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TITLE:
Derivation, Expansion, Cryopreservation and Characterization of Brain Microvascular Endothelial Cells from Human Induced Pluripotent Stem Cells

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KEYWORDS:
Human induced pluripotent stem cells, brain microvascular endothelial cells, blood brain barrier, trans-endothelial electrical resistance, transporter activity, microvascular, schizophrenia, bipolar disorder, passaging, expansion, cryopreservation.

SUMMARY:
This protocol details an adapted method to derive, expand, and cryopreserve brain microvascular endothelial cells obtained by differentiating human induced pluripotent stem cells, and to study blood brain barrier properties in an ex vivo model.

ABSTRACT:
Brain microvascular endothelial cells (BMECs) can be differentiated from human induced pluripotent stem cells (iPSCs) to develop ex vivo cellular models for studying blood-brain barrier (BBB) function. This modified protocol provides detailed steps to derive, expand, and cryopreserve BMECs from human iPSCs using a different donor and reagents than those reported in previous protocols. iPSCs are treated with essential 6 medium for 4 days, followed by 2 days of human endothelial serum-free culture medium supplemented with basic fibroblast growth factor, retinoic acid, and B27 supplement. At day 6, cells are sub-cultured onto a collagen/fibronectin matrix for 2 days. Immunocytochemistry is performed at day 8 for BMEC marker analysis using CLDN5, OCLN, TJP1, PECAM1, and SLC2A1. Western blotting is performed to confirm BMEC marker expression, and absence of SOX17, an endodermal marker[1]. Angiogenic potential is determined via sprouting assay. Trans-endothelial electrical resistance (TEER) is measured using chopstick electrodes and voltohmmeter starting at day 7. Efflux transporter

activity for ATP binding cassette subfamily B member 1 and ATP binding cassette subfamily C member 1 is measured using a multi-plate reader at day 8. Successful derivation of BMECs is confirmed by the presence of relevant cell markers, low levels of SOX17, angiogenic potential, transporter activity, and TEER values ~2000 Ω x cm^2. BMECs are expanded until day 10 before passaging onto freshly coated collagen/fibronectin plates or cryopreserved. This protocol demonstrates that iPSC-derived BMECs can be expanded and passaged at least once. However, lower TEER values and poorer localization of BMEC markers was observed after cryopreservation. BMECs can be utilized in co-culture experiments with other cell types (neurons, glia, pericytes), in three-dimensional brain models (organ-chip and hydrogel), for vascularization of brain organoids, and for studying BBB dysfunction in neuropsychiatric disorders.

INTRODUCTION:

Blood-Brain Barrier Function

The blood-brain barrier (BBB) forms a boundary that limits movement of substances from the blood to the brain. The BBB is comprised of brain microvascular endothelial cells (BMECs) that form a monolayer lining the vasculature. BMECs together with astrocytes, neurons, pericytes, microglia, and extracellular matrix, form the neurovascular unit. BMECs have a tightly regulated paracellular structure that allows the BBB to maintain high trans-endothelial electrical resistance (TEER), which limits passive diffusion and serves as an indicator of barrier integrity[2,3]. BMECs also have proteins that assist with transcellular movement such as endocytosis, transcytosis, and transmigration, as well as extravasation of leukocytes during an immune response[4]. BMECs rely on influx and efflux transporters for nourishment and removal of waste products, in order to maintain a homeostatic balance in the brain[4]. For example, solute carrier family 2 member 1 (SLC2A1) is an influx transporter responsible for the movement of glucose across the BBB[5], while efflux transporters such as the ATP binding cassette subfamily B member 1 (ABCB1) and the ATP binding cassette subfamily C member 1 (ABCC1) are responsible for returning substrates back into the blood stream[4,6–8]. ABCB1 substrates include morphine, verapamil[5], and antipsychotics such as olanzapine and risperidone[9], while the ABCC1 transporter has a variety of substrates including sulfate conjugates, vincristine, and glucuronide conjugates[5].

Application of BBB Models in Psychiatric Disorders

BBB dysfunction has been implicated in a number of neurological and psychiatric disorders, including schizophrenia and bipolar disorder[10,11]. Recently, iPSC-derived ex vivo cellular models are being utilized to interrogate the cellular and molecular underpinnings of psychiatric disorders, but these models currently do not take into account the potential role played by the neurovasculature[12–14]. It is hypothesized that peripheral inflammatory cytokines circulating in the blood can adversely impact the BBB[15–18], but there is also evidence for paracellular[19–23], transcellular[24–30] and extracellular matrix[21,30–33] abnormalities contributing to BBB dysfunction. Disruption of the BBB can result in the contents of the blood entering the brain parenchyma and activating astrocytes and/or microglia to release proinflammatory cytokines, which in turn initiate an inflammatory response[34] that can have detrimental effects on the brain[35]. BMECs are the primary component of the BBB and examining the structure and function of these cells can enhance the understanding of BBB dysfunction in neurological and psychiatric disorders.

Alternative BMEC Models

Prior to the development of efficient protocols for deriving BMECs from iPSCs[2,7,36,37], researchers had employed immortalized BMECs[38] to study BBB function. However, many of these models failed to attain desirable BBB phenotypes, such as the physiological range of TEER values[39,40]. Utilizing iPSCs has the advantage of retaining the genetic background of the individual from which the cells are derived. Scientists are actively working on establishing iPSC-derived ex vivo microenvironment models that recapitulate the structure and function of the human brain. Researchers have developed methods to derive BMECs that are structurally and physiologically similar to BMECs found in vivo. Methods for obtaining purified populations of iPSC-derived BMECs require a number of different steps with protocols being optimized in the last few years[2,7,36,37]. Generally, iPSC-derived BMECs are cultured in Essential 6 (E6) medium for 4 days, followed by 2 days in human endothelial serum-free medium (hESFM) supplemented with basic fibroblast growth factor (bFGF), retinoic acid (RA), and B27 supplement. The cells are then cultured on a collagen IV (COL4) and fibronectin (FN) matrix to obtain >90% homogeneous BMECs[2].

The identity of BMECs are confirmed by immunofluorescence showing the co-expression of BMEC proteins including platelet-endothelial cell adhesion molecule-1 (PECAM1), SLC2A1, and tight junction proteins such as tight junction protein 1 (TJP1), occludin (OCLN), and claudin-5 (CLDN5)[7]. Sprouting assays have been used to confirm the angiogenic potential of iPSC-derived BMECs.[7] The BBB integrity of BMECs is evaluated by the presence of physiologic in vitro TEER values (~2000Ω x cm^2)[38] and measurable activity for efflux transporters such as ABCB1 and ABCC1[2,7,37]. Recent methodological advances by the Lippmann group have led to iPSC-derived BMEC protocols with reduced experimental variability and enhanced reproducibility[2]. However, less is known about whether iPSC-derived BMECs can be derived using a different donor and reagents, and whether they can be expanded and passaged beyond the sub-culturing stage. Our modified protocol aims to address this issue by passaging iPSC-derived BMECs beyond day 8 and whether they can be further expanded to retain BBB properties after cryopreservation. While no studies have described passaging of iPSC-derived BMECs, a protocol exists for BMEC cryopreservation that retains physiologic BBB properties after undergoing a freeze-thaw cycle[41]. However, there is no clear evidence whether this cryopreservation protocol can be replicated in other cell lines or whether post-cryopreservation BMECs can withstand several passaging steps.

BMECs derived from iPSCs using the Lippmann protocol have been utilized to model BBB disruption in neurological disorders such as Huntington's disease[8]. Such iPSC-derived BMECs have also been used to investigate the effects of bacterial infection such as *Neisseria meningitidis* or *Group B Streptococcus* on disruption of blood-CSF barrier and BBB respectively[42,43]. Also, using iPSC-derived BMECs from 22q deletion syndrome patients with schizophrenia, researchers observed an increase in intercellular adhesion molecule-1 (ICAM-1), a major adhesion molecule in BMECs that assist with recruitment and extravasation of leukocytes into the brain[44]. Taken together, these studies demonstrate the utility of iPSC-derived BMECs for studying BBB disruption in complex neuropsychiatric disorders.

PROTOCOL:

Human iPSCs were reprogrammed from the fibroblasts of healthy donors using a protocol approved by the Institutional Review Boards of Massachusetts General Hospital and McLean Hospital, and characterized as described in previous studies[45–47].

NOTE: Briefly, fibroblasts were reprogrammed to iPSC via mRNA-based genetic reprogramming[48]. The iPSCs were maintained in stem cell medium (SCM) (see material list) and stored at a density of ~1.2 x 10^2 cells/mL with 1 mL of SCM, 10 µM with rho-associated protein kinase inhibitor (ROCKi) Y-27632, and 10% (v/v) dimethyl sulfide (DMSO), in cryopreserved vials in liquid nitrogen at -160 °C. All of the following procedures below are carried out in a biosafety cabinet unless stated otherwise.

1. Basement membrane matrix dilution and plate coating

1.1. Dilute (1:50) growth factor reduced basement membrane matrix purified from Engelbreth-Holm-Swarm tumor in Dulbecco's Modified Eagle Medium (DMEM) without phenol red.

1.2. Coat cell culture plates with the appropriate amount of diluted basement membrane matrix (i.e., 6-well plate = 1mL, 12-well plate = 0.5 mL) and incubate these plates at 37 °C for at least 1 hour.

2. iPSC maintenance

NOTE: The maximum confluency per well in a 6-well flat-bottom plate is ~1.2 x 10^6 cells.

2.1. Thaw cryopreserved iPSCs into SCM with 10 µM Y-27632 and plate onto a 6-well plate coated with diluted growth factor reduced basement membrane matrix.

2.2. Maintain iPSCs in SCM with 10 µM Y-27632 for the first 24 hours after thawing. Switch to fresh medium after 24 hours.

2.3. Maintain iPSCs in SCM until cells reach 80-90% confluency before passaging.

2.3.1. Calculate how many iPSCs will be needed for differentiation by multiplying desired density for differentiation (15,600 cells/cm^2) by the surface area of the well. For a 6-well flat-bottom plate, multiply 15,600 cells/cm^2 by 9.6 cm^2 for a total of 149,760 cells/well.

2.4. To passage, wash the cells with Hanks' Balanced Salt Solution (HBBS). Then, incubate the cells with non-enzymatic ethylenediaminetetraacetic acid (EDTA) (see material list) for 5 minutes at 37 °C.

2.4.1. Use a cell scraper to gently lift off the cells. Collect cells in fresh SCM.

2.4.2. Plate cells onto cell culture plates coated with diluted SCM and maintain cells as described in step 3 or store them at ~1.2 x 10^6 cells/mL in 1 mL of SCM, 10 µM Y-27632, and 10% DMSO (v/v) in cryopreserved vials in liquid nitrogen at temperature of -160 °C.

3. Differentiation of iPSCs to BMECs

NOTE: Non-enzymatic EDTA separates cells into clumps. Enzymatic EDTA (see **Table of Materials**) separates cells into single cell suspension. Retinoic acid (RA) should be protected from light.

3.1. Wash iPSCs once with Dulbecco's Phosphate Buffer Saline (DPBS). Incubate with enzymatic EDTA (1 mL for 6-well plate, 0.5 mL for 12-well plate, and 0.25 mL for 24-well plate) for approximately 5 minutes at 37 °C to yield a single cell suspension.

3.2. Collect cells and centrifuge at 300 x g (relative centrifugal force) for 5 minutes at room temperature. Resuspend cell pellets in SCM containing 10 µM Y-27632.

3.3. Determine cell density using Trypan Blue and automated cell counter or a hemocytometer device. Plate cells at a density of 15,600 cells/cm^2 or 149,760 cells/well of a 6-well flat-bottom plate (with a surface area of 9.6 cm^2/well) in SCM containing 10 µM Y-27632 for 24 hours.

3.4. Initiate differentiation after 24 hours by changing SCM to E6 medium. Change E6 medium daily for the next 4 days.

3.5. On day 4 of differentiation, replace E6 medium with hESFM supplemented with diluted (1:200) B27 supplement, 20 ng/mL bFGF, and 10 µM RA. Do not change this medium for the next 48 hours.

