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Supplemental Information

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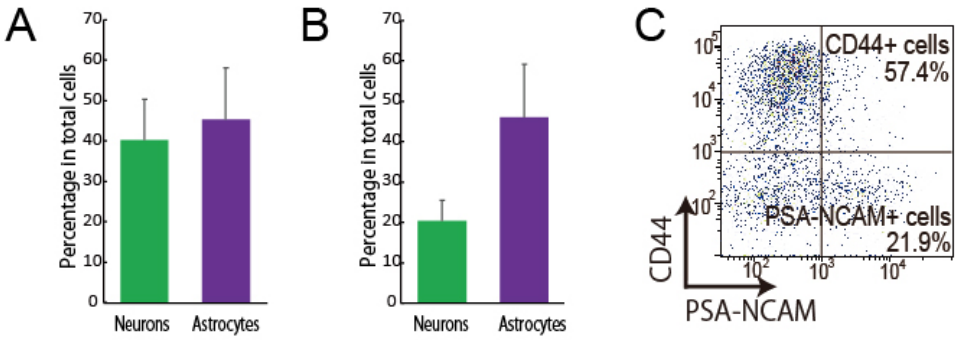
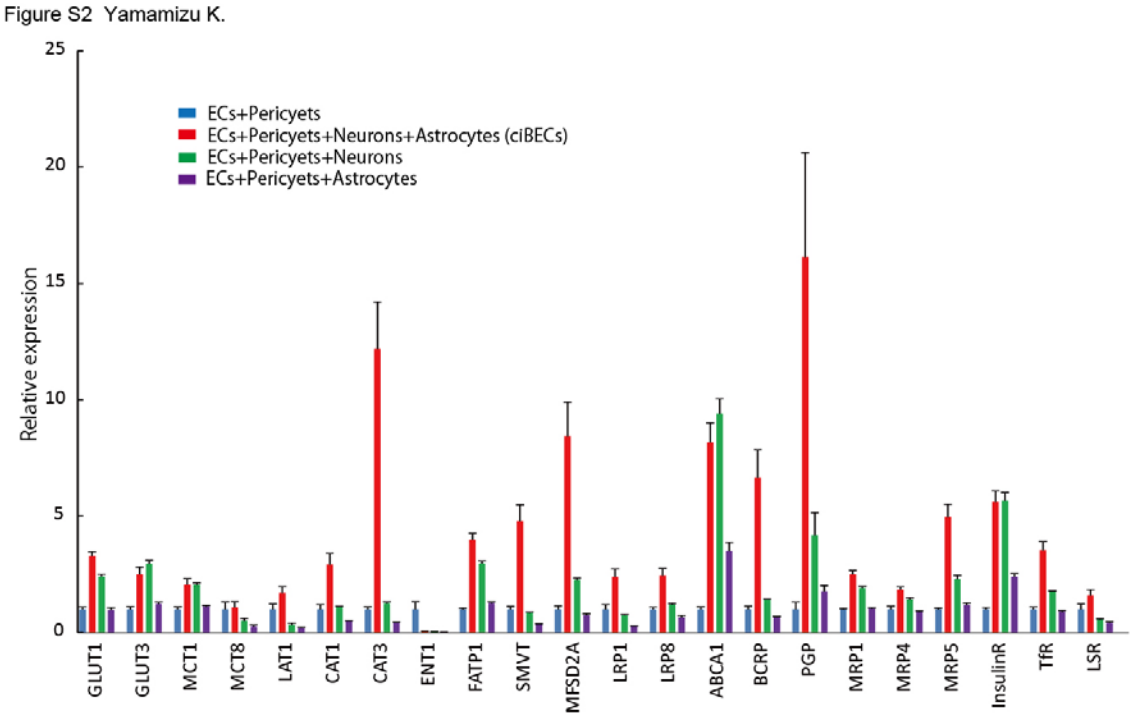
Inventory of Supplementary Information

Figure S1 Yamamizu K.



