \pm 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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Supplemental Information

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

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Inventory of Supplementary Information

1. Supplementary Figure S1-S6

Supplementary Figure S1. Cell populations of neurons and astrocytes – related to Figure 2 and 4.

Supplementary Figure S2. Role of cell types for generating ciBECs – related to Figure 2.

Supplementary Figure S3. ciBECs generation with 836B3 iPSC cell line and chimera differentiation assay with 201B6 and 836B3 iPSC cell lines – related to Figure 2.

Supplementary Figure S4. Effects of three *Dll1* siRNA on *Dll1* mRNA expression – related to Figure 4, and expression of CLAUDIN5 in EC junctions – related to Figure 5.

Supplementary Figure S5. Comparison of barrier function and PGP function with ECs, ciBECs, hCMEC/D3, and HUVEC– related to Figure 5 and 6, and DHA permeability measured by nanoLC-MS/MS – related to Figure 6.

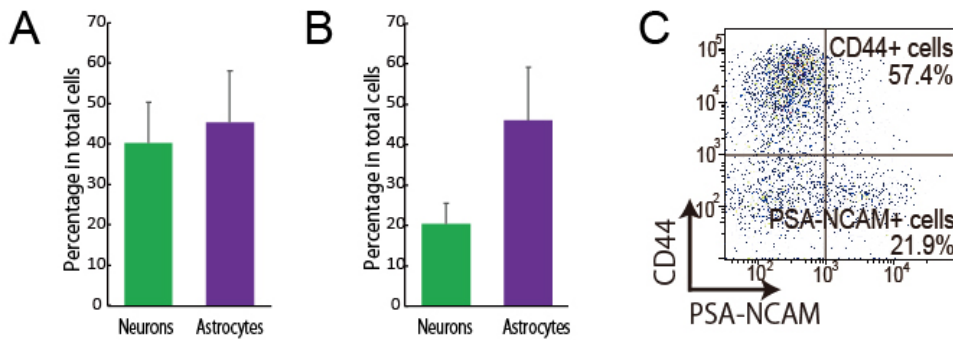
Supplementary Figure S6. Expression level of *Dll1*, *Wnt7a*, *Wnt7b*, *GDNF*, *bFGF*, *EGF*, and *Ang1* mRNA on ECs, pericytes, neurons, and astrocytes, or during cell differentiation

2. Supplementary Table

Supplementary Table 1. Primer list for qPCR.

3. Supplemental experiment procedures

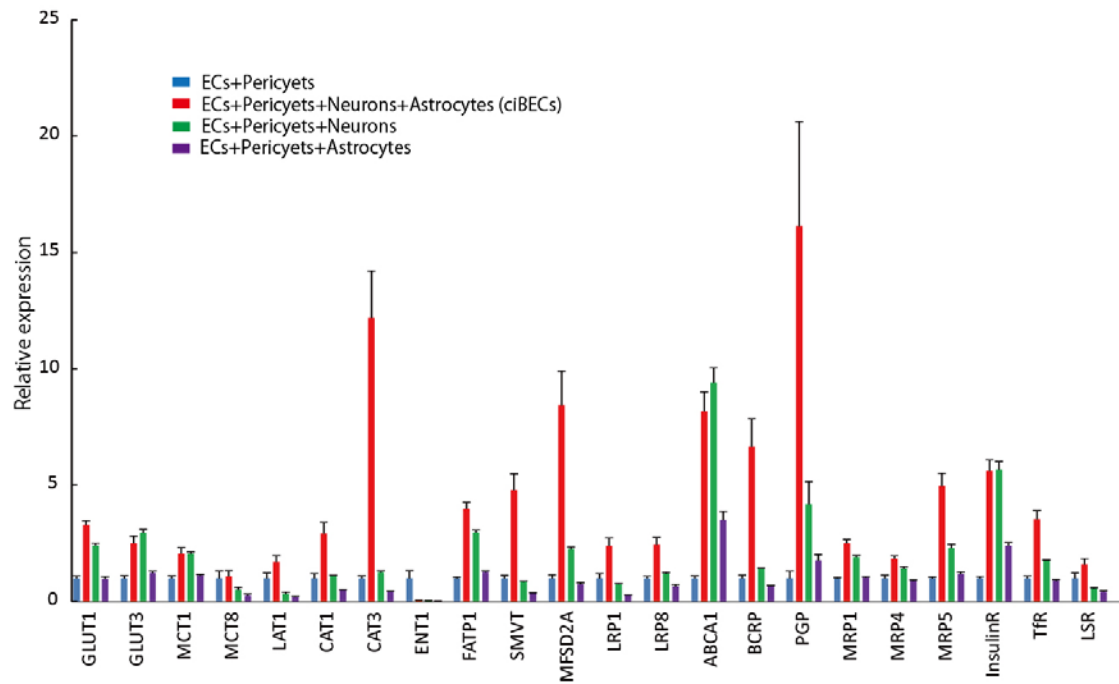
Figure S1 Yamamizu K.



