Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells

Ethan S Lippmann1,3, Samira M Azarin1,3, Jennifer E Kay1, Randy A Nessler2, Hannah K Wilson1, Abraham Al-Ahmad1, Sean P Palecek1 & Eric V Shusta1

The blood-brain barrier (BBB) is crucial to the health of the brain and is often compromised in neurological disease. Moreover, because of its barrier properties, this endothelial interface restricts uptake of neurotherapeutics. Thus, a renewable source of human BBB endothelium could spur brain research and pharmaceutical development. Here we show that endothelial cells derived from human pluripotent stem cells (hPSCs) acquire BBB properties when co-differentiated with neural cells that provide relevant cues, including those involved in Wnt/β-catenin signaling. The resulting endothelial cells have many BBB attributes, including well-organized tight junctions, appropriate expression of nutrient transporters and polarized efflux transporter activity. Notably, they respond to astrocytes, acquiring substantial barrier properties as measured by transendothelial electrical resistance (1,450 ± 140 Ω cm²), and they possess molecular permeability that correlates well with in vivo rodent blood-brain transfer coefficients.

The BBB is composed of specialized brain microvascular endothelial cells (BMECs) that help regulate the flow of substances into and out of the brain. Complex intercellular tight junctions limit the passive diffusion of molecules into the brain and result in blood vessels exhibiting extremely high trans-endothelial electrical resistance (TEER) in vivo. In addition, efflux transporters, such as p-glycoprotein, contribute to barrier properties by returning small lipophilic molecules that diffuse into BMECs back to the bloodstream. Thus, BMECs are endowed with a network of specific transport systems to shuttle essential nutrients and metabolites across the BBB. In addition, because of its substantial barrier properties, the BBB prevents uptake of most small-molecule and virtually all biologic pharmaceuticals delivered intravenously, hampering the development of drugs for neurological disease. Conversely, BBB breakdown and dysfunction is associated with various diseases, including Alzheimer’s disease, stroke, multiple sclerosis and brain tumors. These considerations have led researchers to develop a variety of BBB models to enable detailed mechanistic studies and drug screens in vitro.

Most in vitro BBB models have been established using brain microvessels isolated from primary animal sources such as cow, pig, rat and mouse. However, given inevitable species differences, a robust in vitro BBB model of human origin would be of great utility for high-throughput screening to identify brain-penetrating molecules or for the study of BBB developmental, regulatory and disease pathways in humans. Human BBB models have been established by culturing primary human (BMECs) isolated from autopsy tissue or, more often, from freshly resected brain specimens derived from tumor or epilepsy patients. Issues involving BMEC availability and fidelity limit widespread use of these human BBB models. Another proposed route toward a human BBB model is cell immortalization. However, immortalized BMECs have poor barrier properties, including low baseline transendothelial electrical resistance (TEER) and discontinuous tight junction protein expression. We sought to create a robust, scalable human BBB model using hPSCs, including both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), which exhibit virtually unlimited self-renewal and can differentiate into somatic cell types from all three embryonic germ layers. Human endothelial cells have been generated from hPSCs by a variety of methods, including embryoid body differentiation and OP9 stromal cell coculture. However, endothelial cells develop distinct gene and protein expression profiles that depend on microenvironmental cues during organogenesis, and hPSC-derived endothelial cells with organ-specific properties have yet to be reported.

Here we describe a facile hPSC differentiation method that can reproducibly generate pure populations of endothelial cells with BBB properties. The method employs simultaneous endothelial and neural cell co-differentiation followed by purification of the BBB-like endothelial population on selective matrix. The purified endothelial cells express BBB markers, respond to astrocytic cues, and have barrier and transport properties similar to those of primary BMECs.