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

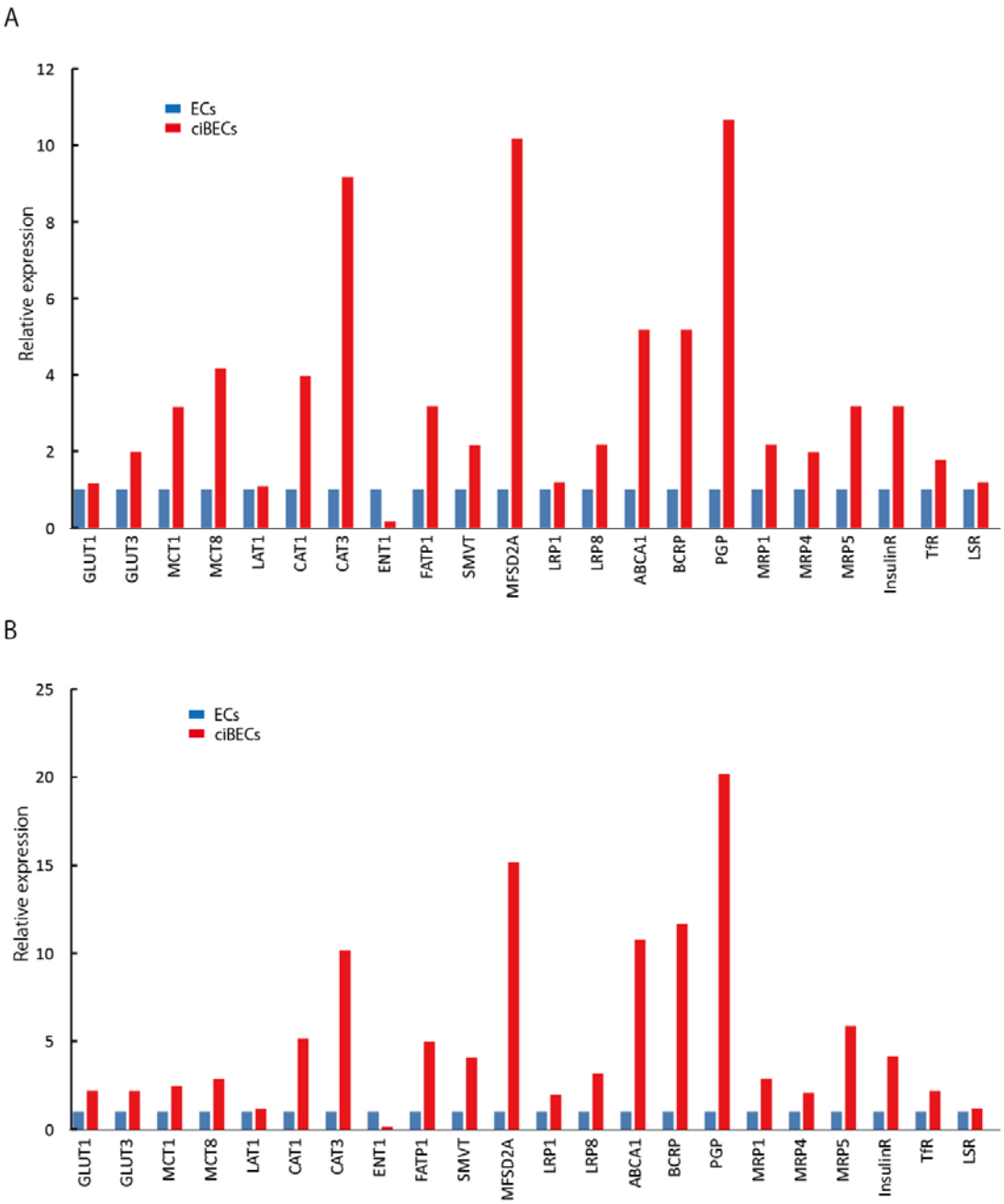
(A) Percentage of TUJ1 positive neurons and GFAP positive astrocytes from 90 days to 120 days (n=3, independent experiments). (B) Percentage of TUJ1 positive neurons and GFAP positive astrocytes after co-culture with ECs and pericytes for 5 days (n=3, independent experiments). (C) FACS analysis for PSA-NCAM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



