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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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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

    ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **35**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Sovannarath Pong**: BMECs generated from iPSCs provide new experimental models for studying blood-brain barrier function and dysfunction. We have used this protocol to examine whether BMECs retain their barrier properties after expansion and cryopreservation **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Sovannarath Pong**: This technique enables the generation of disease-specific BMECs from patients with disorders in which blood-brain barrier disruption plays a role in the pathophysiology of disease **[1]**

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Brain Microvascular Endothelial Cell (BMEC) Differentiation from Human Induced Pluripotent Stem Cell (iPSC)

2.1. To induce BMEC (B-M-E-C) differentiation from human iPSCs (eye-P-S-Cs), wash the confluent iPSC culture with DPBS (D-P-B-S) one time **[1-TXT]** before incubating the cells with the appropriate volume of enzymatic EDTA (E-D-T-A) for approximately 5 minutes at 37 degrees Celsius **[2]**.

2.1.1. WIDE: Talent washing cells, with DPBS container visible in frame **TEXT: DPBS: Dulbecco's Phosphate Buffer Saline**

2.1.2. Talent adding EDTA to culture, with EDTA container visible in frame

2.2. When a single cell suspension has been obtained, collect the cells by centrifugation **[1]-TXT]** and resuspend the pellet in an appropriate volume of stem cell medium supplemented with 10-micromolar rock inhibitor for counting **[2-TXT]**.

2.2.1. Talent placing tube(s) into centrifuge **TEXT: 5 min, 300 x g, RT**

2.2.2. Shot of pellet if visible. **TEXT: See text for all medium and solution preparation details**

2.3. Dilute the cells to a 1.56 x 10^4 cells/cubic centimeter concentration **[1]** and seed 2 milliliters of cells into individual wells of a 6-well, flat-bottom **[2]**.

2.3.1. Talent adding medium to tube, with medium and Y-27632 containers visible in frame *Videographer: Important/difficult step*

2.3.2. Talent adding cells to well(s) *Videographer: Important/difficult step*

2.4. After 24 hours in the cell culture incubator, replace the supernatant in each well with E6 medium **[1]** and return the plate to the incubator for 4 more days **[2-TXT]**.

2.4.1. Talent adding E6 medium to well(s), with medium container visible in frame *Videographer: Important step*

2.4.2. Talent placing plate into incubator *Videographer: Important step* **TEXT: Refresh medium daily**

3. iPSC-Derived BMEC Subculture and Purification

3.1. To purify the iPSC-derived BMECs, coat the subculturing plates with the appropriate volume of a freshly prepared collagen four and fibronectin solution per well **[1]** and incubate the plates for a minimum of 2 hours at 37 degrees Celsius **[2]**.

3.1.1. WIDE: Talent adding solution to well(s), with collagen 4 and fibronectin containers visible in frame *Videographer: Important step*
3.1.2. Talent placing plate into incubator *Videographer: Important step*

3.2. On day 6 of differentiation, collect the cells by centrifugation **[1]** and resuspend the pellets in the appropriate volume of **[2]** fresh human endothelial serum-free medium supplemented with B27 (B-twenty-seven), basic fibroblast growth factor, and retinoic acid **[2B]**.

3.2.1. Talent placing tube(s) into centrifuge *Videographer: Important step*
3.2.2. Shot pellet(s) if visible.
Added shot: 3.2.2B Then medium being added to tube, with medium container visible in frame

3.3. For TEER (tear, rhymes with fear) analysis, plate the differentiated cells into each well of a collagen- and fibronectin-coated 12- transwell filtered plate **[1-TXT]**. For efflux transporter activity analysis, plate the cells into each well of a collagen- and fibronectin-coated 24-well flat-bottom plate for **[2]**.