RESULTS

Differentiation of hPSCs into BBB endothelial cells

In vivo, BBB specification begins as endothelial cells forming the perineurval vascular plexus invade an embryonic brain microenvironment comprising neuroepithelial cells, radial glia, neuroblasts and neurons. Notably, much of this early BBB induction occurs in the absence of astrocytes. The cells of the developing embryonic brain provide relevant BBB induction cues, such as Wnt7a and...
Wnt7b, that signal through the canonical Wnt-β-catenin pathway to help promote BBB specification of endothelial cells.26,27 We therefore hypothesized that a strategy that simultaneously differentiates hPSCs to both neural and endothelial lineages could lead to hPSC-derived endothelial cells with BBB attributes. Both neural and endothelial cells can be generated from pluripotent stem cells by directed differentiation in adherent culture.28–30 Thus, we developed a custom two-dimensional hPSC differentiation strategy that promotes neural and endothelial co-differentiation, in essence providing a microenvironment resembling the embryonic brain in vitro. The strategy was initially implemented using the IMR90-4 hiPSC line13, and protocol robustness was subsequently validated with multiple hiPSC and hESC lines listed. Table 1. UM, purification. hiPSC and hESC lines used in this study and their descriptions are listed. UM, unconditioned medium; EC, endothelial cell medium. (b) βIII tubulin (red) and nestin (green) expression was detected after differentiation of IMR90-4 hiPSCs in unconditioned medium for 4 d (i) and 6 d (ii). Scale bars, 50 μm. (c) Flow cytometric distributions of IMR90-4-derived βIII tubulin+ and nestin+ cells at days 4 and 6 of unconditioned medium treatment. Red dots, βIII tubulin+nestin+ cells; blue dots, βIII tubulin−nestin− cells; green dots, βIII tubulin−nestin+ cells; black dots, βIII tubulin+nestin− cells. Data are representative of two biological replicates. (d) Phase contrast image of IMR90-4 hiPSCs after 3 d in unconditioned medium (left) and 6 d in unconditioned medium with 3 additional days in endothelial cell medium (right). Circle at left, small region with flattened cobblestone endothelial cell morphology typical of region probed with antibodies in e. Morphology at right is widespread and corresponds to regions identified by immunolabeling (f and Supplementary Fig. 3). Scale bars, 200 μm. (e) IMR90-4 hiPSCs cultured for 4 d in unconditioned medium gave rise to PECAM-1+ cells (left) that did not express tight protein claudin-5 (right). Scale bars, 50 μm. (f) After 5–7 d of unconditioned medium treatment, IMR90-4-derived endothelial cells coexpressed PECAM-1 (i, red) and claudin-5 (ii, green, same field). Within these endothelial cell colonies, expression of characteristic BBB markers occludin (iii), p-glycoprotein (iv) and GLUT-1 (v) was also observed. All scale bars, 50 μm. (g) Flow cytometry dot plots of temporal evolution of PECAM-1+GLUT-1+ population within differentiating IMR90-4 hiPSCs or H9 hESCs. Green dots, PECAM-1+GLUT-1− cells; blue dots, PECAM-1+GLUT-1− cells; red dots, PECAM-1+GLUT-1+ cells. Full quantitative results are in Table 1.
We quantified the population of hPSC-derived BMECs by flow cytometry. For this analysis, we defined BMECs as cells having both PECAM-1 expression and elevated GLUT-1 expression compared with the low basal GLUT-1 expression exhibited by cells during the first 4 d of differentiation (GLUT-1+PECAM-1+, see Online Methods for gating details). For IMR90-4-derived BMECs, this population comprised ~30% of the cultures at day 6 of unconditioned medium treatment (Fig. 1g and Table 1). Thus, the neural and endothelial populations combined represented ~95% of the total differentiating culture at day 6 of unconditioned medium treatment. This hPSC-derived BMEC population was further expanded for 2 d in a custom endothelial cell medium that included factors known to facilitate primary BMEC growth with some selectivity (basic fibroblast growth factor (bFGF) and platelet-poor plasma derived serum32), and the percentage of GLUT-1+PECAM1+ cells in the differentiating IMR90-4 culture increased to 66% (Fig. 1d, right and g, d unconditioned medium, 2 d endothelial cell medium, and Table 1). The BMEC differentiation process yielded on average 11.6 PECAM-1+GLUT-1+ cells per input hPSC after 6 d in unconditioned medium and 2 d in endothelial cell medium, indicating proliferation of endothelial cell progenitors and/or endothelial cells during differentiation. At this point, all GLUT-1+PECAM1+ cells coexpressed the requisite BBB markers (Supplementary Fig. 3). Moreover, in endothelial cell medium, the hPSC-derived BMECs exhibited a commensurate increase in BBB properties as indicated by substantially increased expression of GLUT-1 protein (Table 1). In contrast, if instead the cultures were grown for two additional days in unconditioned medium (8 d unconditioned medium) rather than in endothelial cell medium, the percentage of IMR90-4 derived BMECs increased to a lesser extent although the total PECAM-1+ population increased to similar numbers (Fig. 1g and Table 1), indicating the importance of the endothelial cell medium treatment for selective BMEC expansion and conferral of BBB phenotypes on the endothelial cell population. Similar results were observed for the additional hiPSC33 and hESC11 lines tested (Fig. 1a), with cell line-dependent BMEC differentiation efficiency (Supplementary Figs. 4–6 and Table 1). For the H9 hESC line, extended culture (7 d for unconditioned medium and 6 d for endothelial cell medium) was required to increase BMEC yield from 16% to 35% of the population (Fig. 1g and Table 1). Thus, in general, two-dimensional differentiation and expansion of hPSCs for 6–7 d in unconditioned medium followed by 2–6 d in endothelial cell medium generated substantial yields of highly enriched hPSC-derived BMECs.

### Table 1 Quantitative assessment of hPSC-derived BMEC differentiation

| Differentiation time | GLUT-1+ | PECAM-1+ | Mean per cell expression of GLUT-1b (AU) |
|----------------------|---------|----------|-----------------------------------------|
| **IMR90-4 cell line** |         |          |                                         |
| 4 d UM               | 0       | 5        | N/A                                     |
| 6 d UM               | 30      | 36       | 99                                      |
| 6 d UM, 1 d EC       | 36      | 42       | 221                                     |
| 6 d UM, 2 d EC       | 66      | 68       | 300                                     |
| 6 d UM, 2 d EC (+10 µM XAV-939) | 46     | 61       | 272                                     |
| 6 d UM, 2 d EC (+DMSO control) | 64     | 68       | 285                                     |
| 8 d UM               | 39      | 61       | 263                                     |
| C/Fc                 | 100     | 100      | 553                                     |
| **DF19-9-11T cell line** |         |          |                                         |
| 6 d UM, 2 d EC       | 75      | 75       | 70                                      |
| C/Fc                 | 100     | 100      | 76                                      |
| **H9 cell line**     |         |          |                                         |
| 6 d UM, 2 d EC       | 16      | 41       | 110                                     |
| 7 d UM, 6 d EC       | 35      | 63       | 109                                     |

Percentages are representative of at least two independent biological replicates.

*Time in unconditioned (UM) medium (followed by time in endothelial cell medium (EC)).

Mean per cell expression of GLUT-1 in arbitrary units (AU) measured by flow cytometry.

Base single values for GLUT-1 are different for each line tested, and thus should not be used for comparison between different hPSC lines.*Cells subcultured on fibronectin-collagen IV and grown to confluence.

when large percentages of the culture had adopted a BMEC phenotype, the majority of the nestin+ and βIII tubulin+ cells maintained expression of WNT7A, except for some bipotent βIII tubulin+ cells (Supplementary Fig. 7b–e), whereas the WNT7B transcript was no longer detected. The percentage of PECAM-1+ endothelial cells exhibiting detectable nuclear β-catenin substantially increased to 40±6% at 5 d of unconditioned medium treatment (Fig. 2b, middle) and nearly all PECAM-1+ endothelial cells (90±6%) had nuclear β-catenin after 6 d unconditioned medium and 2 d endothelial cell treatment (Fig. 2a, right and Supplementary Fig. 8 for other hPSC lines). Notably, elevated expression of GLUT-1 was nearly exclusively detected in cells that also contained nuclear β-catenin (Fig. 2b, middle), this is consistent with in vivo reports of absence of BBB GLUT-1 in endothelial-specific β-catenin knockout mutants39 and GLUT-1 downregulation in the vascular plexus of WNT7A-WNT7B double knockout mutants37.