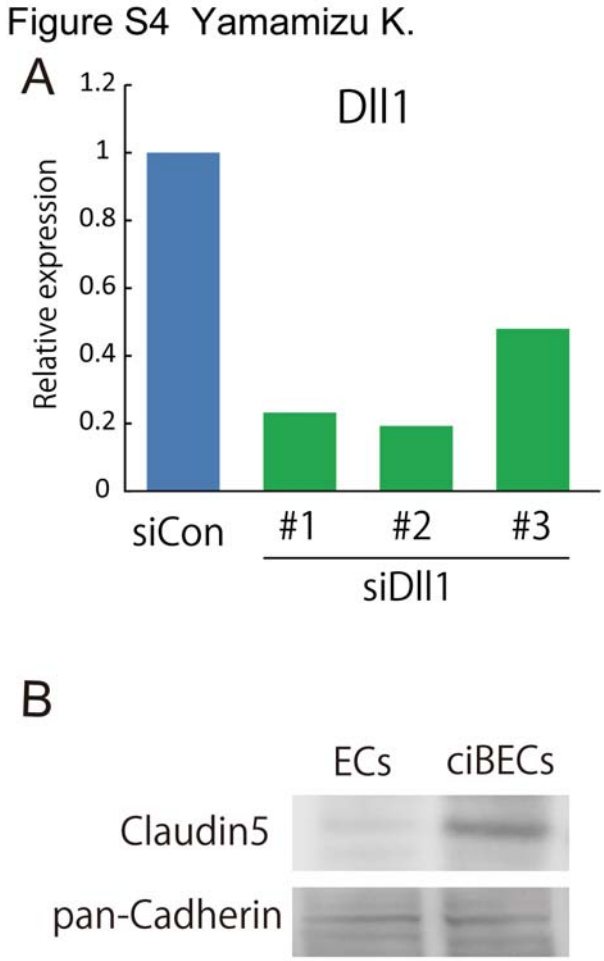
Supplementary Figure 2 Role of cell types for generating ciBECs – related to Figure 2.

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.