Supplementary Figure 1 Cell populations of neurons and astrocytes – related to Figure 2 and 4.

(A) Percentage of TUJ1 positive neurons and GFAP positive astrocytes from 90 days to 120 days (n=3, independent experiments). (B) Percentage of TUJ1 positive neurons and GFAP positive astrocytes after co-culture with ECs and pericytes for 5 days (n=3, independent experiments). (C) FACS analysis for PSA-NCAM positive neurons and CD44 positive astrocytes at 100 days after differentiation. Percentage of PSA-NCAM positive neurons and CD44 positive astrocytes in total cells are indicated.

Figure S2 Yamamizu K.

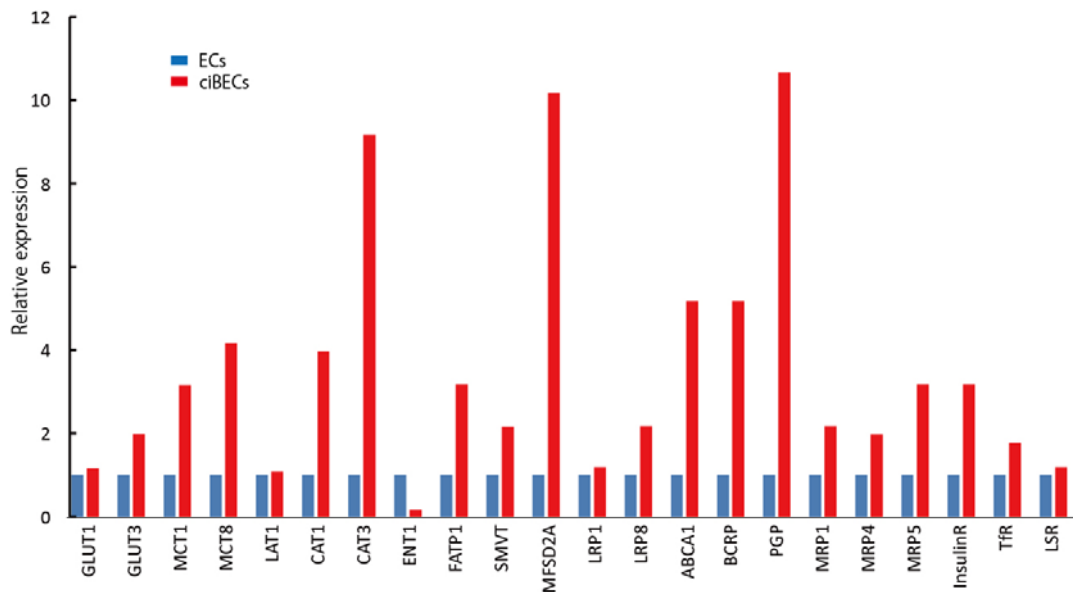


Supplementary Figure 2 Role of cell types for generating ciBECs – related to Figure 2.