As all cell lines tested exhibited nuclear β-catenin localization during BMEC differentiation, we also assessed linkage to Wnt-mediated processes by evaluating transcript expression of Wnt receptors and target genes during IMR90-4 differentiation (Fig. 2c). We compared gene expression in later (7 d, unconditioned medium) cultures containing ~30% PECAM-1+GLUT-1+ BMECs and early (3 d, unconditioned medium) cultures devoid of these cells. As expected, transcripts indicative of BMEC differentiation, such as ABCP1 (β-glycoprotein) and SLCA1 (GLUT-1), were upregulated during this time period (Fig. 2c). Wnt receptors Frizzled4 (FZD4) and Frizzled6 (FZD6) have been implicated in angiogenesis of retinal35–37 and brain endothelial cells26, with the FZD6 transcript being highly expressed in adult brain endothelial cells compared with lung and liver endothelial cells26.
Transcripts of Frizzled4 and Frizzled6 were upregulated in concert with the emergence of IMR90-4-derived GLUT-1+PECAM1+BMECs in the differentiating cultures, whereas the FZD7 gene, which encodes Wnt receptor Frizzled7, which has no known linkage to brain endothelial differentiation, was downregulated during the same time frame. The gene products for β-catenin-associated transcription factor LEF1 and Wnt-downstream gene FST (encoding follistatin) were also upregulated during this time. In addition, the STRA6 gene, which encodes a BBB-resident vitamin A transporter that has been identified as a Wnt target gene and is enriched in adult brain endothelial cells compared with lung and liver endothelial cells, was upregulated during the course of BBB differentiation. APCDD1, which is highly enriched in adult brain endothelial cells compared with lung and liver endothelial cells and is an antagonist of Wnt signaling, was unchanged over the time course of analysis, potentially suggesting a more prominent role of APCDD1 in adult BBB maintenance through Wnt pathway regulation rather than in BBB development. Treatment with the Wnt signaling inhibitors secreted frizzled receptor protein 2 (SFRP2) or XAV-939 led to downregulation of LEF1, FST, STRA6, FZD6, SLC2A1 and ABCB1 (Fig. 2c), further supporting the involvement of Wnt signaling in regulating these transcripts. Moreover, differentiating IMR90-4 cultures treated with XAV-939 showed a reduction in GLUT-1+ cells from 64% to 46% (Fig. 2d and Table 1). Treatment with XAV-939 also led to a slight reduction in the overall percentage of PECAM-1+ cells (68% to 61%), thus giving a 19% net reduction in the overall percentage of PECAM-1+ endothelial cells that became PECAM-1+GLUT-1+ BMECs. Notably, the presence of XAV-939 did not alter claudin-5 or occludin localization even in GLUT-1+ endothelial cells, and western blots indicated equal amounts of claudin-5 and occludin in the differentiating cultures regardless of XAV-939 treatment (data not shown). These data compare favorably with observations in endothelial-specific β-catenin knockout mouse mutants in which brain vascular malformations lacked GLUT-1 expression but still had tight junctions. Thus, although certain aspects of BMEC specification seem to be influenced by the Wnt–β-catenin pathway (for example, elevated GLUT-1 expression), this pathway is probably not exclusively responsible for BMEC specification.

**Purification of hPSC-derived BMECs**

Although the two-dimensional co-differentiation strategy led to many hPSC-derived BMECs as defined by expression of tight junction proteins, p-glycoprotein and elevated GLUT-1, many characteristic BBB properties, including barrier formation and transporter activity, are best evaluated in purified, confluent monolayers. Furthermore, reverse transcription PCR (RT-PCR) analysis indicated that although the IMR90-4-derived BMECs expressed the PECAM1 transcript, they did not express endothelial transcripts encoding von Willebrand factor (VWF) and VE-cadherin during the early unconditioned medium treatment phase (Fig. 3a). During treatment with endothelial cell medium, VE-cadherin expression was detected (Fig. 3a), consistent with sequential expression of PECAM-1 and VE-cadherin endothelial genes observed during endothelial cell differentiation from stem cells. Thus, to stimulate further maturation and facilitate purification, IMR90-4-derived BMECs were subcultured from Matrigel onto plates coated with collagen-fibronectin extracellular matrix.
matrix commonly used for primary BMEC culture. In endothelial cell medium, the cells grew to confluence after 1–2 d, were contact inhibited, had characteristic endothelial cell morphology (Fig. 3b) and could uptake acetylated low-density lipoprotein (aLDL; scale bar, 50 µm). (c) IMR90-4-derived BMECs could uptake fluorescent acetylated LDL (scale bar, 50 µm). (d) Flow cytometry of IMR90-4-derived BMECs after subculture. ZO-1 and PECAM-1 expression are compared with appropriate rabbit IgG control, and occludin, claudin-5 and p-glycoprotein expression are compared with appropriate mouse IgG control in the flow cytometric histograms. (e) Characteristic endothelial cell and BBB markers were expressed by purified IMR90-4-derived BMECs. IMR90-4-derived BMECs expressed PECAM-1 (i; red), claudin-5 (i; green), vWF (ii; red), occludin (ii; green), GLUT-1 (iii), p-glycoprotein (iv), ZO-1 (v) and VE-cadherin (vi). DAPI nuclear stain (blue) is overlaid in i,ii.

For example, when IMR90-4-derived BMECs were subcultured after differentiation IMR90-4 hiPSCs after 3 d on collagen-fibronectin matrix for 2 d (lane 3). This step was critical for obtaining this pure, confluent monolayer of BMECs.

We found similar cultures purified on collagen-fibronectin matrix after 4 d of unconditioned medium treatment did not grow to confluence and areas with malformed or discontinuous claudin-5 expression (green) were observed. Colabel with DAPI is shown (blue). Arrows, continuous claudin-5 expression; arrowheads, defective claudin-5. Scale bar, 50 µm.

Supplementary Fig. 9. Gel electrophoresis of RT-PCR products for transcripts encoding PECAM-1, VE-cadherin and vWF in differentiating IMR90-4 hiPSCs after 3 d of differentiation in unconditioned medium (lane 1), 6 d in unconditioned medium and 2 d in endothelial cell medium (lane 2), or subculture onto a collagen-fibronectin matrix for 2 d (lane 3). (b) Phase contrast image of IMR90-4-derived BMECs on collagen-fibronectin matrix. Scale bar, 100 µm. (c) IMR90-4-derived BMECs could uptake fluorescent acetylated LDL (scale bar, 50 µm).

Supplementary Fig. 10. Characteristic endothelial cell and BBB markers were expressed by purified IMR90-4-derived BMECs. IMR90-4-derived BMECs expressed PECAM-1 (i; red), claudin-5 (i; green), vWF (ii; red), occludin (ii; green), GLUT-1 (iii), p-glycoprotein (iv), ZO-1 (v) and VE-cadherin (vi). DAPI nuclear stain (blue) is overlaid in i,ii.