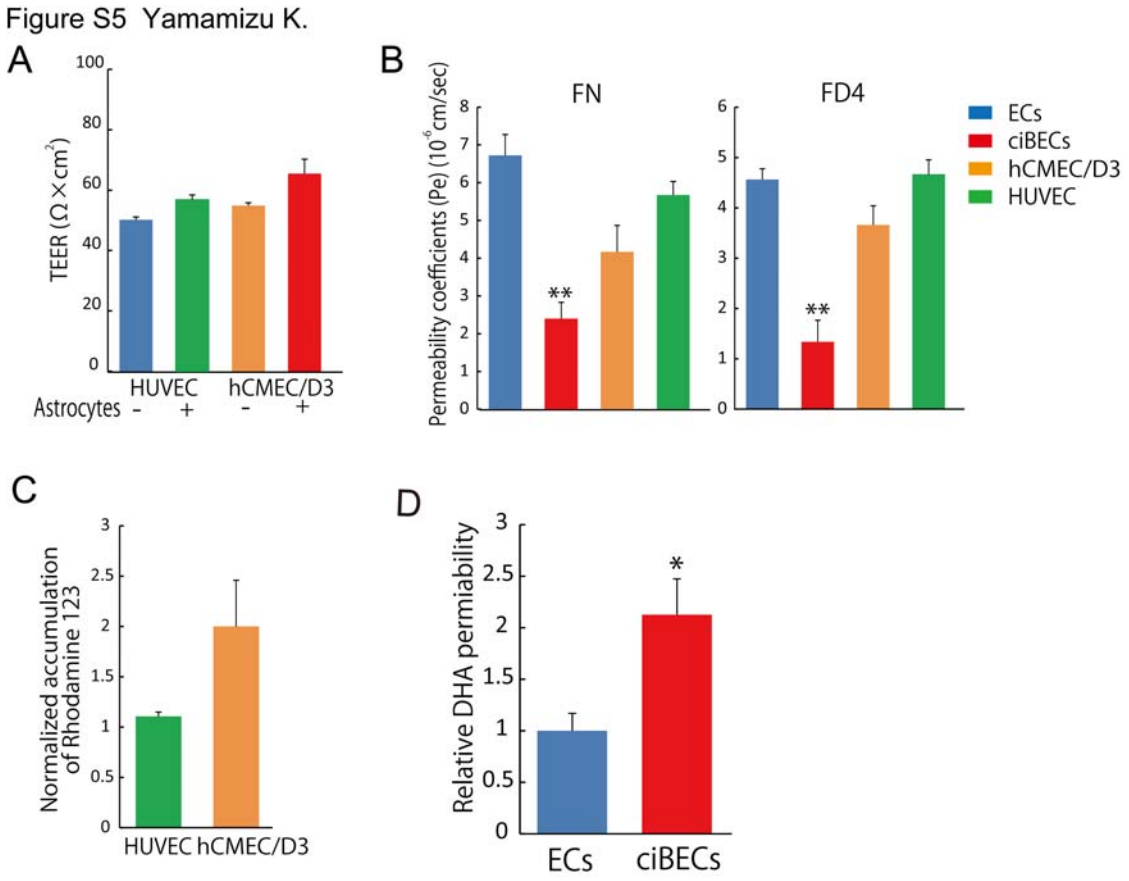


Supplementary Figure 3 ciBECs generation with 836B3 iPSC cell line and chimera differentiation assay with 201B6 and 836B3 iPSC cell lines – related to Figure 2. qPCR for the mRNA expressions of BBB specific transporters and receptors in purified CD31 positive ECs and ciBECs. (A) 863B3 cell line (n=2, independent experiments). (B) ECs and pericytes derived from 863B3 were co-cultured with neurons and astrocytes derived from 201B6 (n=2, independent experiments).



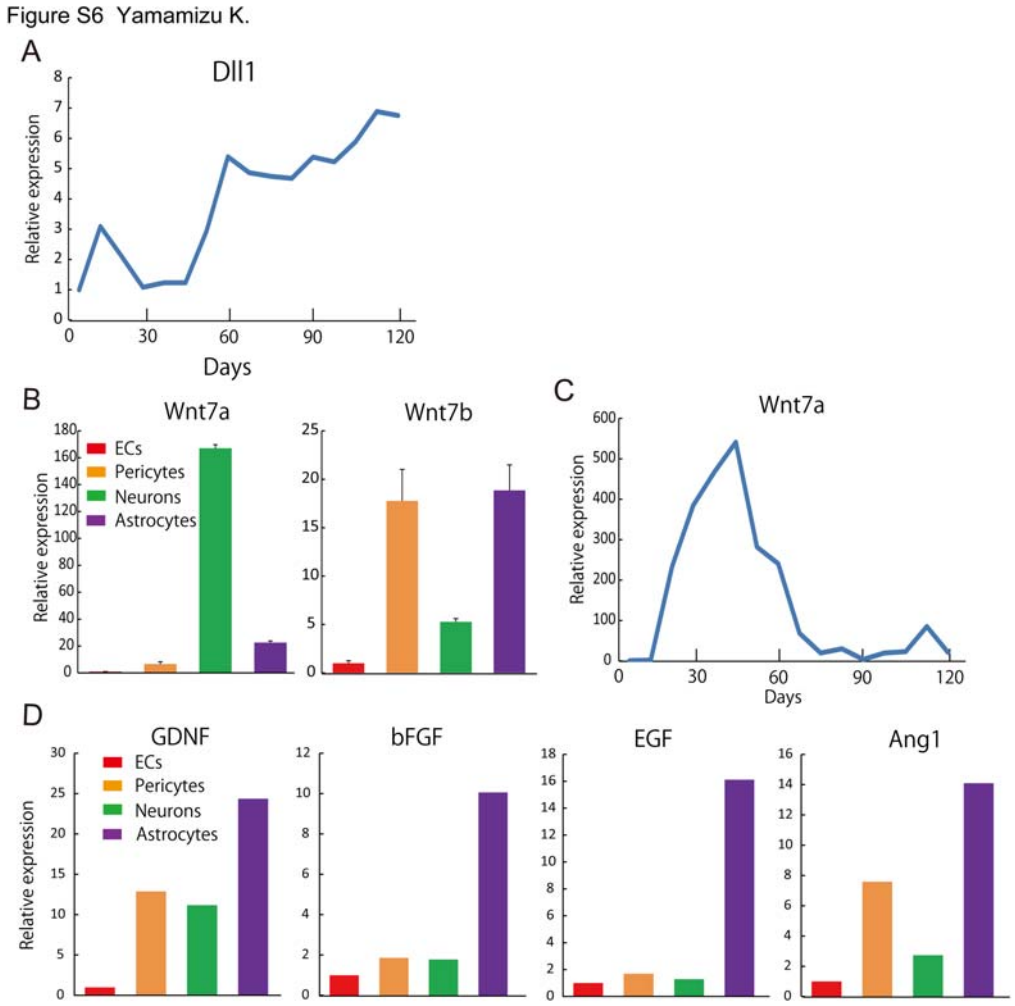
Supplementary Figure 4 Effects of three Dll1 siRNA on *Dll1* mRNA expression – related to Figure 4, and expression of CLAUDIN5 in EC junctions – related to Figure 5.