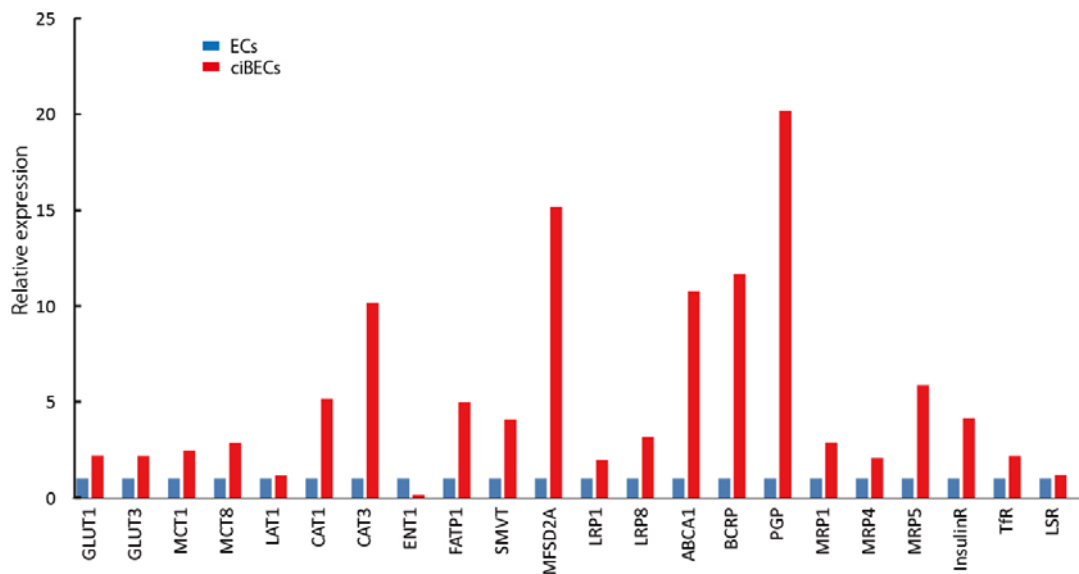
qPCR for the mRNA expressions of BBB specific transporters and receptors in purified CD31 positive ECs and ciBECs after co-culture with neurons and/or astrocytes (n=3, independent experiments).

Figure S3 Yamamizu K.

A



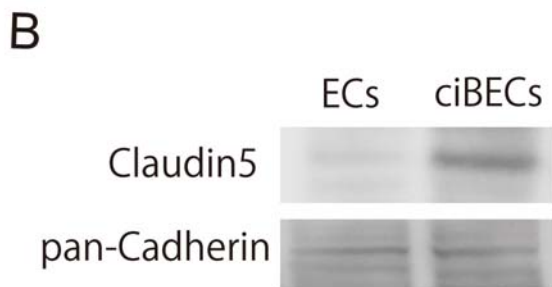
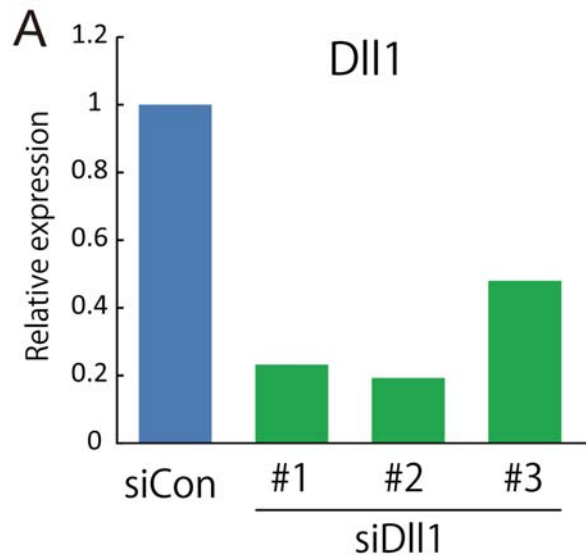
B



Supplementary Figure 3 ciBECs generation with 836B3 iPSC cell line and chimera differentiation assay with 201B6 and 836B3 iPSC cell lines – related to Figure 2.

qPCR for the mRNA expressions of BBB specific transporters and receptors in purified CD31 positive ECs and ciBECs. (A) 863B3 cell line (n=2, independent experiments). (B) ECs and pericytes derived from 863B3 were co-cultured with neurons and astrocytes derived from 201B6 (n=2, independent experiments).