3.3.1. Talent adding cells to well(s) of 12-well plate *Videographer: Important step*
3.3.2. Talent adding cells to well(s) of 24-well plate *Videographer: Important step*
TEXT: Seed undifferentiated iPSC as negative controls

3.4. After 24 hours of sub-culture, replace the supernatant in each well with fresh human endothelial serum-free medium supplemented only with B27 supplement **[1]**.

3.4.1. Talent adding medium to well(s), with medium container visible in frame

4. Trans-Endothelial Electrical Resistance (TEER) Measurement and Analysis

4.1. To measure the blood-brain barrier properties of the BMECs using TEER, charge the TEER instrument overnight and lightly wipe the instrument and chopstick electrodes with 70% ethanol **[1]** before placing them into the safety hood **[2]**.

4.1.1. WIDE: Talent wiping instrument(s)
4.1.2. Talent placing instrument(s) into hood

4.2. Switch on the power **[1]** and calibrate the ohm meter according to manufacturer's specifications **[2]**.

4.2.1. Talent switching on power
4.2.2. Talent calibrating meter

4.3. Plug in the electrodes **[1]** and rinse them one time with 70% ethanol and one time with DPBS **[2]**.

4.3.1. Talent plugging in electrodes
4.3.2. Talent rinsing electrodes, with ethanol and DPBS containers visible in frame *Videographer/Video Editor: shot will be used again*

4.4. Place the shorter end of the electrode into the apical chamber of the insert **[1]** and the longer end into the basolateral chamber of a blank well **[2]**.

4.4.1. Electrode being placed into insert *Videographer: Important step*
4.4.2. Electrode being placed into bottom of well *Videographer: Important step*

4.5. After measuring the negative control response **[1]**, quickly rinse the electrodes as demonstrated **[2-TXT]** and measure the first experimental well **[3]**.

4.5.1. Talent measuring response
4.5.2. Use 4.3.2. Talent rinsing electrode(s) **TEXT: Rinse between experimental conditions**
4.5.3. Talent placing electrode(s) into insert and/or well

4.6. After all of the measurements have been recorded, rinse chopstick electrodes again **[1],** gently wipe the electrodes **[2]**, and let them air dry in the safety hood **[3]**.

4.6.1. Use 4.3.2. Talent rinsing electrode(s)
4.6.2. Talent wiping electrode(s)
4.6.3. Talent placing electrodes onto hood surface to dry

5. Efflux Transporter Activity and Analysis

5.1. To assess the efflux transporter activity of the cells, on day 8 of culturing, treat the appropriate number of wells with a 10-micromolar efflux transporter inhibitor solution and **[1]** place in an incubator for 1 hour at 37 degrees Celsius **[1B]**.

5.1.1. WIDE: Talent adding inhibitor to well(s), with inhibitor containers visible in frame.
Added: 5.1.1B. Incubator shot was added.

5.2. At the end of the incubation, treat the cells with a 10-micromolar efflux transporter substrate solution **[1]** for 1 hour in the incubator **[1B]**.

5.2.1. Talent adding substrate to well(s), with substrate container visible in frame.
Added: 5.2.1B. Incubator shot was added.

5.3. At the end of the incubation, wash each well twice with 500 microliters of DPBS per well **[1]** before lysing the cells with 500 microliters DPBS supplemented with 5% Triton-X per well **[2]**.

5.3.1. Talent washing well(s), with DPBS container visible in frame
5.3.2. Talent adding DPBS + Triton-X to well(s), with DPBS + Trion-X container visible in frame

5.4. After 5 minutes, use a microplate reader to measure the fluorescence of the lysed cells at a 485-nanometer excitation and a 530-nanometer emission **[1]**.

5.4.1. Talent loading plate onto plate reader

5.5. For wells not used in the transporter assay, wash the cells twice with DPBS **[1]** before fixing with 4% paraformaldehyde for cell nuclei quantification **[2]**.