(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.



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 Fluoresein-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).



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	cagacacagccctcacaaac
	Reverse	ggagtatgaggaggtctggc
MAP2	Forward	actcctggaacccctagcta
	Reverse	tgggagtcgcaggagatttt
GFAP	Forward	gatcaactcaccgccaacag
	Reverse	ataggcagccaggttgttct
GLUT1	Forward	ggccatcttttctgttgggg
	Reverse	ccagcaggttcatcatcagc
GLUT3	Forward	ctgggcatcgttgttggaat
	Reverse	agggctgcactttgtaggat
MCT1	Forward	cttgggcttgccttcaactt
	Reverse	gtacagaggaacacagggct
MCT8	Forward	tgggtgctcttggtgtgtat
	Reverse	aggagcaggaaggaaaggac
LAT1	Forward	gtccctgttcacatcctcca
	Reverse	tagagcagcgtcatcacaca
CAT1	Forward	ccctacttctgcctggacaa
	Reverse	ataacccgaggcatgggaaa
CAT3	Forward	ttggggtctggatgctgatt
	Reverse	ttggctctagacttgcgtga
ENT1	Forward	gcacctgggaacgttacttc
	Reverse	ccaggccacatgaatacagc
FATP1	Forward	ctcggcaggaaacatcatcg
	Reverse	gatgtactgaaccaccgtgc
SMVT	Forward	gctctaccatgcttgtcgtg
	Reverse	tagatctctgacggcacacc
ABCA1	Forward	agccctggatgtacaacgaa
	Reverse	tttccttccatacagcgggt

MFSD2A	Forward	cctatgaagcccagcagtct
	Reverse	aaggaggtgaagaggaagcc
LRP1	Forward	caacaacacctcagtctgcc
	Reverse	gcagccaccgttattcagag
LRP8	Forward	ctaccctggctacgagatgg
	Reverse	tcttgagcatggggatgagg
BCRP	Forward	ctcttcggcttgcaacaact
	Reverse	ttctcctccagacacaccac
PGP	Forward	ccttcacccaggcaatgatg
	Reverse	gcaccaaagacaacagctga
MRP1	Forward	caaggtggatgcgaatgagg
	Reverse	tgaggaagtagggcccaaag
MRP4	Forward	tcccatggattctgtggctt
	Reverse	actcgtaacctcatcccagc
MRP5	Forward	tgtccaagcacgagtcttct
	Reverse	gcagaagatccacacaaccc
InsulinR	Forward	gacaacgaggagtgtggaga
	Reverse	tacagatggtcgggcaaact
TfR	Forward	agcccactgttgtatacgct
	Reverse	tttctcaactttgctggccc
LSR	Forward	ctggagacgttgacaggagt
	Reverse	tgtagtacaggacccgcatg
CD31	Forward	attgcagtggttatcatcggagt
	Reverse	ctggttgttggagttcagaagtg
SM22α	Forward	aatggcgtgattctgagcaagc
	Reverse	agtcttgatgaccccatagtcctc
Notch1	Forward	atgcagaacaacagggagga
	Reverse	tatgatccgtgatgtcccgg
Notch2	Forward	ccaggttttgaagggagcac
	Reverse	agcggcagttgtaagtgttg
Notch3	Forward	accgatgtcaacgagtgtct
	Reverse	ttcctgtgaagcctgccata
Notch4	Forward	tcagccaccagtgtcagaat
	Reverse	gggtctcacactcatccaca
Dll1	Forward	cagaaagactcatcagccgc
	Reverse	cagccctctccgtagtagtg

Dll4	Forward	cgctactcttaccgggtcat
	Reverse	atctggctggcacacatagt
Jagged1	Forward	cctgaaggggtgcggtatat
	Reverse	catggcagtatgttcccgtg
Jagged2	Forward	caacccctgtgtgaatggtg
	Reverse	attgtagcaaggcagagggt
Wnt7a	Forward	ttcgggaaggagctcaaagt
	Reverse	ccttgcttctctttgtcgca
Wnt7b	Forward	tactacaaccaagccgaggg
	Reverse	tcttcttgatctcccgagcg

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 μg/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 Subcelluar Proteome Extraction kit (Calbiochem), according to the manufacturer's instructions. Membrane protein were subjected to SDS/polyacrylamide gel electophoresis 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 μL/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).