3.6. Prepare 200 mL of hESFM with diluted (1:200) B27, mix 1 mL of 50x concentrated B27 supplement to 199 mL of hESFM.

3.7. Prepare 20 ng/mL of bFGF by reconstituting 50 µg of bFGF in 250 µL of Tris buffer (5 mM Tris, pH 7.6, 150 mM NaCl) to make 200 µg/mL stock solution. Prepare 200 mL of hESFM containing 20 ng/mL bFGF by mixing 20 µL of 200 µg/mL bFGF with 200 mL of hESFM.

3.8. Prepare 10 µM RA by first making a 40 mg/mL of RA stock solution by adding 2.5 mL of DMSO to 100 mg of RA powder. Dilute this concentration to 3 mg/mL to make 10 mM stock solution. Prepare 200 mL of hESFM containing 10 µM RA by mixing 200 µL of 10µM RA in 200 mL hESFM.

4. Coating collagen IV (COL4) and fibronectin (FN) Matrix for Purification of iPSC-Derived BMEC

4.1. Add 2 mL of sterile water to 2 mg of FN to make 1 mg/mL FN stock solution. Add 5 mL of sterile water to 5 mg of COL4 to make a 1 mg/mL COL4 stock solution.

4.1.1. Allow FN to dissolve for at least 30 minutes at 37 °C and the COL4 to dissolve at room temperature.

4.2. Dilute FN stock solution in sterile water to a final concentration of 100 µg/mL and COL4 stock solution to a final concentration of 400 µg/mL.

4.3. Coat the desired plates (6-well plate = 1 mL of COL4/FN solution, 12-well plate = 0.5 mL, 24-well plate= 0.25 mL, and 12-transwell filtered plate = 0.25 mL) with the mixture of 400 µg/mL COL4 and 100 µg/mL FN.

4.3. Incubate plates for a minimum of 2 hours or overnight at 37 °C; for Transwell filtered plates, a minimum of 4 hours is recommended.

5. Sub-culture and purification of iPSC-Derived BMECs

NOTE: Incubation with enzymatic EDTA may take longer than 15 minutes depending on the confluency of the cells on day 6 of differentiation.

5.1. On day 6 of differentiation, wash cells twice with DPBS. Incubate with 1 mL of enzymatic EDTA for at least 15 minutes at 37 °C until a single cell suspension is obtained.

5.2. Collect cells via centrifugation at 300 x g for 5 minutes at room temperature. Resuspend cell pellets with fresh hESFM with diluted (1:200) B27 supplement, 20 ng/mL bFGF, and 10 µM RA.

5.3. Seed cells onto plates coated with a mixture of 400 µg/mL COL4 and 100 µg/mL FN. Seed cells using a ratio of 1 well of a 6-well plate to 3 wells of a 12-well plate, 3 wells of a 12-transwell filtered plate, or 6 wells of a 24-well plate.

5.4. Seed undifferentiated iPSCs from the same cell line onto COL4/FN coated 12-transwell filtered plate as negative control for TEER analysis.

5.5. After 24 hours of sub-culturing, change medium to hESFM with B27 supplement only. No medium changes are needed after this step.

6. Sprouting assay

6.1. Collect Day 8 iPSC-derived BMECs and seed them at 100,000 cells/well onto a 24-well flat-bottom plate freshly coated with 200 µL/cm^2 of basement membrane matrix.

6.2. Treat these cells with hESFM with diluted (1:200) B27 and 40 ng/mL of vascular endothelial growth factor A (VEGFA165).

6.3. Observe cells every 24 hours and change the medium every two days.

7. Immunocytochemistry (ICC)

NOTE: ICC is carried out on 24-well flat-bottom plates.

7.1. After 48 hours of sub-culturing (day 8), wash cells twice with DPBS. Fix cells with 4% paraformaldehyde (PFA) for 20 minutes.

7.2. Wash cells three times with DPBS, 5 minutes per wash. Pre-block cells for 1 hour at room temperature in DPBS with 5% donkey serum and 0.3% Triton X-100 (v/v).

7.3. Incubate with primary antibodies: mouse anti-human-PECAM1 (1:100, stock 0.5 mg/mL), rabbit anti-human-TJP1 (1:200, stock 0.53 mg/mL), mouse anti-human-CLDN5 (1:200, stock 0.5 mg/mL), mouse anti-human-OCLN (1:200, stock 0.5 mg/mL), and rabbit anti-human-SLC2A1(1:100, stock 0.2 mg/mL) in DPBS containing 5% donkey serum overnight at 4°C.

7.3.1. Rinse cells once with DPBS and then wash five times for 5 minutes per wash with DPBS.

7.4. Incubate cells with secondary antibodies: donkey-anti-rabbit Alexa Fluor 555 (1:200) and donkey-anti-mouse 488 (1:200) in DPBS containing 5% donkey serum for 1 hour.

7.5. Following this incubation, add Hoechst 33342 trihydrochloride trihydrate diluted (1:1000) in DPBS for 10 minutes.

7.5.1. Remove Hoechst 33342 solution and rinse once with DPBS and wash four times with DPBS for 5 minutes per wash.

7.6. Visualize cells on fluorescence microscopes to look for expression and localization of cell makers.

8. TEER Measurement and Analysis

NOTE: Corning 12-Transwell filtered plates are equipped with filters consisting of 1.1 cm^2 polyethylene terephthalate membranes and 0.4 micrometer pores. TEER measurements are obtained in technical (3 per well) and biological replicates (3 wells per cell line and/or condition).

8.1. 24 hours after sub-culturing (day 7), measure TEER using chopstick electrodes and a voltohmmeter every 24 hours. Refer to voltohmmeter user manual for specific instructions on obtaining measurements.

8.1.1. To measure TEER, charge the voltohmmeter instrument the night before. Lightly wipe the instrument and chopstick electrodes with 70% ethanol before placing them in the safety hood.

8.1.2. Switch the power on and calibrate the ohm meter as recommended by the manufacturer.

8.1.3. Plug in the chopstick electrodes and rinse electrodes with 70% ethanol followed by DPBS.

8.1.4. Place the shorter end electrode into the trans-well insert (the apical chamber) and the longer end into the basolateral chamber.

8.1.5. First measure a blank well that is coated with COL4/FN only. Then measure the other wells.

8.1.6. Quickly rinse chopstick electrodes with 70% ethanol followed by DPBS when measuring different conditions (i.e. measuring different cell lines).

8.2. After all measurements (in Ω) have been recorded, rinse chopstick electrodes with 70% ethanol and then sterile water. Gently wipe electrode and let it air dry in the safety hood.

8.3. Average the triplicate TEER values (in Ω) from the blank well and subtract this average value from each raw TEER value by condition.

8.3.1. Average the subtracted values and multiply them by 1.12 cm^2 (the surface area of the 12-transwell insert).

8.3.2. Use transformed values from step 6 to generate the graph showing TEER value and standard errors for each day of TEER measurement.

9. Efflux Transporter Activity and Analysis

NOTE: Efflux transporter activity assay is performed on a 24-well flat-bottom plate. Efflux transporters of interest include ABCB1 and ABCC1. It is recommended that each condition should be performed in triplicate with control wells (i.e. blank wells without the respective inhibitors).

9.1. After 48 hours of sub-culturing (day 8), incubate cells with 10 µM Valspodar (ABCB1 inhibitor) or 10 µM MK571 (ABCC1 inhibitor) for 1 hour at 37 °C.

9.1.1. Prepare 10 mM Valspodar stock by dissolving 5 mg of powder (1214.64 g/mol) in 412 µL of DMSO and dilute to working concentration of 10 µM. For example, to make 10 mL of hESFM with 10 µM Valspodar, mix 10 µL of 10 mM Valspodar stock with 10 mL of hESFM.

9.1.2. Prepare 10 mM MK571 stock by dissolving 5 mg powder (537.07 g/mol) in 931 µL and dilute to working concentration of 10 µM. For example, to make 10 mL of hESFM with 10 µM MK571, mix 10 µL of 10 mM MK571 stock with 10 mL of hESFM.

9.2. After 1 hour, incubate cells with 10 µM rhodamine 123 (ABCB1 substrate) or 10 µM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, ABCC1 substrate) with or without their respective inhibitors for 1 hour at 37 °C.

9.2.1. Prepare 10 mM rhodamine 123 by dissolving 10 mg of powder (380.82 g/mol) in 875 µL of DMSO and dilute to working concentration of 10 µM. For example, to make 10 mL of hESFM with 10 µM rhodamine 123, mix 10 µL of 10 mM rhodamine 123 stock with 10 mL of hESFM.

9.2.2. Prepare 10 mM H2DCFDA by dissolving 50 mg of powder (487.29 g/mol) in 10.26 mL of DMSO and dilute to working concentration of 10 µM. For example, to make 10 mL of hESFM with 10 µM rhodamine 123, mix 10 µL of 10 mM rhodamine 123 stock with 10 mL of hESFM.

9.3. Wash cells twice with 0.5 mL of DPBS and lyse using DPBS containing 5% Triton-X (v/v).

9.4. Measure fluorescence of the lysed cells using a multi-plate or microplate reader (see material list).

9.4.1. Set fluorescent plate reader instrument to 485 nanometer excitation and 530 nanometer emission and measure fluorescence at these wavelengths.

9.4.2. For wells not used in the transporter assay, wash cells twice with DPBS before fixing them with 4% PFA for cell nuclei quantification.

9.5. Incubate cells with Hoechst 33342 trihydrochloride trihydrate diluted (1:1000) in DPBS for 10 minutes. Image multiple visual fields in each well to calculate average cell nuclei counts using fluorescence microscopes.

9.6. Count nuclei using Fiji and normalize fluorescence values on a per-cell basis to these counts.

9.6.1. Calculate average accumulation of fluorescence by subtracting raw fluorescence accumulation value for each condition from its respective blank value.

9.6.2. Average subtracted values for each condition.

9.6.3. Divide average values from step 9.6.1 by the average cell counts. Use these values to normalize fluorescence values on a per-cell basis.

9.6.4. Use normalized values to generate a graphical representation for each inhibitor condition and perform any necessary statistical analysis.

10. Passaging, Expanding, and Cryopreserving BMECs

10.1. Replenish day 8 BMEC cultures with fresh hESFM supplemented with diluted (1:200) B27 and allow cells to expand for two more days on the COL4/FN matrix.

10.2. Coat a new 12-Transwell filtered plate and a 24-well flat-bottom plate with 400 µg/mL COL4 and 100 µg/mL FN and incubate for 4 hours.

10.3. On day 10, wash cells with DPBS and incubate with 1 mL of enzymatic EDTA for at least 15 minutes at 37°C until a single cell suspension is obtained.

10.4. Collect cells via centrifugation at 300 x g for 5 minutes at room temperature.

10.4.1. To cryopreserve these cells, resuspend cell pellets with fresh hESFM with 30% Fetal Bovine Serum (FBS) and 10% DMSO.

10.4.2. Store iPSC-derived BMECs in cryopreserved vials in an isopropanol container for the first 24 hours at -80 °C, then place in liquid nitrogen for long-term storage at -160 °C.

10.4.3. To passage these cells, resuspend cell pellets with fresh hESFM supplemented with diluted (1:200) B27.

10.5. Seed cells onto coated plates prepared in Step 10.2. Seed cells using a ratio of 1 well of a 6-well plate to 3 wells of a 12-transwell filtered plate and to 6 wells of a 24-well plate. Allow cells to grow and expand for 24 hours.

10.6. On day 11, measure TEER by following steps listed in Step 8.

10.7. On day 12, perform ICC by following steps listed in Step 9.

10.8. To thaw cryopreserved BMECs, place the cryopreserved vials in a warm water or bead bath at 37°C. Then transfer the thawed BMECs to 5 mL of hESFM supplemented with diluted (1:200) B27.

10.8.1. Collect cells via centrifugation at 300 x g for 5 minutes at room temperature. Resuspend the cells in hESFM supplemented with diluted (1:200) B27, 10 µM RA and 10 µM Y-27632.