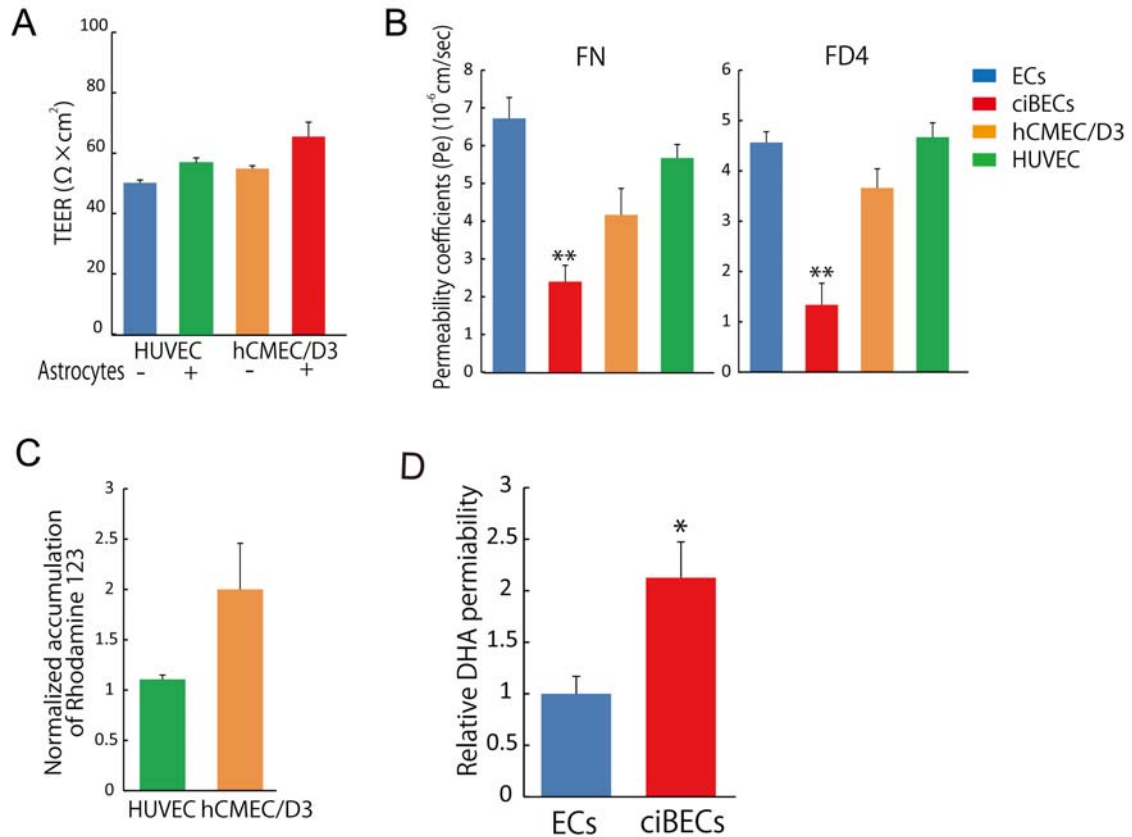
Figure S4 Yamamizu K.



Supplementary Figure 4 Effects of three Dll1 siRNA on *Dll1* mRNA expression – related to Figure 4, and expression of CLAUDIN5 in EC junctions – related to Figure 5.

(A) Quantitative RT-PCR showing mRNA expression of *Dll1* in neurons and astrocytes treated with Dll1 siRNA after 2 days. Dll1 mRNA expression treated siControl was set as 1.0. (n=1) (B) Western blotting for CLAUDIN5 and pan-CADHERIN using the purified membrane protein from ECs and ciBECs.