BBB phenotype of hiPSC-derived BMECs

In addition to BBB marker expression and a vascular phenotype, hiPSC-derived BMECs should also respond to astrocytic cues, exhibit tight barrier properties and express functional transport systems. A hallmark of the BBB is the high TEER—a consequence of tight junction protein interactions between adjacent BMECs. To measure the TEER for the BMEC monolayers, we seeded IMR90-4-derived BMECs onto Transwell filters coated with collagen-fibronectin matrix and grew them to confluence in endothelial cell medium to create a standard two-compartment BBB model. (Fig. 4a). Immunofluorescent detection of ZO-1, occludin and claudin-5 showed maintenance of continuous cell-cell contacts between BMECs on the filter surface, similar to the tight junctions observed on fibronectin-collagen-coated polystyrene culture dishes (Supplementary Fig. 11). Initial TEER measurements taken at confluence were 150–175 Ω cm², indicating a tightening endothelial monolayer (Fig. 4b; time 0 h). TEER measurements after coculture with either primary rat astrocytes or non-neural human embryonic kidney 293 (HEK293) cells in endothelial cell medium were compared with monocultured IMR90-4-derived BMECs. After 24 h, TEER in the astrocyte cocultures (412 ± 38 Ω cm²) was nearly twice that observed in both HEK cell coculture (236 ± 23 Ω cm²).
Figure 4 Functional barrier properties and BBB characteristics of purified hiPSC-derived BMECs. (a) Schematic of two-compartment BBB model. hiPSC-derived BMECs were seeded on a Transwell filter coated with collagen-fibronectin and cocultured with rat astrocytes to assay for induction of BBB properties. Apical (blood side) and basolateral (brain side) chambers are denoted with respect to transport assays. (b) hiPSC-derived BMECs responded to soluble cues from astrocytes. IMR90-4-derived BMECs were cultured alone (monoculture) or cocultured with either rat astrocytes or HEK cells for 96 h and TEER was monitored. Values are mean ± s.d. of triplicate filters. Preferential TEER response in astrocyte coculture compared with HEK coculture was observed for more than ten biological replicates. See Table 2 for TEER values from experiments with optimized medium and seeding density. (c) Freeze-fracture electron microscopy of IMR90-4-derived BMECs after coculture with rat astrocytes for 24 h. P, P-face; E, E-face. Red arrows, E-face groove largely devoid of tight junction particles; blue arrow, infrequent tight junction particle found at E-face; yellow arrows, complex network of tight junction particles associated with P-face. Scale bar, 0.2 μm. (d) RT-PCR detection of representative BBB transcript expression in IMR90-4-derived BMECs cocultured with rat astrocytes. Transcript presence was confirmed for LDLR, LRPI, INSRI, LEPR, BCAM, TFR2, AGER, STRA6, SLCTA5, SLCA14, SLCB5A5, SLC16A1, SLC2A1, ABCB1, ABCG2, ABCC1, ABCG2, ABCG4 and ABCG5. PLVAP and SLCO1C1 transcripts were not detected. Monocultured IMR90-4-derived BMECs had a similar transcript profile except that SLC16A1 transcripts were not detected. Monocultured IMR90-4-derived BMECs were cultured alone (monoculture) or cocultured with either HEK293 cells or astrocytes for their induction. (e) Correlation between IMR90-4-derived BMEC permeability coefficients ($P_{\text{app}}$, x axis) and rodent in vivo transfer coefficients ($K_{\text{trans}}$, y axis). $P_f$ values (cm min$^{-1}$) were calculated from flux experiments using triplicate filters as described in Online Methods. Values are mean ± s.d. for each compound measured in at least three such experiments. To accumulate these data, five individual coculture models (independently differentiated from undifferentiated hiPSCs) were assembled and three to six compounds were measured at a time. Sucrose $P_f$ values were also acquired from efflux transporter inhibition assay controls. Colchicine was the only compound with large variability across biological replicates (see Supplementary Table 1 for numerical values). $K_m$ values (μL s$^{-1}$ g$^{-1}$) were extracted from plotted in situ rodent brain perfusion data reported earlier$^{45}$. (f) Functional expression of efflux transporters in IMR90-4-derived BMECs. Accumulation of rhodamine 123 or [14C]doxorubicin into monocultured IMR90-4-derived BMECs was measured with and without cyclosporin A, Ko143 or MK 571 (top). Transport of rhodamine 123 or doxorubicin from apical to basolateral chambers was measured in two-compartment astrocyte coculture model with and without cyclosporin A, Ko143 or MK 571 (bottom). For all plots, lane 1, control; lane 2, cyclosporin A addition; lane 3, Ko143 addition; lane 4, MK 571 addition. Values are mean ± s.d. of triplicate wells or filters. Data are representative of two biological replicates for each inhibition assay. ***P < 0.01; **P < 0.05; *P < 0.1 (Student’s unpaired t-test).

and BMEC monolocyte (222 ± 51 Ω cm$^2$) and remained elevated for up to 96 h, indicating a specific response to astrocytic cues, as we expected of BMECs (Fig. 4b and Table 2). When astrocyte coculture was done in medium containing 10% FBS, TEER of the IMR90-4-derived monolayer reached 696 ± 8 Ω cm$^2$ after 24 h, whereas TEER in the HEK cell cocultures was again substantially lower at 364 ± 53 Ω cm$^2$ (Table 2). Optimization of seeding density to generate a more uniform monolayer yielded IMR90-4-derived BMECs with a maximum TEER of 1,450 ± 140 Ω cm$^2$ upon astrocyte coculture (Table 2). DF19-9-11T-derived BMECs exhibited a maximum TEER of 777 ± 112 Ω cm$^2$ during astrocyte coculture (Table 2). The hiPSC-derived BMEC monolayers typically showed maximum TEER between 24 h and 48 h of coculture and maintained elevated TEER levels up to 8 d (Supplementary Fig. 12). In terms of robustness, hiPSCs of differing passage number consistently yielded TEER in a range of 700–1,450 Ω cm$^2$ over the course of 30 individual experiments, with an average value of 860 ± 260 Ω cm$^2$. For comparison, one of the highest documented TEER values for primary human BMECs obtained from fresh biopsy is 339 ± 107 Ω cm$^2$ (ref. 45) and the immortalized human BMEC cell line hCMEC/D3 reaches a maximum TEER of 199 ± 5 Ω cm$^2$ in response to hydrocortisone$^9$, whereas peripheral endothelium exhibits a TEER in the 2–30 Ω cm$^2$ range.

To further validate the fidelity of the tight junctions formed by the IMR90-4-derived BMECs after coculture with astrocytes, we analyzed the cells by freeze-fracture electron microscopy (Fig. 4c). Freeze-fracture showed networks of tight junction strands whose complexity mimicked that of high-resistance BBB endothelium in vivo. The tight junction particles were primarily associated with the protoplasmic fracture face (P-face), in contrast with lower-resistance in vitro BBB models that often undergo a switch of tight junction particles from the P-face to the E-face$^{46}$.

The BBB in vivo is characterized not only by its high TEER but also by its impermeability to passive diffusion, its expression of various transport systems used for import and export of nutrients and metabolites and its ability to act as an active barrier to small hydrophobic molecules by using key efflux transporters like p-glycoprotein, breast cancer resistance protein (BCRP) and members of the multidrug resistance protein (MRP) family. Purified IMR90-4-derived BMECs...
Table 2 Evaluation of hiPSC-derived BMEC TEER

| Cocultured cell | Coculture medium | Maximum TEER (Ω cm²⁻¹) |
|-----------------|------------------|-------------------------|
| IMR90-4-derived BMECs |                 |                         |
| Monoculture     | 1% POS           | 222 ± 51                |
| HEKs            | 1% POS           | 236 ± 23                |
| HEKs (optimized) | 10% FBS          | 364 ± 53                |
| HEKs (optimized) | 10% FBS          | 899 ± 132               |
| Astrocytes      | 1% POS           | 412 ± 38                |
| Astrocytes (optimized) | 10% FBS      | 696 ± 8                 |
| Astrocytes (optimized) | 10% FBS      | 1,450 ± 140              |
| DF19-9-11T-derived BMECs | 10% FBS | 777 ± 112               |

*Serum component of culture medium. Full media details are in Online Methods. 1% plasma-derived bovine serum (POS) coculture medium is equivalent to endothelial cell medium. *Measured after 74 h of coculture. TEER is mean ± s.d. measured from triplicate filters. **Optimized BMEC subculture density to reduce nonspecific cell adherence and debris accumulation.

cocultured with astrocytes expressed transcripts encoding a variety of receptors and transporters found at the BBB (Fig. 4d), such as LDLR (low-density lipoprotein receptor), LR1P (low-density lipoprotein receptor–related protein 1), INSR (insulin receptor), LEPR (leptin receptor), BCAM (lutheran glycoprotein), TFFR (transferrin receptor) and AGER (receptor for advanced glycation end products, RAGE). Transcripts were also detected for members of amino acid and peptide transporter families that are highly enriched at the BBB compared with other endothelium, including STRA6 (retinol-binding protein), SLC2A1 (GLUT-1), SLC7A5 (LAT1), SLC1A1 (EAAT3), SLC38A5 (SNAT5) and SLC6A1 (MCT). Efflux transporter transcripts were also detected, including ABCB1 (MDR1 or p-glycoprotein), ABCG2 (BCRP), ABC1 (MRP1), ABC2 (MRP2), ABC4 (MRP4) and ABC5 (MRP5). Notably, two assayed transporters were not detected: PLVAP and SLC1A1 (also known as SLC21A1). PLVAP (plasmalemma vesicle–associated protein) is initially expressed at the BBB during development and becomes downregulated with onset of BBB polarity, but its expression remains in peripheral vessels throughout adulthood. Thus, the absence of PLVAP in the IMR90-4-derived BMECs further indicates BBB specification and maturation. SLC1O1C1 encodes Oatp14, an organic anion transporter whose transcript and protein product is highly enriched at the rodent but not human BBB. Taken together, the gene expression profile of receptors and transporters is highly representative of the BBB.

Next, we screened radiolabeled small molecules of varying sizes, lipophilicity and efflux transporter recognition for relative permeability using the IMR90-4-derived BMEC-astrocyte coculture model (Fig. 4e and Supplementary Table 1). The measured permeability (Pe) values were highly correlated (R² = 0.98) with in vivo rodent brain uptake measured by in situ brain perfusion. Notably, sucrose permeability (Pe = 3.4 ± 10⁻⁵ cm min⁻¹), which is often used to benchmark the passive barrier of in vitro models, was on the order of that in high-fidelity animal BBB models (10⁻⁴ to 10⁻⁵ cm min⁻¹). Glucose, a small (180 Da) hydrophilic molecule that is actively imported across the BBB by the GLUT-1 transporter, showed a seven-fold higher permeation compared with sucrose (Pe = 2.2 ± 10⁻⁴ cm min⁻¹). Inulin, a large, hydrophilic polysaccharide polymer, showed similar permeation to sucrose (Pe = 2.9 × 10⁻⁵ cm min⁻¹). Diazepam, a very lipophilic small molecule that shows high BBB penetration, showed a seven-fold higher permeation compared with sucrose (Pe = 2.9 × 10⁻⁵ cm min⁻¹). Diazepam and vincristine, which are also lipophilic small molecules but have limited brain uptake in vivo as they are substrates of p-glycoprotein and MRP family members, had lower Pe values in vitro (9.2 × 10⁻⁵ and 6.2 × 10⁻⁵ cm min⁻¹, respectively). Prazosin, which is a substrate for efflux by BCRP but also thought to be imported by an organic cation transporter, showed slightly higher Pe (2.9 × 10⁻⁴ cm min⁻¹) than colchicine and vincristine, consistent with its in vivo ranking. Notably, the 40-fold dynamic range of Pe values (diazepam to sucrose) indicates the potential use of the hiPSC-derived BBB model as a drug screening tool, although future testing with a broader spectrum of compounds is needed to validate the predictive power of the model.

Although the relative exclusion of aforementioned lipophilic small molecules like colchicine, vincristine and prazosin suggests functionality of the relevant BBB efflux transporters described in Figures 3e and 4d, we also assessed their functionality and polarity using selective inhibitors (full compound details are in Supplementary Table 2). First, p-glycoprotein function in monocultured IMR90-4-derived BMECs was probed using rhodamine 123, a cell-permeable, fluorescent p-glycoprotein substrate. There was a 1.4-fold increase in cellular accumulation of rhodamine 123 (decrease in efflux) in the presence of cyclosporin A, a known p-glycoprotein inhibitor, but not with Ko143 or MK 571, inhibitors of BCRP and the MRP family, respectively, indicating the presence of functional p-glycoprotein in IMR90-4-derived BMECs (Fig. 4f, top). When doxorubicin, a substrate for p-glycoprotein, BCRP and the MRPs, was added to monocultured IMR90-4-derived BMECs, it accumulated to a greater extent in the presence of cyclosporin A, Ko143 or MK 571, demonstrating the functional efflux activity of each class of transporter (Fig. 4f, top). Next, we used directional transport assays with the IMR90-4-derived BMEC-astrocyte coculture model to demonstrate preferential efflux function in the brain-to-blood direction (basolateral to apical). For p-glycoprotein, transport of rhodamine 123 from the apical to basolateral chamber was 2.3-fold higher with cyclosporin A, but not with Ko143 or MK 571, because of inhibited efflux in the basolateral-to-apical direction (Fig. 4f, bottom). Conversely, transport of rhodamine 123 in the basolateral-to-apical direction slightly decreased upon treatment with cyclosporin A because p-glycoprotein extrusion of rhodamine 123 was blocked at the apical interface (Supplementary Fig. 13). Transport of doxorubicin from the apical to basolateral chamber was increased 1.3-fold, 1.3-fold and 1.2-fold by cyclosporin A, Ko143 and MK 571, respectively, indicating net functional polarization of p-glycoprotein, BCRP and MRP family efflux transporters (Fig. 4f, bottom). Together, these data demonstrate both function and polarization of efflux transporters. Notably, the degree of polarization, as measured by increased flux in the presence of inhibitors, is consistent with that found in primary cultured in vitro rodent BBB systems. Overall, the combination of BBB gene and protein expression, tight junction fidelity, relative permeability for small molecules and polarized efflux transporter activity indicates that the hPSC-derived BMECs have considerable BBB character.

DISCUSSION

Compared with primary animal and human BMEC culture and immortalized cell lines, the approaches described here for generating hPSC-derived BMECs that can form robust BBB models are relatively easy and scalable given the efficiency of endothelial differentiation (>60%) and the possibility of facile extracellular matrix–based purification. Other hPSC differentiation strategies using embryoid bodies, OP9 coculture and two-dimensional differentiation with endothelial factors have lower scalability given the efficiency of endothelial differentiation (>60%) and the possibility of facile extracellular matrix–based purification. Other hPSC differentiation strategies using embryoid bodies, OP9 coculture and two-dimensional differentiation with endothelial factors have lower reported endothelial cell differentiation efficiencies (1–43%) and must be coupled with antibody-assisted purification methods to yield pure populations of endothelial cells.44–46. Our differentiation process leads to a high yield of 11.6 PECAM-1⁺GLUT-1⁺ BMECs per input hPSC after 8 d of differentiation, whereas recent publications using
OP9 coculture and embryoid body or directed differentiation methods have reported yields of 0.6 and 7.4 endothelial cells, respectively. Thus, our method is highly scalable and can generate sufficient cells for thousands of filters for drug screens. In addition, to our knowledge no previous methods for differentiating hPSCs have generated organ-specific endothelial cells. In our study, BBB specification occurred in the presence of co-differentiating neural cells, which probably supplied many of the necessary cues normally provided by the embryonic brain microenvironment in vivo. The model could therefore help elucidate the specification process seems to involve Wnt-β-catenin signaling, which is consistent with in vivo mechanisms of brain development demonstrated in mice. A better understanding of human BBB development may be clinically relevant for promoting BBB repair after stroke or for inhibiting recruitment of blood vessels by brain tumors. As hPSC-derived BMECs have good barrier characteristics with appropriate molecular exclusion and functional transport systems, this cellular platform should also be useful in drug screens to develop pharmaceuticals with desired brain permeability.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

This work was funded in part by US National Institutes of Health (NIH) grants NS056249 (E.V.S.), AA020476 (E.V.S.) and EB007534 (S.P.P.) and US National Science Foundation (NSF) grant EURI-0735903 (S.P.P.). E.S.L. is a recipient of a NIH Chemistry Biology Interface traineeship (T32 GM008505) and S.M.A. is the recipient of a NSF Graduate Research Fellowship. We thank the WiCell Research Institute for providing research support and W.M. Partridge (University of California–Los Angeles) for the gift of GLUT-1 antisera.

AUTHOR CONTRIBUTIONS

E.S.L., S.M.A., S.P.P. and E.V.S. conceived the hPSC-derived BMEC strategy, designed all experiments, analyzed all data and wrote the paper. E.S.L. and S.M.A. did all experiments, I.E.K. did RT-PCR experiments, R.A.N. carried out freeze-fracture microscopy and contributed to its interpretation, H.K.W. did fluorescence in situ hybridization experiments and A.A.-A. contributed to characterization of the BMECs.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nbt.2247.
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ONLINE METHODS

hPSC culture and differentiation. hESCs (H9) and hiPSCs (iPS(IMR90)-4 (ref. 13), iPS-DF19-9-11T and iPS-DF6-9-9T (ref. 13)) were maintained on irradiated mouse embryonic fibroblasts in standard unconditioned medium: DMEM/Ham’s F12 containing 20% Knockout Serum Replacer (Invitrogen), 1x MEM nonessential amino acids (Invitrogen), 1 mM L-glutamine (Sigma), 0.1 mM β-mercaptoethanol (Sigma) and human basic fibroblast growth factor (bFGF; 4 ng ml⁻¹ for hESCs and 100 ng ml⁻¹ for hiPSCs; Waisman Clinical Biomaterializing Facility, University of Wisconsin-Madison). Before differentiation, cells were passed onto Matrigel (BD Biosciences) in mTeSR1 medium (STEMCELL Technologies). After 2–3 d in mTeSR1 (ref. 51), medium was switched to unconditioned medium lacking bFGF (referred to as unconditioned medium throughout the manuscript) to initiate differentiation. Major morphological changes were observed by day 5–7 of unconditioned medium treatment, at which point the medium was switched to endothelial cell medium: human Endothelial Serum-Free Medium (Invitrogen) supplemented with 20 ng ml⁻¹ bFGF and 1% platelet-poor plasma-derived bovine serum (PDS; Biomedical Technologies). After 1–2 d of EC medium treatment, cells were dissociated with dispase (2 mg ml⁻¹; Invitrogen) and plated onto 12-well tissue culture polylysine plates or 1.12 cm² Transwell-Clear permeable inserts (0.4 µm pore size) coated with a mixture of collagen IV (400 µg ml⁻¹; Sigma) and fibronectin (100 µg ml⁻¹; Sigma). Culture plates were incubated with the coating for at least 30 min at 37 °C, whereas the inserts were incubated for a minimum of 4 h at 37 °C. One well of differentiated hPSCs from a standard 6-well tissue culture plate (9.6 cm²) could be seeded on either three wells of a collagen–fibronectin-coated 12-well plate (11.4 cm²) or four collagen–fibronectin-coated inserts (4.48 cm²). Cells were then cultured in endothelial cell medium until they reached confluence (typically 1–2 d). Over the course of dozens of differentiation and purification experiments, multiple lots of PDS (five lots), Knockout Serum Replacer (at least three lots), Matrigel (at least three lots), collagen IV (new batches every 2–3 months) and fibronectin (new batches every 2–3 months) were used with no observable effects on differentiation efficiency or BMEC barrier fidelity. None of the aforementioned materials were qualified or prescreened for their capacity to promote efficient differentiation.

hPSC-derived BMEC coculture experiments. For coculture experiments, primary astrocytes were isolated as described. Briefly, cortices were isolated from P6 neonatal Sprague Dawley rats (Harlan) and minced in Hank’s Balanced Salt Solution (HBSS; Sigma). This tissue was digested in HBSS containing 0.5 mg ml⁻¹ trypsin (Mediatech) in a 37 °C shaker bath for 25 min, followed by digestion in HBSS containing 114 U ml⁻¹ DNase I ( Worthington Biochemical) in a 37 °C shaker bath for 5 min. After trituration and filtration, cells were cultured on collagen-I-coated flasks (100 µg ml⁻¹; Sigma) in DMEM containing 10% qualified heat-inactivated FBS (FBS; Invitrogen), 10% heat-inactivated horse serum (Sigma), 2 mM L-glutamine and 1% antibiotic-antimycotic (Invitrogen). HEK293 cells (HEK cells; ATCC) were cultured in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate (Sigma), 2 g l⁻¹ sodium bicarbonate ( Fisher Scientific), 30 mM HEPES (Sigma) and 1% antibiotic-antimycotic, and used as a non-neural cell control. Coculture of hPSC-derived BMECs was initiated with primary rat astrocytes or HEK cells in either endothelial cell medium (called 1% PDS medium in Table 2) or 70:30 (v/v) DMEM/F12 (Sigma/Invitrogen) supplemented with 1% antibiotic-antimycotic, 2% B27 (Invitrogen) and 10% FBS (called 10% FBS medium in Table 2). TEER measurements were carried out using an EVM voltohmmeter (World Precision Instruments) at the start of coculture and every 24 h thereafter. The resistance value (Ω cm²) of an empty filter coated with collagen–fibronectin was subtracted from each measurement. To determine the Pₒ of radiolabeled ligands, compounds were diluted to 0.4 µCi in transport buffer (distilled water with 0.12 M NaCl, 25 mM NaHCO₃, 3 mM KCl, 2 mM MgSO₄, 2 mM CaCl₂, 0.4 mM K₂HPO₄, 1 mM HEPES and 0.1% BSA; Sigma) and 0.5 ml were added to the upper chamber. Aliquots (200 µl) were extracted from the basolateral chamber every 15 min and replaced by fresh transport buffer. The rate of accumulation of radioactive ligand in the basolateral or apical chambers over the course of 1 h was calculated for Pₒ values for [³H]Collagen, [³H]Linulin, [⁵¹]Colchicine, [³H]Diazepam, [³H]Prazosin, [¹⁴C]Glucose and [³H]Vincristine. [³H]Vincristine was purchased from American Radiolabeled Chemicals, whereas all other radiolabeled compounds were acquired from PerkinElmer. All compound incubations were conducted at 37 °C, and the radioactive permeability experiments were carried out on a rotator. Triplicate filters were used for all permeability studies.

Efflux transport assays. P-glycoprotein, BCRP and MRP functionality were assessed using rhodamine 123 (Sigma), a preferred substrate for P-glycoprotein, and [¹⁴C]doxorubicin (PerkinElmer), a substrate for all aforementioned efflux transporters. To assess activity, hPSC-derived BMEC monolayers cultured on polystyrene (absent astrocyte coculture) were preincubated for 30 min on a rotator at 37 °C with or without 5 µM cyclosporin A (Sigma), 1 µM Ko143 (Sigma), or 10 µM MK 571 (Sigma), which are inhibitors of P-glycoprotein, BCRP or various MRPs, respectively. BMECs were then incubated with rhodamine 123 (10 µM) or doxorubicin (0.25 µCi) for 1 h on a rotator at 37 °C with or without inhibitors. Cells were then washed three times with ice-cold PBS and lysed with 5% Triton X-100 (TX-100; Fisher). Fluorescence (485-nm excitation and 530-nm emission) was measured using a plate reader and normalized to cell counts obtained using a hemacytometer, whereas radioactivity was measured using a liquid scintillation counter. To quantify apical-to-basolateral transport, hPSC-derived BMEC monolayers on Transwell filters were cocultured with astrocytes for 24 h and then preincubated with or without inhibitors for 60 min, followed by addition of rhodamine 123 or doxorubicin to the upper chamber. After another 60 min, aliquots were extracted from the bottom chamber and transport was quantified on a plate reader or scintillation counter. To quantify basolateral-to-apical transport, hPSC-derived BMEC monolayers on Transwell filters were preincubated with or without cyclosporin A for 60 min, followed by addition of rhodamine 123 to the lower chamber. After 3 h, aliquots were extracted from the upper chamber and fluorescence was quantified on a plate reader. All measurements of accumulation and transport were normalized to accumulation and transport without inhibitor. Rhodamine accumulation and transport studies were carried out in the 10% FBS coculture medium, whereas doxorubicin studies were conducted in transport buffer described above. Sucrose permeability and TEER measurements were used to confirm monolayer integrity in the presence of inhibitors.

Tube-forming and acetylated low-density lipoprotein uptake assays. 24-well tissue culture plates were coated with 500 µl of Matrigel for 1 h at 37 °C. Collagen-fibronectin-purified hPSC-derived BMECs were dissociated using trypsin, and 100,000 cells were plated into each Matrigel-coated well in endothelial cell medium supplemented with 40 ng ml⁻¹ VEGF (R&D Systems) and imaged after 12 h. A control sample of cells lacking VEGF was also used. For acetylated LDL uptake, purified hPSC-derived BMECs were incubated with 10 µg ml⁻¹ acetylated LDL conjugated to Alexa Fluor 488 (Invitrogen) for 4 h at 37 °C, washed twice with PBS and visualized immediately with an Olympus epifluorescence microscope. Images were taken using a Diagnostic Instruments camera run by MetaVue software.