10.8.2. After 24 hours, switch medium to hESFM supplemented with diluted (1:200) B27 and 10 µM Y-27632 without RA.

REPRESENTATIVE RESULTS:

BMEC Differentiation

A few critical steps in this protocol should be precisely followed (**Figure 1**). E6 medium use on day 1 is important, since it is often used for deriving neuroectoderm lineage from iPSCs within a relatively short period of time yielding reproduceable results across multiple cell lines[37]. Another important step is on day 4 of differentiation, where E6 medium should be switched to hESFM with diluted (1:200) B27, 20 ng/mL bFGF and 10 µM RA to expand iPSC-derived BMECs. The addition of B27 supplement is used as an alternative to bovine serum to support serum-free cell culturing[2], bFGF is added to facilitate growth of iPSC-derived BMECs[7], and RA is used to facilitate the development of the BBB phenotype[36]. The last important step involves the purification stage, where day 6 iPSC-derived BMECs are sub-cultured onto a COL4/FN coated plate to select iPSC-derived BMECs[2,7,36,37]. **Figure 2** demonstrates the morphological transition from iPSCs to BMECs.

After one day of E6 medium (day 1), cellular morphology is similar to that of iPSCs. By day 4 of E6, cells begin to appear visibly distinct from iPSCs and cover most of the well (~90% confluency). By day 6, while cultured in hESFM with diluted (1:200) B27, 20 ng/mL bFGF and 10 µM RA, cellular morphology begins to have an elongated and cobblestone appearance. At day 8, each individual cell is distinct in a large cobblestone pattern. A sprouting assay was performed to demonstrate the angiogenic potential of iPSC-derived BMECs, which resulted in tube-like structures after 3 days of VEGFA165 treatment (**Figure 3**).

[Place **Figure 1** Here]

[Place **Figure 2** Here]

[Place **Figure 3** Here]

Purified iPSC-derived BMECs were seeded at 100,000 cell/cm^2 onto basement membrane matrix in hESFM with (1:200) B27 supplement and 40ng/mL VEGFA165. Tube-like structures appeared after 3 days of VEGFA165 treatment.

BMEC characterization was performed using immunocytochemistry for cell-specific markers. iPSC-derived BMECs were assessed for the presence of tight junction proteins (OCLN, TJP1, and CLDN5), which are commonly expressed in the tight junctions of brain endothelial cells[4] and endothelial cells in the lung, liver, and kidney[19]. Other markers such as PECAM1 and SLC2A1, have been previously used as markers for purified BMECs[7]. PECAM1[4] and SLC2A[5] are both expressed in vascular endothelial cells of the BBB. The iPSC-derived BMECs generated using this protocol co-expressed all five of these markers (**Figure 4**).

[Place **Figure 4** Here]

To characterize BBB function of BMECs, TEER was measured 24 hours (day 7) after sub-culturing and the medium was changed to hESFM with diluted (1:200) B27 without bFGF and RA. TEER measurements were obtained starting at day 7 of differentiation (day 0 of TEER measurement) and peaked at ~2000 Ω x cm^2 on day 8 or 48 hours after sub-culturing BMECs (**Figure 5**). These TEER values are within the range described for co-cultured iPSC-derived BMECs with rat primary astrocytes[38]. The iPSC line did not have any discernable BBB function according to their low TEER values.

[Place **Figure 5** Here]

To evaluate ABCB1 and ABCC1 efflux transporter activity, the amount of fluorescent substrate taken up for ABCB1 and ABCC1 were quantified following incubation with their respective inhibitors. As expected, inhibition of ABCB1 and ABCC1 efflux transporters with PSC833 (ABCB1 inhibitor) or MK-571 (ABCC1 inhibitor) led to an increase in rhodamine 123 (R123) or 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), respectively (**Figure 6**). This evidence suggests that BMECs derived using this protocol have efflux transporter activity.

[Place **Figure 6** Here]

Passaging, Expanding and Cryopreserving iPSC-Derived BMECs

Another aim was to investigate whether iPSC-derived BMECs could be passaged and cryopreserved after sub-culturing. For this purpose, day 7 iPSC-derived BMECs were allowed to expand until day 10 before passaging them onto newly coated COL4/FN 12-transwell filtered plates for TEER measurement and 24-well flat-bottom plate for ICC analysis (**Figure 7**). Using this condition iPSC-derived BMECs continued to proliferate, maintained the expression of OCLN, TJP1, CLDN5, SCL2A1, and PECAM1 (**Figure 8**), and continued to sustain proper TEER values (peak at~2000 Ω x cm^2) after passaging (**Figure 9**). Cryopreserved BMECs were later thawed, expanded, and then passaged (**Figure 7**). TEER measurements of BMECs were obtained 24 hours after thawing and several more days after that. TEER measurements of these post-thawed BMECs were reduced (peaking at only 800 Ω x cm^2) when compared to freshly derived BMECs. A second passaging of post thawed BMECs exhibited even lower TEER values (peaking at only 200-300 Ω x cm^2) (**Figure 9**) and showed frayed and/or freckled patterns of the tight junction formation (**Figure 10**). Western blot analysis[49] revealed that iPSCs primarily expressed an endodermal marker (SOX17)[1] and some tight junction markers (OCLN), but not other BMEC related markers (TJP1, CLDN5, and SLC2A1) (**Figure 11**). BMECs primarily expressed endothelial related markers (TJP1, CLDN5, OCLN and SLC2A1), with low levels of the endodermal marker, SOX17.

[Place **Figure 7-11** Here]

[Place **Figure 8** Here]

Figure 1: Outline for Differentiation of Human iPSCs to BMECs. Human iPSCs were initially cultured in stem cell medium containing 10 μM Y-27632 for 24 hours before changing medium to E6 for 3 days. On day 4, medium was changed to hESFM with (1:200) B27 supplement, 20 ng/mL bFGF, and 10 μM RA for 2 days. On day 6, cells were sub-cultured onto COL4/FN coated plates. On day 7, medium was changed to hESFM with B27 supplement without bFGF and RA and TEER was measured. On day 8, ICC and efflux transporter activity assays were performed. iPSC-derived BMECs were expanded until day 10 before being passaged to a trans well plate or a 24-well flat bottom plate for TEER measurement and ICC analysis, respectively. Day 8 BMECs were used for the sprouting assay (not depicted). 2 wells of a 6-well plate of iPSC-derived BMECs were collected and stored in hESFM with 10% DMSO and 30% FBS at -80 °C and then in liquid nitrogen for long-term storage at -160°C. On day 12, a peak in TEER value was observed in expanded iPSC-derived BMECs at which point ICC was performed.

Figure 2: Bright-field Images Depicting Differentiation of iPSCs to BMECs. After one day of culture in E6 medium, iPSCs retain their characteristic morphology. On day 4 in E6 medium, cellular morphology appears distinctly different from iPSCs. On day 6, cellular morphology changes to an elongated and cobblestone appearance. By day 8, cells appear large and with a cobblestone pattern.

Figure 4: Marker Analysis of iPSC-Derived BMECs. Human iPSC-derived BMECs were stained for tight junction (OCLN, TJP1, CLDN5), influx transporter (SLC2A1), and adherens junction (PECAM1) proteins. OCLN, TJP1, and CLDN5 proteins are primarily localized in the cell membrane. SLC2A1 and PECAM1 are localized in both the nuclei and cell membrane. Hoechst 33342 trihydrochloride trihydrate was used for nuclear staining.

Figure 5: TEER Measurements in iPSC-Derived BMECs. TEER values peaked after one day of sub-culturing on COL4/FN matrix (on day 8 of differentiation). TEER measurements were obtained in technical (3 measures per well) and biological replicates (3 wells per cell line). The technical average value from a blank well was subtracted from raw TEER values. These values were averaged for each day and multiplied by 1.12 cm^2 (surface area of the 12-transwell insert). Error bars represent standard error.

Figure 6: Efflux Transporter Activity in iPSC-Derived BMECs. Efflux transporter activity in BMECs was determined by quantifying the accumulation of rhodamine 123 (R123) or 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) in the presence or absence of PSC833 (ATP binding cassette subfamily B member 1 (ABCB1) inhibitor) or MK-571 (ATP binding cassette subfamily C member 1 (ABCC1) inhibitor). Technical triplicates were performed for each condition (N=1). Fluorescence values from the control condition (i.e. without inhibitors) were deducted from raw fluorescence values. These fluorescence accumulation was normalized on a per-cell basis for each technical replicate. Statistical significance was determined using student t-test from the three technical replicates. No statistical significance was observed between the accumulation of R123 with and without ABCB1 inhibitor (t-stat= -1.66, p=0.11). Statistical significance was observed between the accumulation of H2DCFDA with and without ABCC1 inhibitor (t-stat=-7.23, p=0.04). *p<0.05. Error bars represent standard error.

Figure 7: Bright Field Images of Expanded and Cryopreserved iPSC-derived BMECs. A) Day 10 (4 day after initial sub-culture) cells reached maximum confluency. B) Day 11, 24 hours after passaging onto COL4/FN coated 24-wells plate. C) Day12, 48 hours after passaging onto COL4/FN coated 24-wells plate; peak TEER values was observed and ICC was performed. D) 48 hours post-thawed iPSC-derived BMECs on COL4/FN coated 6-wells plate; cells were previously cryopreserved at 1.2 x 10^2 cells/mL. E) 24 hours after post-thawed iPSC-derived BMECs were passaged onto COL4/FN coated 6-wells plate. F) 48 hours after post-thawed iPSC-derived BMECs were passaged.

Figure 8: ICC of iPSC-Derived BMECs after Passaging. BMECs were passaged and maintained on COL4/FN matrix until day 12, when TEER values peaked. BMECs on day 12 were stained for tight junction (OCLN, TJP1, CLDN5), influx transporter (SLC2A1) and adherens junction (PECAM1) proteins. The expression pattern and localization resemble those observed in conditions where passaging was not performed, as shown in Figure 4.

Figure 9: Comparing TEER Measurements in iPSCs, non-passaged BMECs, passaged BMECs, cryopreserved BMECs, and cryopreserved & passaged BMECs. On day 1, TEER values peaked for non-passaged and passaged BMECs, but not iPSCs or cryopreserved BMECs. Cryopreserved

BMECs had moderate TEER values between day 3 and 7, with even lower TEER values for the cryopreserved & passaged BMECs. iPSCs did not demonstrate any measurable TEER values between day 0 and 9. TEER measurements were obtained in technical (3 measurements per well) and biological replicates (3 per cell line). The technical average value from a blank well was subtracted from the raw TEER values. These values were averaged for each day and multiplied by 1.12 cm2 (surface area of the 12-transwell insert). Error bars represent standard error.

Figure 10: ICC of cryopreserved & passaged BMECs. BMECs were passaged and maintained on COL4/FN until peak TEER values were observed. BMECs were stained for tight junction (OCLN, TJP1, CLDN5), influx transporter (SLC2A1) and adherens junction (PECAM1) proteins. The expression pattern of tight junction markers appeared frayed and/or freckled when compared to non-passaged BMECs (**Figure 4**) and passaged BMECs (**Figure 8**).

Figure 11: Western blot analysis of iPSCs, non-passaged BMECs, passaged BMECs, and cryopreserved & passaged BMECs. Western blots showing levels of TJP1, OCLN, SOX17, SLC2A1, CLDN5, and loading control (GAPDH).

DISCUSSION:

In this protocol, we made some modifications in using a commonly used extracellular matrix and cell culture media during iPSC culturing for derivation of BMECs (**Figure 1**). These changes did not impact the ability to derive BMECS from human iPSCs as described in the Lippmann protocol[2]. An iPSC line from a different healthy donor was used to demonstrate that this modified protocol shows results comparable to previous studies with other lines[2]. For cryopreservation, B27 supplement was used in lieu of 1% platelet poor plasma-derived serum (PDS)[41], but this affected BMEC fidelity in subsequent culturing. In regards to troubleshooting, TEER values may fluctuate rapidly before stabilizing. This fluctuation may result from temperature changes occurring when the plates are moved from 37 °C to room temperature[38]. To overcome this issue, measurements should be taken rapidly, efficiently, and consistently. If necessary, temperature effects can be factored in by using a mathematical formula provided by Blume et al. 2009[50] to obtain temperature corrected TEER values.