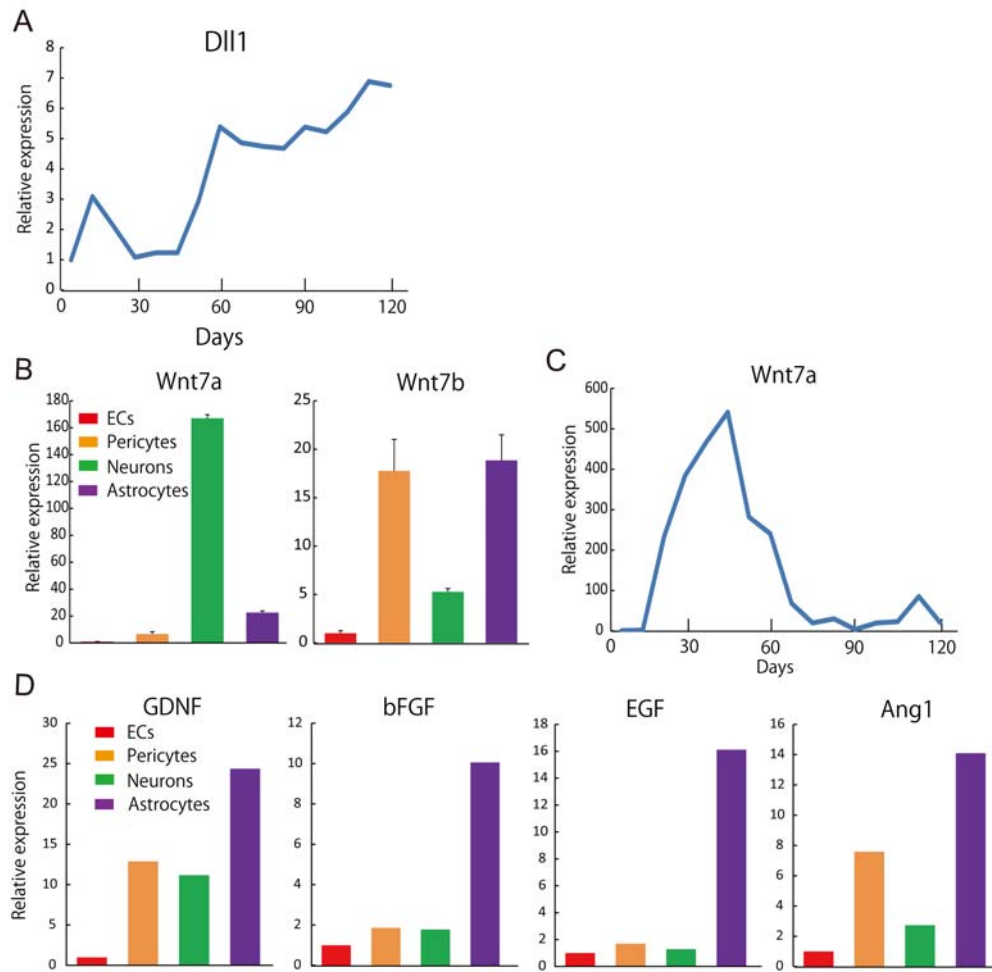
Figure S5 Yamamizu K.



Supplementary Figure 5 Comparison of barrier function and PGP function with ECs, ciBECs, hCMEC/D3, and HUVEC – related to Figure 5 and 6, and DHA permeability measured by nanoLC-MS/MS – related to Figure 6.

(A) Measurement of TEER at 3 days after plating onto the Transwell filter with HUVEC and hCMEC/D3. (B) Permeability assay using Fluorescein-Na and 4 kDa FITC-labelled dextran (n=3 (independent experiments); **P<0.01 vs. ECs). (C) PGP functional efflux assay using rhodamine123. Cell accumulation ratio of rhodamine123 with verapamil treatment and vehicle (0.5% DMSO). (D) DHA permeability measured by nanoLC-MS/MS. DHA permeability in ECs was set as 1.0 (n=3, independent experiments).

Figure S6 Yamamizu K.



Supplementary Figure 6 Expression level of *Dll1*, *Wnt7a*, *Wnt7b*, *GDNF*, *bFGF*, *EGF*, and *Ang1* mRNA on ECs, pericytes, neurons, and astrocytes, or during cell differentiation

(A) Quantitative RT-PCR showing mRNA expression of *Dll1* during neurons and astrocytes differentiation. *Dll1* mRNA expression in undifferentiated hiPSCs was set as 1.0 (n=1). (B) Quantitative RT-PCR showing mRNA expression of *Wnt7a* and *Wnt7b* in ECs, pericytes, neurons, and astrocytes. *Wnt7a* and *Wnt7b* mRNA expression in ECs was set as 1.0 (n=3, independent experiments). (C) Quantitative RT-PCR showing mRNA expression of *Wnt7a* during neurons and astrocytes differentiation. *Wnt7a* mRNA expression in undifferentiated hiPSCs was set as 1.0 (n=1). (D) Quantitative RT-PCR showing mRNA expression of *GDNF*, *bFGF*, *EGF*, and *Ang1* in ECs, pericytes, neurons, and astrocytes. *GDNF*, *bFGF*, *EGF*, and *Ang1* mRNA expression in ECs was set as 1.0 (n=1).

Supplementary Table 1: Primer list for qPCR

Gene	Sequence
RPS18 Forward	Actcaacacgggaaacctca
Reverse	aaccagacaaatcgctccac
Oct3/4 Forward	cgagaggattttgaggctgc
Reverse	cgaggagtacagtgcagtga
Pax6 Forward	cagacacagccctcacaac
Reverse	ggagtatgaggaggtctggc
MAP2 Forward	actcctggaaccctagcta
Reverse	tgggagtcgcaggagatfff
GFAP Forward	gatcaactcaccgccaacag
Reverse	ataggcagccaggttgttct
GLUT1 Forward	ggccatctttctgttgggg
Reverse	ccagcaggttcacatcagc
GLUT3 Forward	ctgggcatcgttgttggaa
Reverse	agggctgcactttgtaggat
MCT1 Forward	cttgggcttccttcaact
Reverse	gtacagaggaacacagggct
MCT8 Forward	tgggtgctcttggtgtgat
Reverse	aggagcaggaaggaaaggac
LAT1 Forward	gtccctgttcacatcctcca
Reverse	tagagcagcgtcatcacaca
CAT1 Forward	ccctactctgcctggacaa
Reverse	ataaccgaggcattgggaaa
CAT3 Forward	ttgggtctggatgctgatt
Reverse	ttgctctagacttgcgtga
ENT1 Forward	gcacctgggaacgttacttc
Reverse	ccaggccacatgaatacagc
FATP1 Forward	ctcggcaggaacatcatcg
Reverse	gatgtactgaaccaccgtgc
SMVT Forward	gctctaccatgcttgcgtg
Reverse	tagatctctgacggcacacc
ABCA1 Forward	agccctggatgtacaacgaa
Reverse	ttcctccatacagcgggt

MFSD2A	Forward	cctatgaagcccagcagtct
	Reverse	aaggaggtgaagaggaagcc
LRP1	Forward	caacaacacctcagtctgcc
	Reverse	gcagccaccgttattcagag
LRP8	Forward	ctacctggctacgagatgg
	Reverse	tcttgagcatggggatgagg
BCRP	Forward	ctcttcggcttgaacaact
	Reverse	ttctctccagacacaccac
PGP	Forward	cctcaccaggcaatgatg
	Reverse	gcaccaaagacaacagctga
MRP1	Forward	caaggtggatgcaatgagg
	Reverse	tgaggaagtagggcccaag
MRP4	Forward	tcccatggattctgtggctt
	Reverse	actcgtaacctcatcccagc
MRP5	Forward	tgtccaagcacgagtcttct
	Reverse	gcagaagatccacacaacc
InsulinR	Forward	gacaacgaggagtgtggaga
	Reverse	tacagatggtcgggcaaact
TfR	Forward	agcccactgtttatagct
	Reverse	tttctcaactttgctggccc
LSR	Forward	ctggagacgttgacaggagt
	Reverse	tgtagtacaggacccgcatg
CD31	Forward	attgcagtggttatcatcggagt
	Reverse	ctggttgttgagttcagaagtg
SM22 α	Forward	aatggcgtgattctgagcaagc
	Reverse	agtcttgatgacccatagtctctc
Notch1	Forward	atgcagaacaacaggaggga
	Reverse	tatgatccgtgatgtcccgg
Notch2	Forward	ccaggttttgaaggagcac
	Reverse	agcggcagttgtaagtgttg
Notch3	Forward	accgatgtcaacgagtgtct
	Reverse	ttctgtgaagcctgccata
Notch4	Forward	tcagccaccagtgcagaat
	Reverse	gggtctcacactcatccaca
Dll1	Forward	cagaaagactcatcagccgc
	Reverse	cagccctctccgtagtagtg