Immunocytochemistry and in situ hybridization. Cells were washed once with PBS and fixed in either 2% paraformaldehyde or 10% ice-cold methanol for 15 min. The cells were then blocked with 40% goat serum in PBS (40% PBST). When probing for an intracellular antigen, 0.1% TX-100 was present in the 40% PBST. The cells were then incubated in 40% PBST containing primary antibodies (see Supplementary Table 3 for list) for 1 h at room temperature or overnight at 4 °C. After three washes in PBS, cells were incubated in 40% PBST containing goat anti-rabbit Texas Red (1:500; Invitrogen) or goat anti-mouse Alexa Fluor 488 (1:500; Invitrogen) for 1 h at room temperature. Cell nuclei were labeled with 300 nM 4′,6-Diamidino-2-phenylindolehydrochloride (DAPI) for 10 min. Cells were washed three times in PBS and visualized. Vascular and BBB markers were tested at the time points indicated in Results. Markers characteristic of basal epithelial and epithelial progenitor cells, cyto-keratin K14 and the p63 transcription factor were tested in IMR90-4-derived cells after 6 d of unconditioned medium and 2 d of endothelial cell medium treatment or in purified IMR90-4-derived BMECs and were not detected. The simple epithelial cytokeratin K18 was expressed in undifferentiated IMR90-4 hiPSCs and its expression was still detected in all cells of the mixed differentiating population after 6 d of unconditioned medium and 2 d of endothelial cell medium treatment. In situ hybridization for detection of WNT7A and WNT7B
transcripts was conducted as described. Briefly, cells were washed once with PBS and fixed in 2% paraformaldehyde for 10 min, followed by permeabilization in PBS containing 0.1% TX-100 for 5 min. Prehybridization was done with a water-based solution containing 3% BSA and 4× saline-sodium citrate buffer (SSC; Fisher), followed by a 1 h hybridization in 4× SSC and 10% dextran sulfate (Fisher) at room temperature. DIG-labeled locked nucleic acid probes were purchased from Exiqon and DIG-labeled locked sense probes were used as negative controls (sequences found in Supplementary Table 4). After three washes in 4× SSC and 0.1% Tween-20, one wash in 2× SSC, one wash in 1× SSC, and one wash in PBS (all washes conducted at 50°C), cells were blocked in PBS containing 4% BSA for 20 min and labeled overnight at 4°C with a monoclonal antibody to digoxigenin (Sigma). Secondary antibody and DAPI labeling were carried out as described above.

Flow cytometry. Cells were harvested via Accutase (Invitrogen) incubation for 2–3 min, fixed in 100% ice-cold methanol for 20 min and blocked with 40% PBSG for 20 min at room temperature. Primary antibody labeling (Supplementary Table 3) was done in 10% PBSG for 1 h at room temperature. IgG controls were used at the same concentration. After a wash with 5% FBS in PBS, secondary antibodies (goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 647; 1:200) in 10% PBSG were added to each sample for 30 min at room temperature. After two washes with 5% FBS in PBS, the samples were analyzed on a FACS calibur flow cytometer. For quantification purposes, PECAM-1+ events were quantified using a PECAM-1/forward scatter dot plot referenced to a rabbit IgG control. Events demonstrating elevated GLUT-1 expression (GLUT-1+) were quantified using a GLUT-1/forward scatter dot plot referenced to a 4D unconditioned medium culture that lacked BMECs to account for low basal GLUT-1 expression in the differentiating culture. Events that were found in both of these positive gates were classified as PECAM-1+GLUT-1+ cells. PECAM-1+ events having basal GLUT-1 expression were referred to as PECAM-1+GLUT-1−. For inhibition of BMEC differentiation, 10 µM XAV-939 (Sigma) or equivalent volume of DMSO vehicle control was added to IMR90-derived BMECs at the start of unconditioned medium treatment, and PECAM-1 and GLUT-1 expression was evaluated as described above. Data are presented as two-dimensional dot plots with color codes for ease of viewing. βIII tubulin and nestin expression was quantified versus rabbit and mouse IgG controls, respectively, using two-color flow cytometry.

RT-PCR, quantitative RT-PCR and gel electrophoresis. Cells were differentiated as described. For inhibition of Wnt signaling, cells were treated with 250 ng ml−1 of mouse secreted frizzled-related protein 2 (SFRP2; R&D Systems) in unconditioned medium for 4 d, followed by 750 ng ml−1 of SFRP2 in unconditioned medium for an additional 3 d, or with 10 µM XAV or equivalent volume of DMSO vehicle control starting at day 2 of unconditioned medium treatment. For RNA collection, cells were washed once with PBS and dissociated with trypsin or accutase. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) and oligo-dT primers (Invitrogen). Quantitative PCR (qPCR) was then done using 1 µl of cDNA and IQ SYBR Green Supermix (Bio-Rad) on an iCycler (Bio-Rad). RT-PCR was also done using GoTag Green Master Mix (Promega). Primer sequences are supplied in Supplementary Table 4. Relative quantification was quantified using the comparative cycle threshold (Ct) method, normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Fold difference was calculated as 2x−ΔΔCT, where x refers to primer efficiency calculated according to LinRegPCR version 12.3 (ref. 55). Transcript amplification was analyzed by 2% agarose gel electrophoresis of the qPCR or RT-PCR products.

Freeze-fracture electron microscopy. After 24 h of coculture with rat astrocytes, IMR90-4-derived BMECs were washed once with PBS, fixed in 1.5% glutaraldehyde (Sigma) for 60 min, washed several times with PBS, cryoprotected with glycerol (50%), scraped from the filters, loaded into gold specimen carriers (Bal-Tec part LZ 02125 VN), and plunged in liquid ethane. Frozen specimens were transferred to a Balzers 301 Freeze Fracture Apparatus for fracturing and 1 min of sublimation at −110 °C. Etched fracture surfaces were replicated with platinum at 45° supported by carbon deposited from 90°. Replicas were cleaned for 1 h using commercial household bleach followed by rinsing with double distilled water. Bare 400 mesh copper grids were used to pick up the cleaned replicas. Images were obtained using a JEOL 1230 TEM equipped with a Gatan Model 894 2KX2K CCD camera.

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