Despite obtaining BMECs with a robust BBB phenotype (i.e. high TEER value), maintaining this BBB property for an extended period of time continues to be a major challenge. As shown here, peak TEER values (~2000 Ω x cm^2) using this protocol were much lower than previously reported peak TEER values (~8000 Ω x cm^2)[2]. Despite this observation, TEER values fell within the usual range (2000-8000 Ω x cm^2) of previously reported values[2]. A second limitation is the variability in peak TEER values that results from different iPSC lines used, which has been observed in other versions of this protocol[37]. The variation between different cell lines may be due to the growth and expansion rate of each iPSC line, which is impacted by environmental factors[51]. Another limitation is related to cryopreservation, as the use of B27 did not maintain BMEC fidelity. It is possible that 1% PDS[41] provides greater stability of BMECs during cryopreservation compared to supplementation with B27.

Significance with Respect to Existing Methods

The iPSC-derived BMECs provide cells that have the genetic background of specific individuals, which is valuable in utilizing these cells for the study of disease biology. This is not the case when using animal models or primary BMEC cultures extracted from other animals[38]. Moreover, in vitro primary BMEC cultures show low TEER values (~100 Ω x cm^2 [38]), much lower than those achieved with human iPSC-derived BMECs with the protocol described here. This modified protocol provides a detailed method for obtaining human BMECs from iPSCs, with the potential to expand and maintain them for longer use. The ability to passage and store differentiated BMECs can provide versatility and flexibility in experimental design, especially when studying multiple cell lines at once. Based on these results, iPSC-derived BMECs can be expanded and passaged after the initial sub-culturing step (**Figures 7-9**), but cryopreservation needs further investigation. This protocol can also be used in conjunction with other stem cell-based cellular models to develop innovative approaches such as vascularization of brain organoids. Current methods to generate brain organoids result in an incomplete reconstitution of cell types of the human brain since they lack endothelial cells and critical elements of the neurovascular unit that comprise the BBB[52,53]. The protocol described here can provide a tractable and reproducible approach using two-dimensional Transwell/co-culturing systems that is less expensive and easier to implement than 3D models.

This protocol can be utilized to study the role of the neurovasculature and BBB in neuropsychiatric disorders such as schizophrenia and bipolar disorder, where deficits in the neurovasculature have been hypothesized to play a role[10,11,21,54,55]. Previous versions of this protocol[37] had been used to derive BMECs from iPSC lines of patients with Huntington disease[8] and 22q deletion syndrome patients with schizophrenia[44]. Both studies[8,44] demonstrated successful utilization of this method to investigate paracellular and/or transcellular function related to the BBB. Some concerns have been raised about the potential confounding effects of animal serum in prior protocols[2]. The serum-free protocol used here removes this concern while producing similar results to prior protocols for deriving BMECs.

There are four critical steps when implementing this protocol. First, seeding iPSCs at an optimal density (i.e., seeding at ~15,600 cells/cm^2) is important for efficient BMEC differentiation. If the cell density is too high or too low by day 4 of differentiation, cultures may display greater rates of cellular heterogeneity[56]. A second important step is the induction of differentiation between day 1 and day 4, where E6 medium is used to initiate differentiation. E6 is utilized in place of the "unconditioned medium" which was used in prior protocols[7,36]. Not only does E6 medium cut down the differentiation time from 13 days to 8 days, but it also promotes the expression of tight junction proteins (TJP1, OCLN, CLDN5) and BMEC markers (PECAM1 and SLC2A1)[37]. The use of E6 medium also resulted in proper TEER values (**Figure 5**) and ABCB1 and ABCC1 efflux transporter activity (**Figure 6**)[7,36]. Thirdly, the use of B27 in lieu of bovine serum allowed for serum-free condition, which improved the consistency and reliability of BMEC differentiation[2]. Lastly, in terms of expanding the iPSC-derived BMECs, cells should be passaged when they reach ~100% confluency. Based on this protocol, iPSC-derived BMECs can be further expanded and passaged after the initial sub-culturing stage (**Figures 7-9**).

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DISCLOSURES:

The authors have nothing to disclose.

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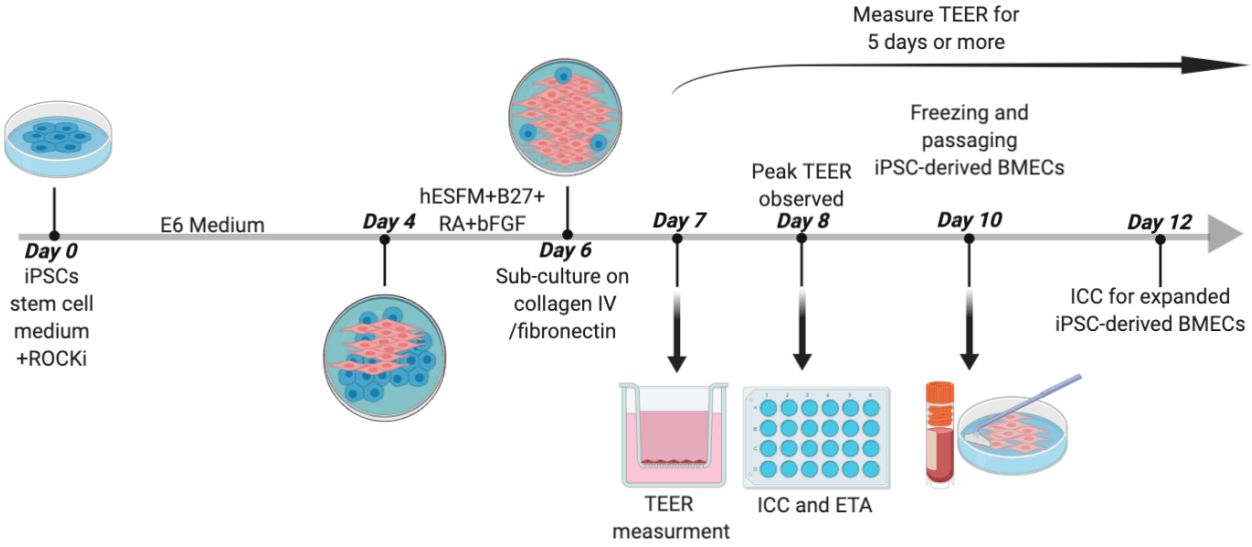
Figure 1



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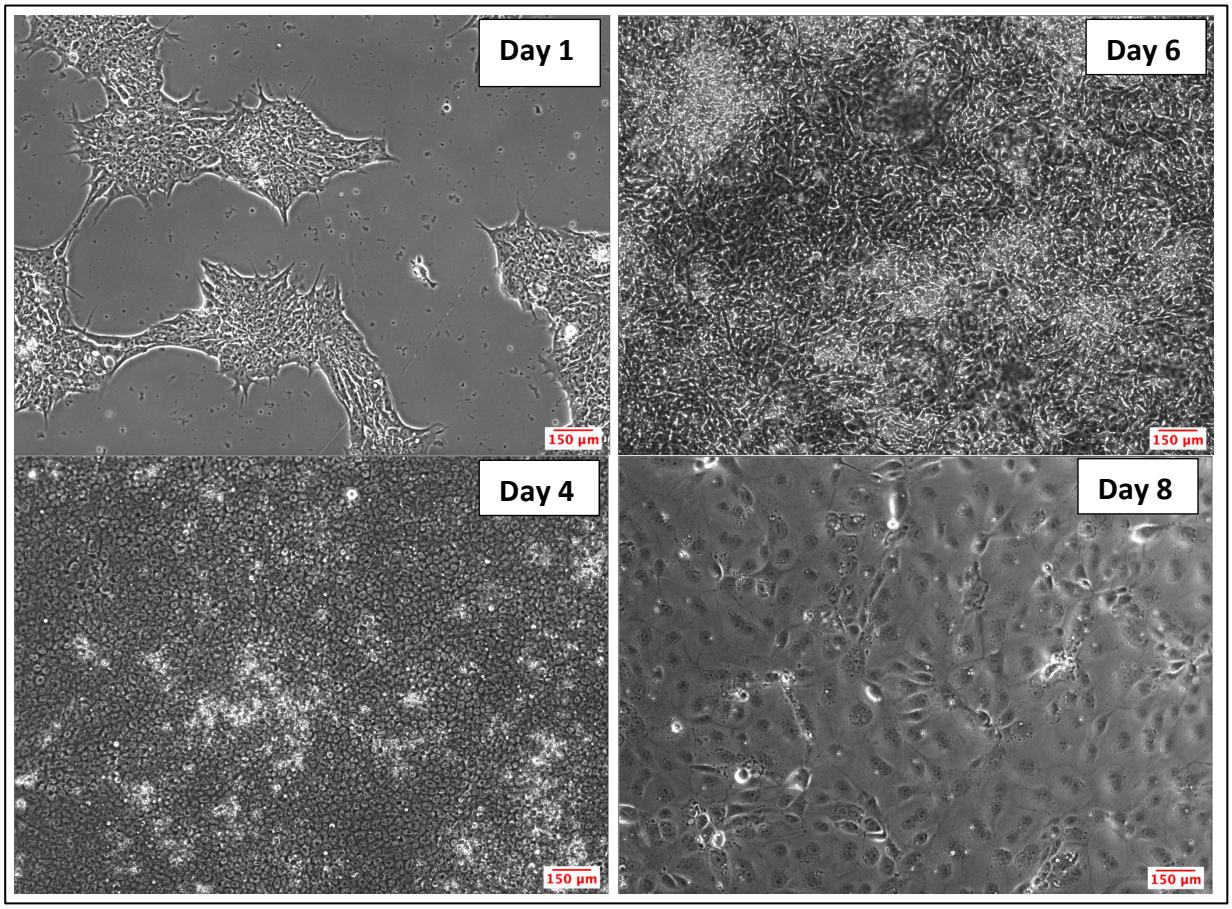

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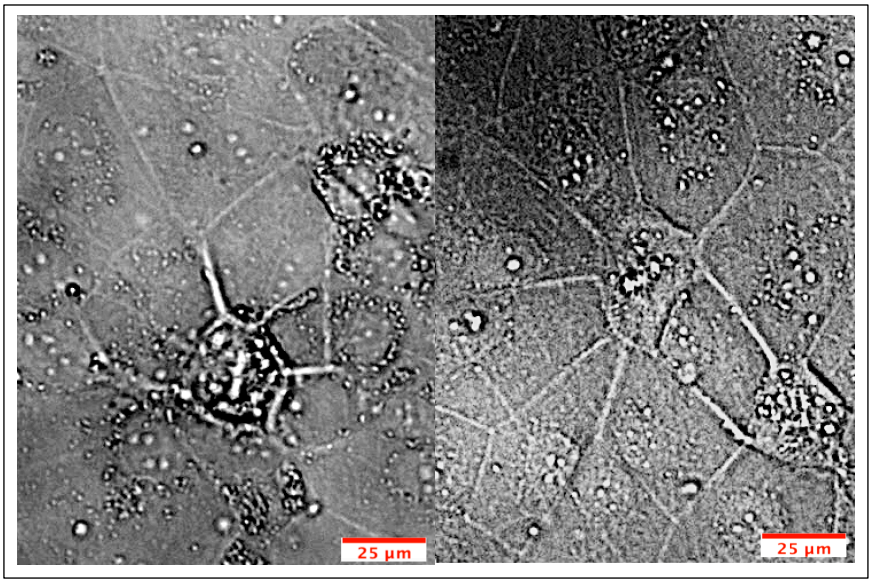

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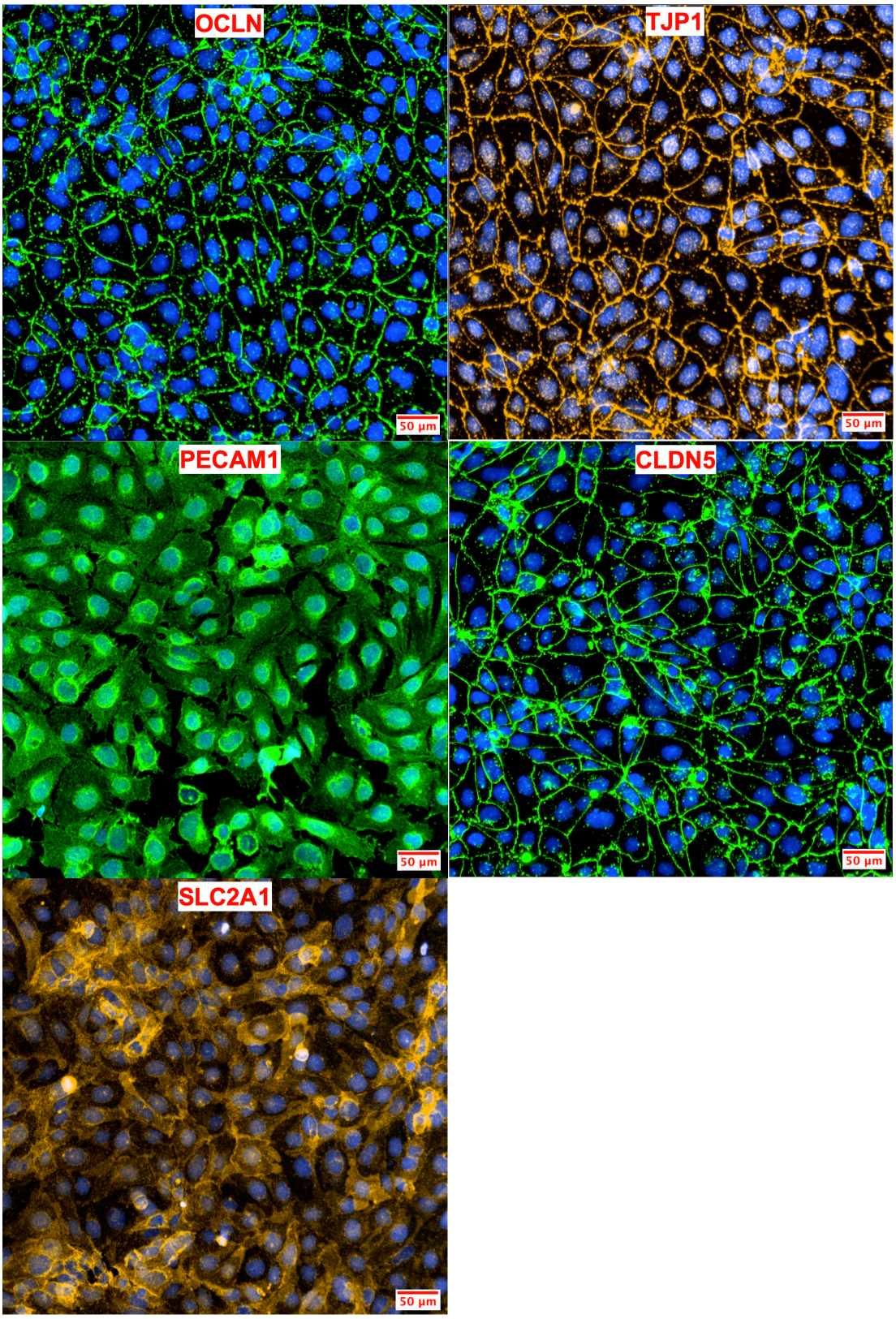

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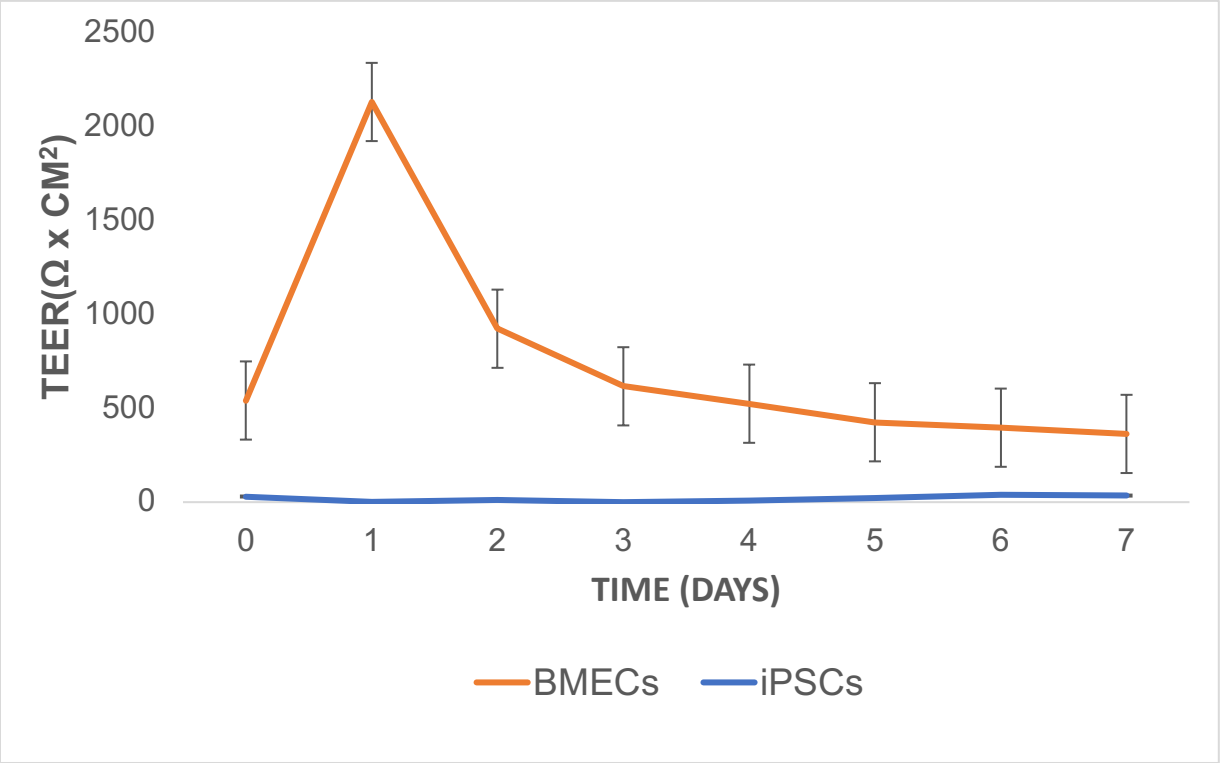

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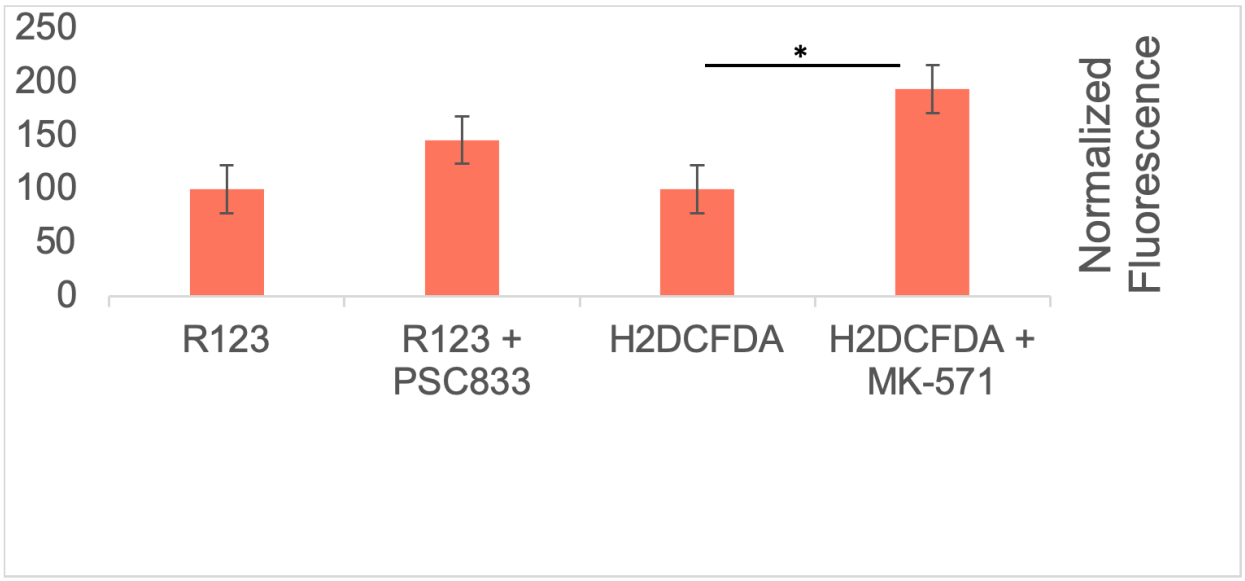

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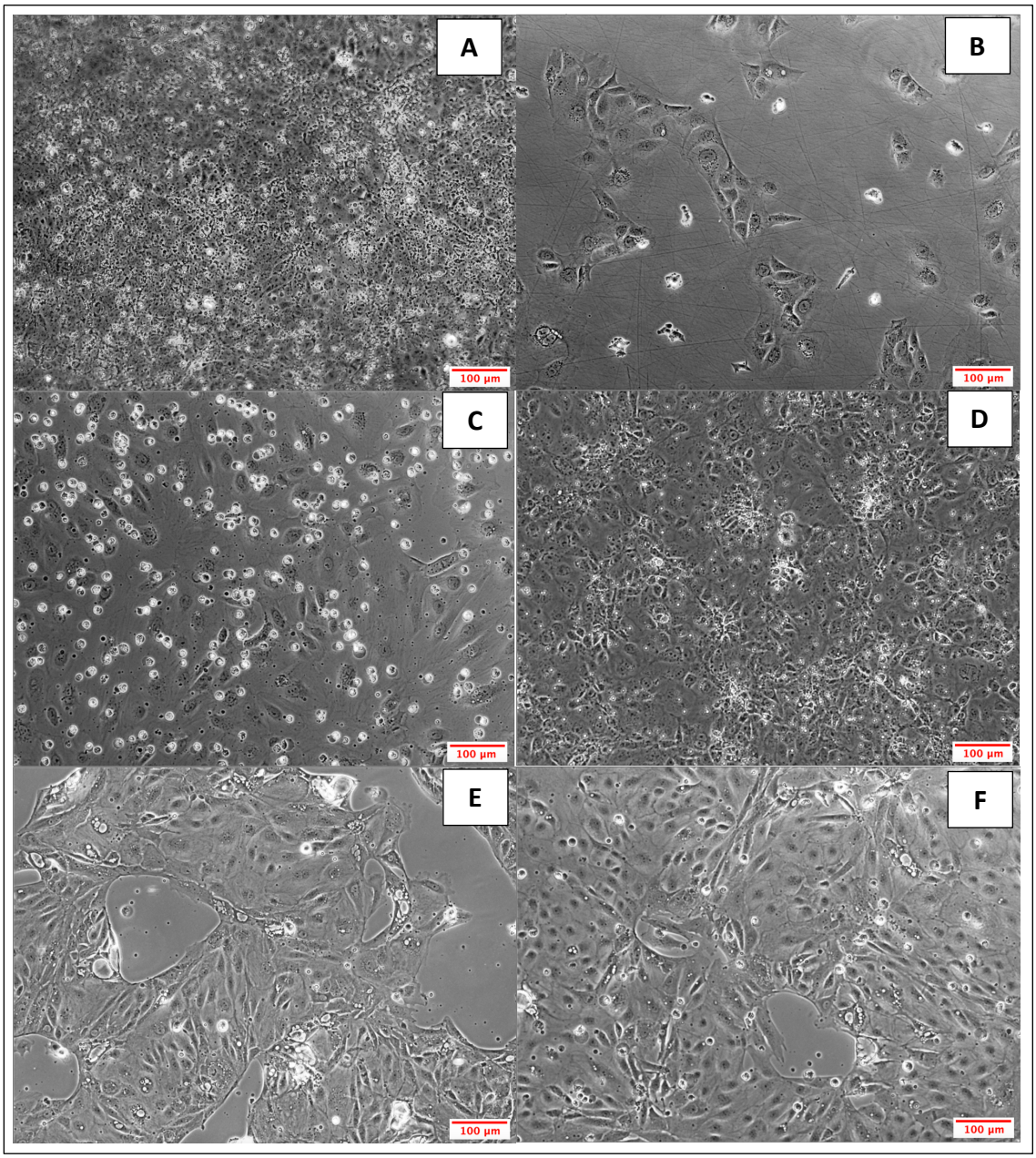

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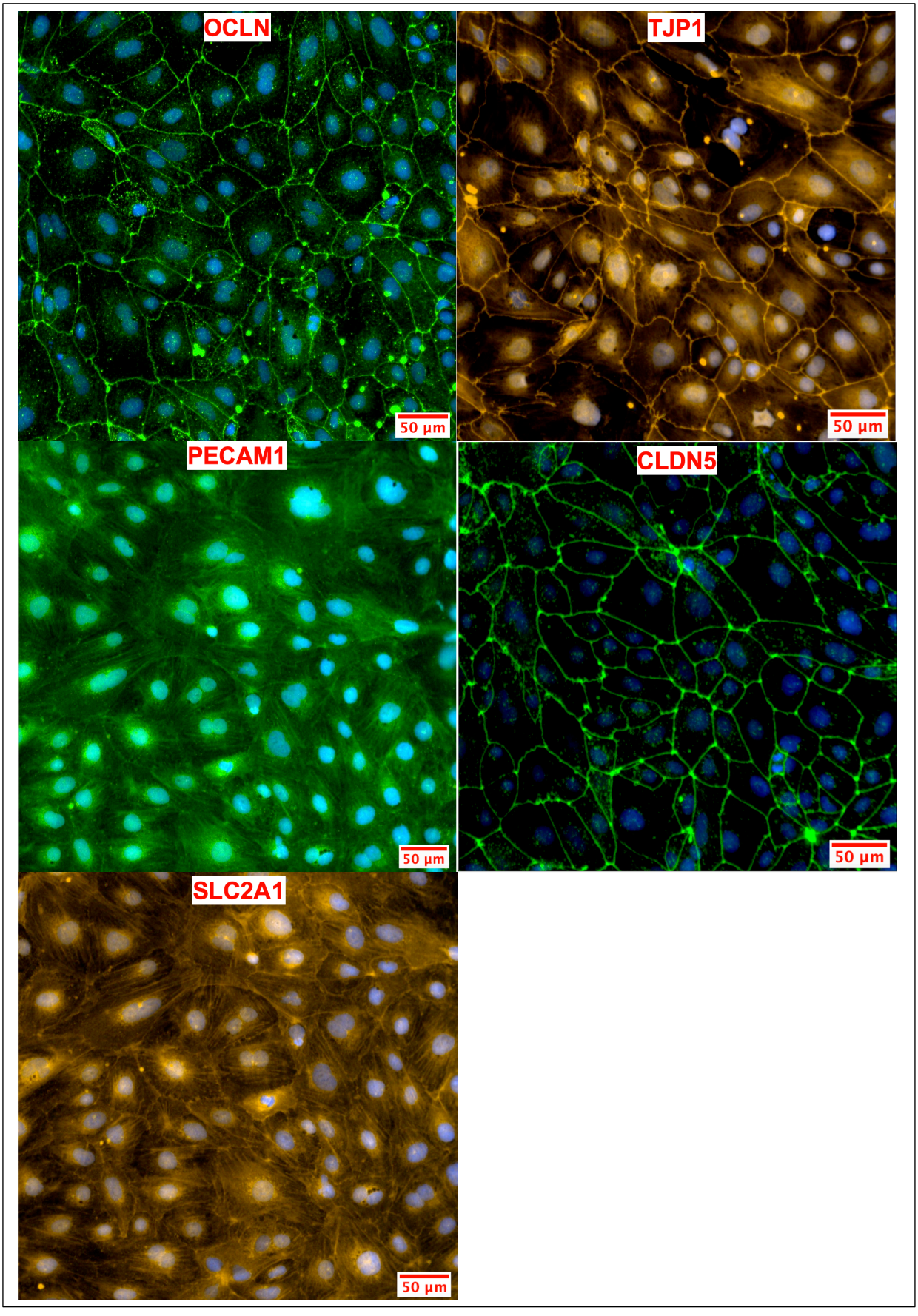


Figure 9

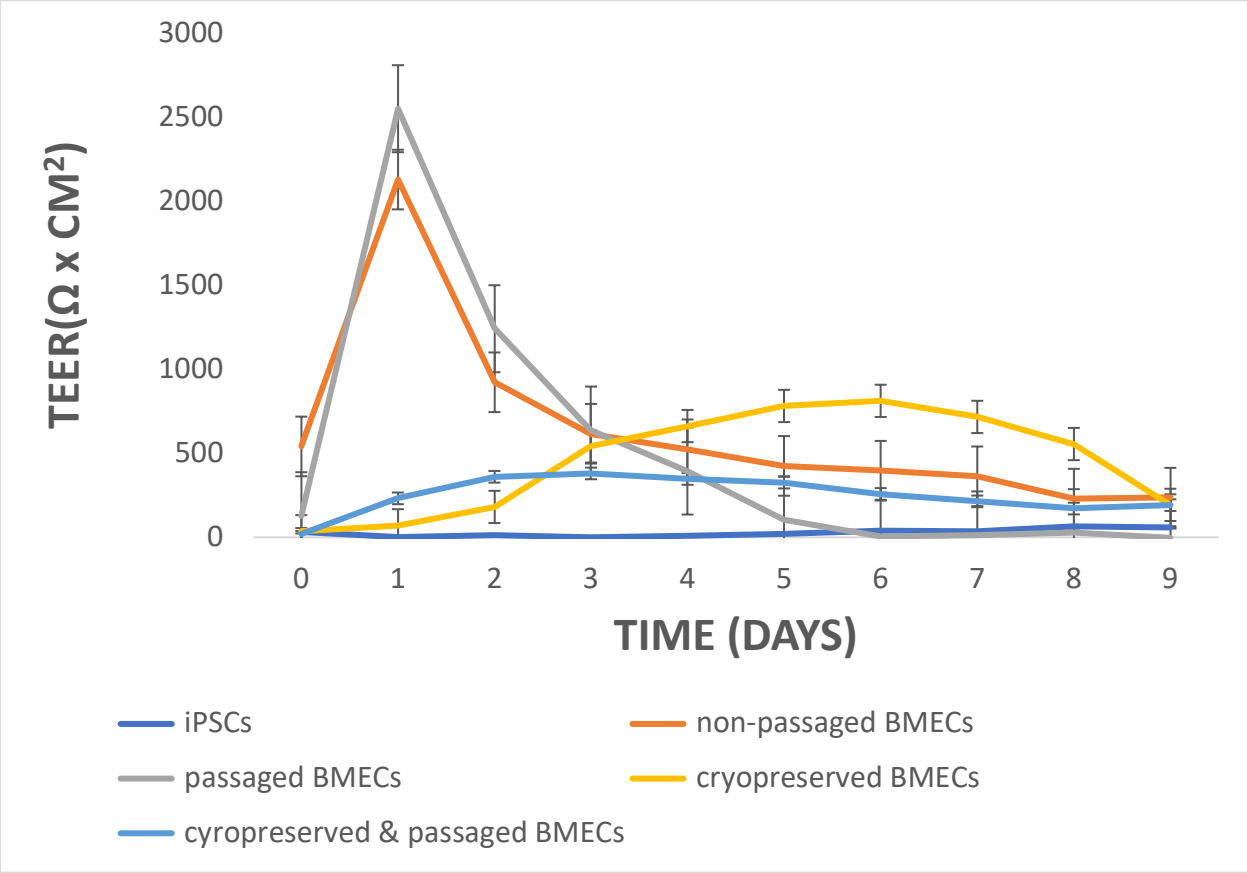


Figure 10

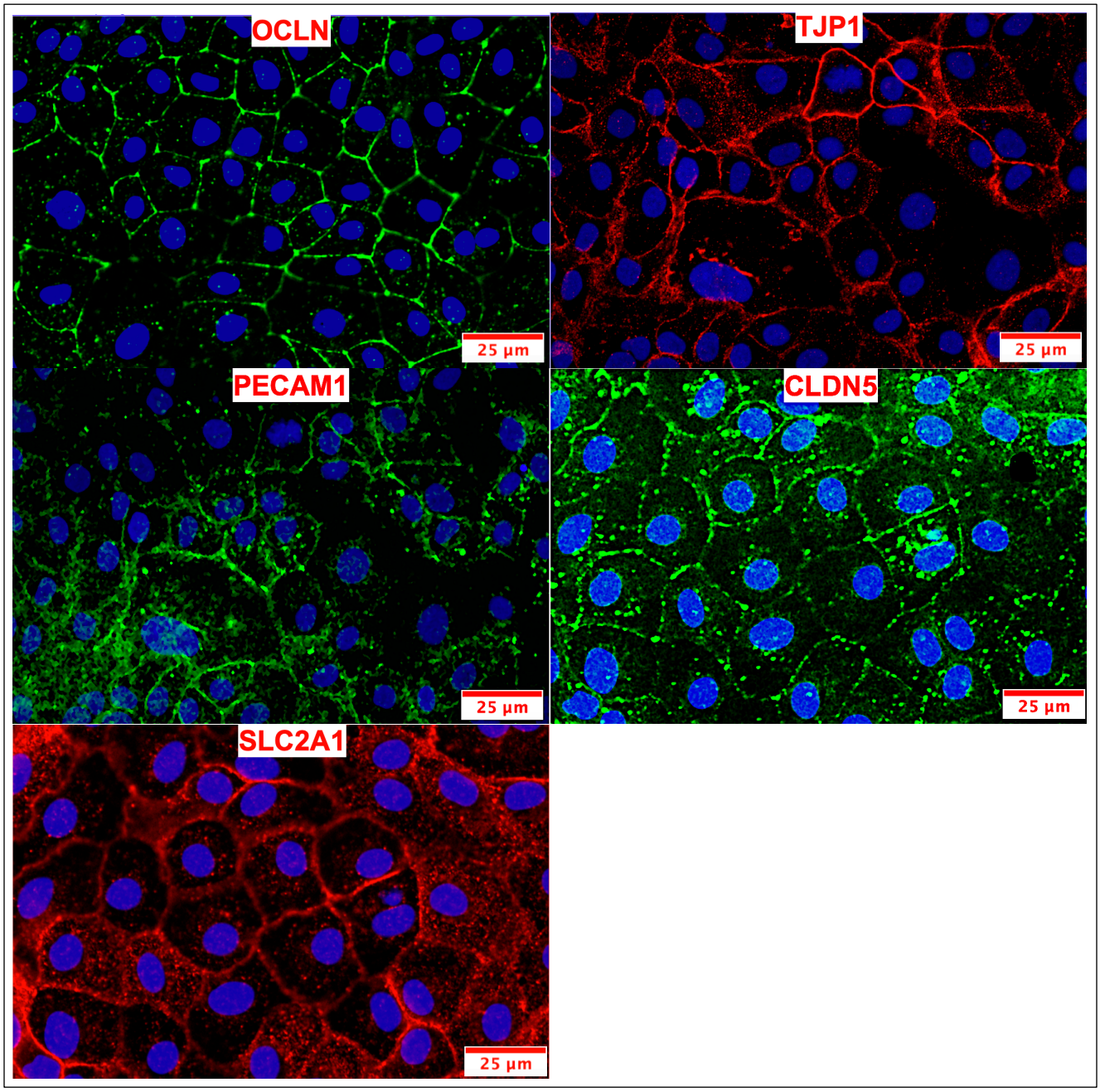
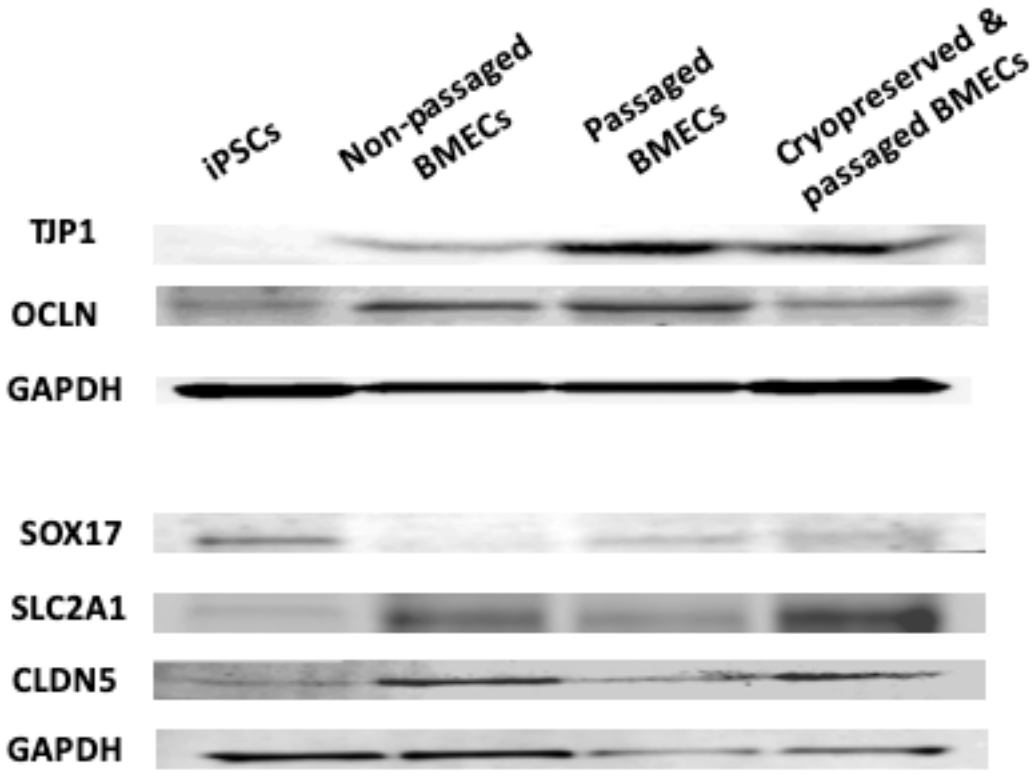


Figure 11



Name of Material/ Equipment

2′,7′-dichlorodihydrofluorescein diacetate
Accutase
Alexa Fluor 488 Donkey anti-Mouse IgG
Alexa Fluor 555 Donkey anti-Rabbit IgG
B27 Supplement
CD31 (PECAM-1) (89C2) Mouse mAb
CLDN5 (Claudin-5)
Collagen IV from human placenta
Corning 2 mL Internal Threaded Polypropylene Cryogenic Vial
Corning Costar Flat Bottom Cell Culture Plates (6-wells)
Corning Falcon Flat Bottom Cell Culture Plates (24-wells)
Corning Transwell Multiple Well Plate with Permeable Polyester Membrane Inserts (12-wells)
Countess slides
DMEM/F12 (without phenol red)
DMSO
Donkey serum
DPBS (+/+)
Epithelial Volt/Ohm (TEER) Meter (EVOM2) STX2
Essential 6 Medium (Thermo Fisher)
Fetal Bovine Serum (FBS)
Fibronectin
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix
Hanks' Balance Salt Solution with calcium and magnesium
Hoechst 33342, Trihydrochloride, Trihydrate
Human endothelial serum-free medium
InCell Analyzer 6000
Invitrogen Countess Automated Cell Counter
MK-571
NutriStem
Occludin
Paraformaldehyde 16%

Perkin Elmer Envision 2103 multi-plate Reader
Recombinant Human VEGF 165
Recombinant Human FGF-basic (154 a.a.)
Retinoic acid
Rhodamine 123
SLC2A1 (GLUT-1)
SOX17
TJP-1 (ZO-1)
Triton X-100
Trypan Blue Stain (0.4%) for use with the Countess Automated Cell Counter
Valspodar (Sigma) (cyclosporin A)
Versene solution
Y-27632 dihydrochloride (ROCK inhibitor)

Company	Catalog Number	Comments/Description
Sigma Aldrich	D6883-50MG	
Sigma Aldrich	A6964-100mL	
Life Technologies	A-21202	
Life Technologies	A-31572	
Thermo Fisher Scientific	17504044	
Cell Signaling	3528S	
Thermo Fisher Scientific	35-2500	
Sigma Aldrich	C5533-5mg	
Corning	8670	
Corning	353046	
Corning	353047	
Corning	3460	
Thermo Fisher Scientific	C10228	
Thermo Fisher Scientific	A1413202	
Sigma Aldrich	D2438-50mL	
Sigma Aldrich	D9663-10ML	
Gibco/Thermo Fisher Scientific	14040-117	
World Precision Instruments	N/A	
Thermo Fisher Scientific	A1516401	
Sigma Aldrich	F2442	
Sigma Aldrich	F2006-2mg	
Thermo Fisher Scientific	A1413202	
Thermo Fisher Scientific	24020-117	
Thermo Fisher Scientific	H3570	
Thermo Fisher Scientific	11111044	
General Electric	N/A	
Thermo Fisher Scientific	N/A	
Sigma Aldrich	M7571-5MG	
Stemgent	01-0005	
Thermo Fisher Scientific	33-1500	
Electron Microscopy Services	15710	

Perkin Elmer	N/A
Peprotech	100-20
Peprotech	100-18B
Sigma Aldrich	R2625-100MG
Sigma Aldrich	83702-10MG
ThermoFisher	PA1-21041
Cell Signaling	81778S
ThermoFisher	PA5-28869
Sigma Aldrich	T8787-50ML
Thermo Fisher Scientific	T10282
Sigma Aldrich	SML0572-5MG
Thermo Fisher Scientific	15040066
Tocris/Thermo Fisher Scientific	1254

We thank the editors and reviewers for their thoughtful and constructive comments. We have addressed the issues mentioned by the editors/reviewers and added new information and/or data to address the issue raised. With these changes, we believe that the manuscript has been significantly improved. We have outlined our point-by-point responses below in bold.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have proofread the manuscript to ensure there are no spelling or grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc.) have been added to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added the requested details. Since reviewer #1 asked for sprouting assay data, we have included the steps needed to perform a sprouting assay in this protocol (line 254-262 in Step 6). Additional details regarding TEER measurement collection have been added to the manuscript (line 307-315 in Step 8). Steps on cell passaging, expansion, and cryopreservation of iPSC-derived BMECs have been added as well (line 404-411 and 421-429 in Step 10).

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

We have formatted the numbering and margins accordingly.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3-page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

We have highlighted the filmable regions and confirm that they are less than 3 total pages.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The discussion section has been re-organized along this format with the subsections provided above. Since the reviewers had asked for additional experiments and protocol details, we have provided additional information on 1) expanding BMECs in the "Critical steps within the protocol" (line 657-659) section, 2) troubleshooting TEER measurement in the "Modification and troubleshooting" (line 596-600) section, and 3) peak TEER variations, cryopreservation and passaging in the "Limitation of the technique" (line 604-613) sections of the discussion.

• **Figures:** Please remove the figure/table legends from the figure files and place them directly below the Representative Results text.

The figure legends have been removed from the figure file and placed directly below the representative results text.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are NutriStem, geltrex, Falcon, cryovials, InCell Analyzer 6000, Olympus,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

The commercial terms were changed as follows: NutriStem to stem cell medium; Versene to nonenzymatic EDTA; Accutase to enzymatic EDTA; Geltrex to basement membrane matrix. Other commercial names such as Falcon, Cryovials, STX2, EVOM2, InCell Analyzer 6000, and Olympus have been removed from the manuscript.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

The figures and tables included in this manuscript are original and have not been previously published.

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
In this study the authors report the development of in vitro high-throughput brain angiogenesis assay. The authors differentiated IPS line into putative brain endothelial cell using the method introduced in Lippmann et al. (Nature Biotech, 2012; Sicen 2014), where it was demonstrated that addition of 10µM retinoic acid (RA) during by day 6 of dhBMECs differentiation induces VE-cadherin expression prior to purification step and enhances BBB properties (e.g. expression of tight junction proteins and TEER). dhBMEC are then expanded and their ability to retain BBB traits tested. While some aspects explored in this paper are pertinent to the field, the nature of this paper (use of IPS differentiated cells) is always challenging to interpret in absence of relevant controls / cellular identity.

We agree that there are challenges with interpreting iPSC-derived BMECs in the absence of relevant controls/cellular identity. Prior studies that laid the foundation for generating BMECs from human iPSCs had used expression of BMEC relevant markers, the ability to sprout, transcriptomic expression of the angiogenic/blood development pathway, physiologic TEER values, and transporter assays for characterization. We adapted the protocol developed by the Lippmann group (described in Neal et al 2019) by using a different healthy human donor as well as using different reagents at the start of the protocol. Using this adapted protocol, we differentiated a human iPSC line into BMECs and examine the same markers (CLDN5, Occludin, PECAM1, SLC2A1, TJP1) and assays (TEER, Efflux transporter) performed in the Lippmann protocol. As recommended by the reviewers, we also performed experiments to assess for endodermal markers by using SOX17 in our different conditions: (iPSCs, non-passaged-BMECs, passaged-BMECs and cryopreserved-passaged-BMECs). We found SOX17 levels to be high in the iPSCs but not in the BMECs (Figure 11). We also performed a sprouting assay and showed that the BMECs we generated are capable of forming tube-like structures (Figure 3), adding more experimental support for the BMEC phenotype.

Major Comments:
- The authors based their study on previously published protocols. Did the authors use all the specific reagents and the specific concentrations of those reagents in appropriate temporal modes to exactly replicate the study conditions of the prior work? Were the same PSC lines used as in the prior published work? To what extent could these variables influence the interpretation of the present report?

We thank the reviewer for pointing this out and have made several efforts throughout the manuscript to clarify that we performed an adapted assay based on Lippmann's protocol. We also tried to clarify in what ways our protocol differed from the Lippmann protocol. Two of the reagents (Geltrex and NutriStem) used in this proposed protocol were different from those used by Neal et al. 2019 (Matrigel and mTeSR). In terms of reagent concentrations and temporality, we followed the same concentrations and temporal approaches reported by Neal et al. 2019. These reagent/matrix changes did not impact maximum TEER values or BMEC cell markers. We used a different human iPSC line than was used in the previous publications, which is a reassuring validation of the previously described methods.

- Recent studies suggested that protocol used in this study could yield cells harboring either an epithelial/endothelial (Delsinger al., StemCells, 2018) mixed phenotype or an epithelial phenotype (Lu et al., BiorXiv, 2019). Since endothelial identity is primordial to interpret the present data (do the authors study brain angiogenesis, or sprouting of any other cell type arising from their differentiation protocol). The authors should obtain the transcriptome of bulk dhBMECs by RNA-sequencing and compare it to previously published IPS-derived brain endothelial cells, and to appropriate endothelial and non-endothelial (hematopoietic, epithelial, fibroblastic) controls.

We agree that differentiating endothelial and epithelial cells in this protocol is important. While the presences of a high TEER value is usually a phenotypic hallmark of BMEC blood brain barrier function, there are some epithelial cells that can attain TEER values as high as 3000 Ω x cm^2 (Srinivasan 2015). As suggested by the Reviewer, we performed a sprouting assay and found that the iPSC-derived BMECs demonstrated sprouting capability after 3 days of VEGFA165 treatment (Figure 3). While we did not undertake transcriptomic studies, Lim et al 2017 had performed a transcriptomic analysis of BMECs derived using a similar protocol and found that BMEC express gene networks that are involved in angiogenesis and blood development.

Minor Concerns:
- Do the dhBMECs show any endothelial cells specific protein markers (i.e. CDH5, SOX7, SOX17, ERG,

ENOS) or markers highly expressed specifically in brain endothelium (i.e. ZIC3, FOXF2, FOXQ1, TBX1) by microscopy or western blot analysis?

We have not tested those specific markers since we focused primarily on the expression of OCLN, CLDN5, SLC2A1, PECAM1, and TJP1 to characterize the BMECs, as described in prior studies to (Lippmann 2012, Lippmann 2014, Hollmann 2017, and Neal 2019). We have added western blot data as well for OCLN, CLDN5, SLC2A1, PECAM1, and TJP1. These proteins were present in BMECs, but not in iPSCs. We also tested for the endodermal marker, SOX17, and found that this marker was expressed by iPSCs and not in BMECs.

- How does the expression of brain specific vascular gene transcripts (ZIC3, FOXF2, FOXQ1, TBX1) vary between dhBMECs and the control HUVECs? Markers such as Occludin and GLUT1 have been shown to be highly expressed in various other cell types from a variety of tissues and may not serve as good brain specific endothelial markers.

We agree that while individually occludin and GLUT1 are not purely brain endothelial specific markers, the co-expression of these markers is unique to brain endothelial cells. When the presence of these markers is combined with high TEER values, efflux transporter activity, and angiogenic potential, we can be more confident that the derived cells are BMECs.

Reviewer #2:
Manuscript Summary:
The manuscript entitled, "Derivation, Expansion and Characterization of Brain Microvascular Endothelial Cells from Human Induced Pluripotent Stem Cells" described a detailed method to differentiate induced BECs (using reported E6, EM+B27 strategy) and a strategy to how these cells can be passaged during differentiation.

Major Concerns:
None

Minor Concerns:
1. The first part of the manuscript provides a detailed protocol adapted from Lippman's published work. A newer strategy to cryopreserve the cells is introduced using Nutristem medium+RI+10%DMSO. Some details as to the post-thaw recovery should be highlighted and how this compares to the published serum-containing cryo protocols described. In addition to viability, what is the time-window to establish TEER for the cryopreserved cells?

As note for reviewer one, we have clarified the ways in which our protocol overlaps and differs from the Lippmann protocol. Additionally, we have made changes to the manuscript to focus on the cryopreservation protocol, to compare this to other cyro protocols, and provide additional data regarding the post-thaw recovery of BMECs. In brief, we cryopreserved iPSC-derived BMECs in hESFM with 10% DMSO and 30% bovine serum as described in Wilson et al. 2016. We thawed these cells and re-plated them onto COL4/FN coated plates and TEER value peaked at ~800 Ω x cm^2 six days after thawing, which continued to decrease thereafter (Figure 9). Further passages of the thawed iPSC-derived BMECs resulted in lowering of the TEER values, ranging from 20-380 Ω x cm^2 (Figure 9).

2. To plate single cells for differentiation, the authors incubate iPSC in Accutase for 15mins at 37C. This is a long time, rationale for this should be detailed or caution statement inserted that this may need to be optimized for individual iPSC lines.

We apologize for this typo. We incubated the iPSCs in Accutase for 5 minutes, not 15 minutes. For the iPSC-derived BMECs at the end of day 6, the cells were incubated for at least 15 minutes. We have made these changes in the manuscript.

3. For their ICC, the authors showed OCLN, TJP1, CLDN5, SLC2A1 and PECAM1 (Fig3), SLC2A1 and PECAM1 are really patchy, faint and discrete membrane staining is poor (this is common for this protocol). It appears that after passaging, the SLC2A1 and PECAM1 expression are better and more uniform (Fig6). Is this indeed the case? The authors should describe this more clearly - is this due to the second selection process with passaging which increases purity of BEC cultures?

The issue with Figure 3 (now Figure 4 in the revised manuscript) was primarily due to poor image quality, which has been replaced with higher quality images. BMEC marker expression is similar under the two conditions (Figure 3 & Figure 8). Therefore, the second selection process did not improve the purity of the BMEC cultures.

4. The BECs were passaged on Day10 (4days after re-plating), all the functional tests were done on Day 8 - why did the authors choose Day10 to passage (need rationale and window-time frame).

We chose to passage the BMECs when they had reached ~ 100 % confluency to optimize successful cell passaging, which happened on Day 10, 4 days after re-plating.

5. Is it possible to re-passage the BECs for multiple times and establish BEC cryo-stocks?

To address this question, we thawed previously cryopreserved iPSC-derived BMECs and found that TEER values peaked at ~800 Ω x cm^2 six days after thawing, and continued to decrease thereafter (Figure 7). Upon further passaging, the BMECs exhibited lower TEER values, ranging from 20-380 Ω x cm^2 (Figure 9) and showed frayed and freckled patterns of tight junction formation (Figure 10).

6. Areas of optimization should be highlighted as this will be required for different iPSC lines. Furthermore, it will validate passaging strategy across multiple lines.

To optimize the passaging protocol, we performed a 1:3 split of BMECs that had reached 100% confluency on COL4/FN, which provided a cell count greater than twice the density used during the original sub-culturing steps (400,000 cells/well vs 156,000 cells/well). The 1:3 split used was effective for maintaining BMEC fidelity after passaging. Further optimization of BMEC passaging may depend on the cell line being used, potentially requiring longer periods in endothelial serum-free culture medium and/or a longer sub-culturing stage to enhance BMEC differentiation.

Summary
Overall, this manuscript is well written and has a detailed protocol which is really clear and straight forward to follow.

Reviewer #3:
Manuscript Summary:
The manuscript is reporting on a protocol for the derivation and expansion of brain microvascular endothelial cells from hiPSCs. The auyhors have optimized the induction process by using E6 medium and showed the possibility to passage once the culture in order to increase the number of cells that can be obtained. The manuscript is well written and the protocol clearly described in all of its parts and details.

Major Concerns:
Beside the limited novelty of the induction protocol, authors should concentrate a bit more on the expansion part that should represent the real novel part of the work. They do not perform cell counting experiments in order to make clear how this expansion step could improve the cells that it is posible to obtain. It is not clear what happens to the cells if a further passaging step is performrd. Do they stop proliferating? Do they die? Do they change their antigenic and/or functional properties?

We thank the reviewer for this comment. We have addressed this above for reviewer 1 and 2 in regards to the induction and cryopreservation protocol. We have made several changes throughout the text to highlight the cell expansion steps. In regards to performing cell counting prior to expansion, please see the response to reviewer 2 question 6. In brief, 100% confluency of BMECs resulted in proliferating cells that when split 1:3 provided a cell density that is greater than twice what is recommended for the initial sub-culturing stage. This expansion technique resulted in the reliable expansion of BMECs. We also examined the expansion potential of cryopreserved BMECs and found that the passaged BMECs continued to proliferate (Figure 7), but resulted in much lower TEER values compared to freshly-derived BMECs (Figure 9). ICC analysis of these cryopreserved & passaged BMECs also showed frayed and freckled patterns of the five markers (OCLN, TJP1, PECAM1, CLDN5, and SLC2A1) (Figure 10).

The quality of the staining on expanded cultures is very low with strong nuclear specific signal; I suggest to improve these. Also, analysis by qRT-PCR should be presented to corroborate the results of the IF.

We agree with the reviewer regarding the cell staining and image quality. We have improved the quality of these images in Figure 4 and 8. Previous results using this protocol had shown the expression of tight junctional markers found in brain endothelial cells (Lippmann et al. 2012, 2014, Hollmann et al. 2017, and Neal et al. 2019). We confirmed the expression of these BMEC proteins by western blot analysis as described above (Figure 11).

Minor Concerns:
The authors should perform a better comparison with existing protocols and clearly state where they have introduced novel aspects with respect to the existing.

As suggested by reviewers, we have noted the differences between our protocol and previous protocols for deriving BMECs. We also shifted the focus of our protocol to highlight the expansion and cryopreserving aspects of the protocol.

Reviewer #4:
Manuscript Summary:
The authors describe a detailed protocol for the derivation of brain microvascular endothelial cells from human induced pluripotent stem cells (hiPSC-BMECs), as well as putative evidence that hiPSC-BMECs can be passaged and expanded during the sub-culturing phase. While the manuscript is well written, the innovation and impact seem very low. Most of the protocol is derived from other published studies and the results for passaging of hiPSC-BMECs are not fully developed. The protocol would greatly benefit from additional experiments that show the utility and characteristics of the expanded hiPSC-BMECs.

Thank you so much for raising this concern. We have addressed this above for reviewers 1-3. We agree that BMEC passaging and cryopreservation are the new elements in this protocol and should be the focus the current protocol. We have included additional data to show that iPSC-

derived BMECs can be passaged, cryopreserved and re-passaged, as descried above and shown in Figures 7,9,10, and 11.

Major Concerns:
*The authors state in the Discussion that "This current protocol provides a streamlined method for obtaining human BMECs from iPSCs." However, the methods detailed in this protocol are virtually identical to those published in Neal et al. Stem Cell Reports. 2019 (reference 1). The steps taken to streamline the protocol should be described clearly along with this statement.

We have adjusted the language to make it clear that the initial method for human BMEC generation is not different from the Neal et al report.

*The immunocytochemistry results in Figure 3 for PECAM1 and SLC2A are not convincing. It appears that less than 50% of the cells express SCL2A. This should be quantified and addressed in the discussion. Very few cells appear to express PECAM1 above background levels. No primary controls should be included to better demonstrate positive PECAM1 signal.

We have replaced the images for PECAM1 and SLC2A1 (Figure 4 in the revised manuscript) which provide a better representation of cellular staining. We also performed western blotting for SLC2A1 and found high protein levels in BMECs compared to iPSCs (Figure 11).

*In the Results section, the authors state "inhibition of ABCB1 and ABCC1 efflux transporters with PSC833 (ABCB1 inhibitor) or MK-571 (ABCC1 inhibitor) led to an increase in rhodamine 123 (R123) or 2',7'- dichlorodihydrofluorescein diacetate (H2DCFDA) respectively", however, no statistical analysis is shown. Appropriate statistical analysis should be performed for Figure 5.

We ran a student's t-test analysis for the technical replicates in the experiment. We have included this in the revised figure (now Figure 6 in the revised manuscript).

*The authors claim robust expression of OCLN, TJP1, CLDN5, SLC2A1, and PECAM1 after passaging of hiPSC-BMECs, however, the ICC shown in Figure 6 is not convincing. The background is much higher and/or the signal is lower in these images. The expression levels for most of the markers appear to be diminished. The expression of each marker should be quantified (by flow cytometry, western blotting, and/or ICC image analysis) and compared to the levels before passaging (Figure 3).

While we observed the expression of these endothelial markers, we agree that "robust" may not be the apt description for the level of expression. We have also replaced the ICC images with ones that more clearly show the expression of OCLN, TJP1, CLDN5, SLC2A1, and PECAM1 (Figure 8), along with western blotting data for these proteins (Figure 11). The ICC and western blotting results also compare the levels in non-passaged BMECs (first induction), passaged BMECs (after expansion), and cryopreserved passaged BMECs (after freeze/thaw and expansion. We also performed similar experiments for the measurement of TEER.

*In many cases, the TEER values for BMECs decrease up to day 8 of subculturing before recovering to higher values at later timepoints. In Figure 6, the authors should compare the TEER values for their passaged BMECs with values for BMECs that remain in the original subculture.

We have included TEER values from non-passaged BMECs, passaged BMECs, cryopreserved BMECs, and cryopreserved & passaged BMECs, which are shown in Figure 9.

*The authors should describe how many times the cells can be passaged before high TEER values can no longer be obtained.

While we had not passaged the original BMECs further to determine their ability to retain high TEER values, we performed further passaging of the cryopreserved BMECs and found that the TEER values decreased significantly after two additional passages (Figure 9).

Minor Concerns:
*Some of the units are missing or incorrectly displayed in several sections of the protocol.

We have corrected these errors.

*In the abstract, the authors state that the "This protocol also demonstrates expansion capability of iPSC-derived BMECS beyond 8 days, which overcomes previous limitation of necessitating freshly derived BMECs for each experiment". However, since their cells are not cryopreserved, the BMECs still need to be freshly derived from hiPSCs even with the single passage and expansion. There is insufficient evidence that the cells can be maintained long enough to provide sufficient flexibility that would eliminate the need to derive the BMECs for each experiment.

We thank the reviewer for pointing this out. We have thawed cryopreserved BMECs, passaged them and recorded TEER measurements as described above (Figures 7, 9, 10).

The authors state that "Current protocols recommend that iPSC-derived BMECs should be freshly derived each time due to decreasing TEER values observed after day 8." A reference should be provided and the authors should address the fact that publications by the Lippmann group have shown high TEER values up to 22 days of sub-culturing.

We agree that high TEER values were indeed observed in the Lippmann group after 22 days of sub-culturing and we have removed that statement to avoid any confusion. When we followed this protocol with a different iPSC line, we did not observe sustained high TEER values beyond day 8 of sub-culturing. We have made changes in the discussion section of the manuscript (line 604-613) to address the reviewer's comment.

*Add the end of the third paragraph in the results section there is a "(2019)", which appears to be a note for a reference.

We have addressed this.

*The authors achieve TEER values of ~2000 Ω x cm2, yet, greater TEER values are obtained without the need for co-cultures by the Lippmann group. Is this dependent on the clone of hiPSC used? This should be addressed in the discussion.

We agree with the reviewer that TEER values may depend on the hiPSC used. Compared to Neal et al. 2019, our peak TEER values are lower than those reported in their study. However, our peak TEER values fall within the range of 2000-8000 Ω x cm^2, which is similar to those values reported in Neal et al. 2019. We have discussed this in the discussion section of the revised manuscript.

*The legend for Figure 1 states: "On day 4, medium was changed to hESFM with 200x B27 supplement...". Was the B27 supplement diluted to 1x from 200x (1:200)?

Thanks for pointing this out. We have corrected this.