Dll4	Forward	cgctactcttaccgggtcat
	Reverse	atctggctggcacacatagt
Jagged1	Forward	cctgaaggggtgcggtatat
	Reverse	catggcagtatgttcccgtg
Jagged2	Forward	caaccctgtgtgaatggtg
	Reverse	attgtagcaaggcagagggt
Wnt7a	Forward	ttcgggaaggagctcaaagt
	Reverse	ccttgcttctttgtcgca
Wnt7b	Forward	tactacaaccaagccgaggg
	Reverse	tcttcttgatctcccagcg

EXPERIMENTAL PROCEDURES

Co-culture ECs and pericytes with neurons or astrocytes

To investigate the roles of different cell types in the generation of ciBECs, neurons (3.5×10^6 cells) from 50 days to 65 days after differentiation (Stage 3), neurons and astrocytes (total 3.5×10^6 cells) from 90 days to 120 days after differentiation (Stage 4), or astrocytes (3.5×10^6 cells) from 210 days to 240 days after differentiation (Stage 5) were co-cultured on differentiated ECs and pericytes (about 3.5×10^6 cells) at day 7 in serum free medium (SFM) (Life Technologies) supplemented with 20 ng/ml bFGF (Wako Pure Chemicals Industries), 10 ng/ml EGF (Life Technologies), and 10 μ g/ml human plasma fibronectin (Life Technologies) for 5 days. We refreshed the medium every 3 days. ECs were purified with FACS AriaII (Becton Dickinson) using anti-CD31 (PECAM1) conjugated with APC and analyzed BBB-specific transporters and receptors with qPCR.

Subcellular proteome extraction and Western blotting for CLAUDIN5

Membrane protein was isolated from ciBECs and ECs using ProteoExtract Subcellular Proteome Extraction kit (Calbiochem), according to the manufacturer's instructions. Membrane protein were subjected to SDS/polyacrylamide gel electrophoresis using

gradient gels (Life Technologies) followed by electrophoretic transfer onto nitrocellulose membranes. The blots were incubated for 1 hr in Blocking One blocking agents (Nacalai Tesque). Then, the membranes were incubated overnight with the respective first antibodies (1:1000) for CLAUDIN5 (352500; Life Technologies) and pan-CADHERIN (Sigma). Horseradish peroxidase (HRP)-conjugated anti-mouse Ig antibody (PI-1000; Zymed Laboratories, San Francisco, CA, USA) was used as the secondary antibody (1:10,000). Can Get Signal Immunoreaction Enhancer Solution kit (Toyobo, Osaka, Japan) was used to enhance the signal. Immunoreactivity was detected with Immobilon Western (Millipore).

Analysis of DHA permeability using nanoLC-MS/MS

Samples were desalted with an ODS column and suspended in methanol for subsequent LC-MS analysis. LC-MS analysis was performed using a TripleTOF 4600 System (AB Sciex, Foster City, CA). Loaded samples were separated on a COSMOSIL 3C18 AR2 packed column (50 mm length, 20 mm i.d.) using a Prominence UFLC XR System (SHIMADZU, Kyoto, Japan). The mobile phases consisted of (A) water with 0.1% formic acid and (B) 100% methanol with 0.1% formic acid. The linear gradient condition was 5–100% B for 10 min, 100% B for 2 min and 5% B for 3 min at a flow rate of 300 μ L/min. A spray voltage of -4500 V was applied. The MS scan range was

300–450 m/z every 0.25 s. All raw data files from the TripleTOF 4600 system were analyzed by PeakView software version 1.2 (AB Sciex, Foster City, CA, USA). Extracted-ion chromatograms (XIC) were observed with ± 0.004 m/z width for DHA (m/z = 327.2331).