5.5.1. Talent washing well(s), with DPBS container visible in frame
5.5.2. Talent adding PFA to well(s), with PFA container visible in frame

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.3., 2.4., 3.1.-3.3., 4.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
2.3. is the most difficult and important aspect of this procedure since the density of cells can affect the efficiency of differentiation and formation of blood-brain barrier properties. Investigators should verify the accuracy of their cell counting by taking multiple measurements at this step.

Results

6. Results: Representative BMEC Differentiation and Characterization After Passaging, Subculture, and Cryopreservation

6.1. After one day of culture in E6 medium **[1]**, the cell morphology is similar to that of iPSCs **[2]**.

6.1.1. LAB MEDIA: Figure 2 Day 1 image
6.1.2. LAB MEDIA: Figure 2 Day 1 image *Video Editor: please outline at least one cell*

6.2. By day 4, the cells are visibly distinct from iPSCs and cover most of the well **[1]**.

6.2.1. LAB MEDIA: Figure 2 Days 1 and 4 images

6.3. Two days of culture in human endothelial serum-free medium **[1]** induces an elongated and cobblestone appearance in the cells **[2]**.

6.3.1. LAB MEDIA: Figure 2 Days 1, 4, and 6 images
6.3.2. LAB MEDIA: Figure 2 Days 1, 4, and 6 images *Video Editor: please emphasize elongated cell(s) in Day 6 image*

6.4. At day 8, individual cells form a mostly large cobblestone pattern **[1]**.

6.4.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize cell(s) in Day 8 image*

6.5. A sprouting assay can be performed to demonstrate the angiogenic potential of iPSC-derived BMECs **[1]**. This leads to the development of tube-like structures after 3 days of growth factor treatment **[2]**.

6.5.1. LAB MEDIA: Figure 3
6.5.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize tube-like structures*

6.6. The iPSC-derived BMECs generated using this protocol co-express tight junction proteins expressed in endothelial cells in the brain, lung, liver, and kidney **[1]** as well as vascular endothelial markers expressed at the blood brain barrier **[2]**.

6.6.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize green and yellow signals in OCLN, TJP1, and CLDN5 images*
6.6.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize green and yellow signals in PECAM1 and SLC2A1 images*

6.7. As observed **[1]**, 48 hours after sub-culture and medium change, iPSC-derived BMECs demonstrate TEER values within the range described for iPSC-derived BMECs that were co-cultured with rat primary astrocytes. **[2]** Efflux transporter activity was also observed at this stage **[3]**.

6.7.1. LAB MEDIA: Figure 5
6.7.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize orange data line*
6.7.3. LAB MEDIA: Figure 6 *Video Editor: please emphasize R123 + PSC833 and H2DCFDA + MK-571 data bars*

6.8. After cryopreservation **[1]**, TEER measurements are lower **[2]** compared to freshly derived BMECs **[3]**. Western blot analysis reveals reduced levels of tight junction markers, such as occludin **[4]**, while immunocytochemistry shows freckled and/or frayed patterns of tight junction proteins **[5]**.

6.8.1. LAB MEDIA: Figures 9 and 11
6.8.2. LAB MEDIA: Figures 9 and 11 *Video Editor: please emphasize yellow data line*
6.8.3. LAB MEDIA: Figures 9 and 11 *Video Editor: please emphasize orange data line*
6.8.4. LAB MEDIA: Figures 9 and 11 *Video Editor: please emphasize OCLN band in Cryopreserved & Passaged BMECs lane*
6.8.5. LAB MEDIA: Figures 9 and 11 *Video Editor: please emphasize TJP1, SLC2A1, and CLDN5 bands in Cryopreserved & Passaged BMECs lane*

Conclusion

7. Conclusion Interview Statements

7.1. **Sovannarath Pong**: A too high or too low iPSC density will affect the differentiation efficiency and impact the development of appropriate blood-brain barrier properties **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.3.)

7.2. **Sovannarath Pong**: This method enables the investigation of barrier properties, cellular signaling, and transporter activity using individualized BMECs to model the physiology and pathophysiology of the blood-brain barrier function **[1]**.

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera