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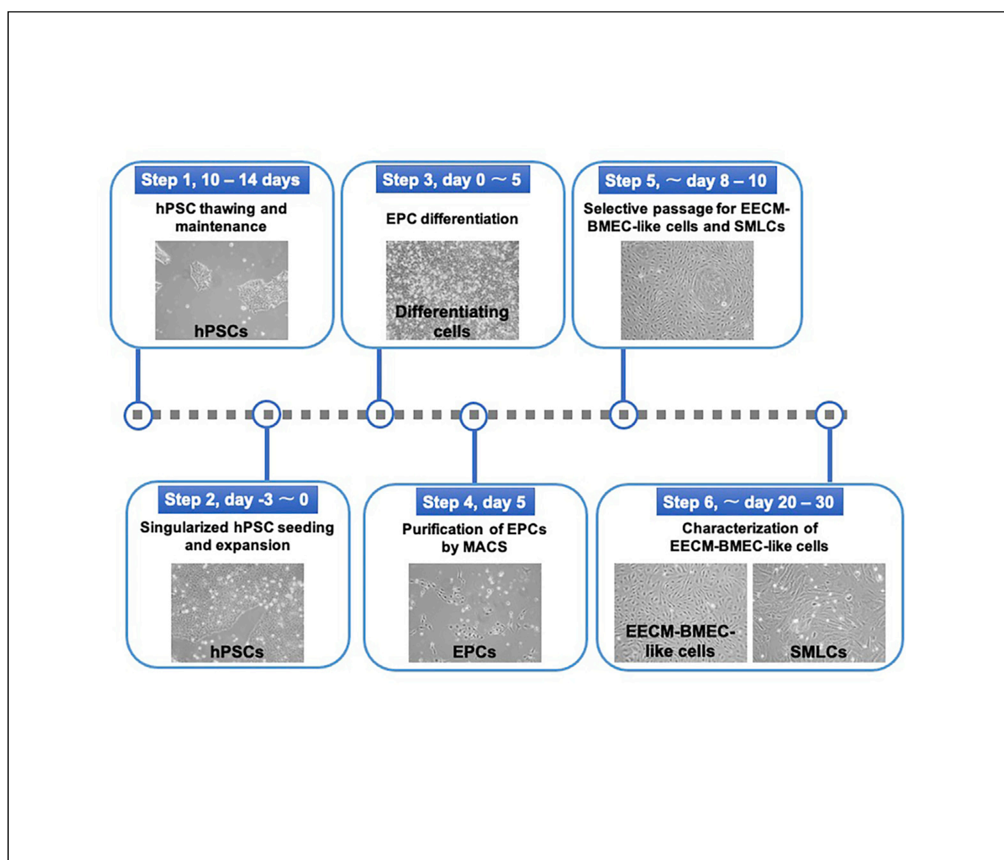
Protocol

Differentiation of human pluripotent stem cells to brain microvascular endothelial cell-like cells suitable to study immune cell interactions



We describe the extended endothelial cell culture method (EECM) for the differentiation of human pluripotent stem cells (hPSCs) into brain microvascular endothelial cell (BMEC)-like cells. EECM-BMEC-like cells resemble primary human BMECs in morphology, molecular junctional architecture, and diffusion barrier characteristics. A mature immune phenotype with proper endothelial adhesion molecule expression makes this model distinct from any other hPSC-derived *in vitro* BBB model and suitable to study immune cell migration across the BBB in a disease relevant and personalized fashion.

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Highlights

EECM-BMEC-like cells: a human pluripotent stem cell-derived BBB model

Detailed protocol to differentiate human EECM-BMEC-like cells

In-depth description of quality control assays for EECM-BMEC-like cells

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Differentiation of human pluripotent stem cells to brain microvascular endothelial cell-like cells suitable to study immune cell interactions

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SUMMARY

We describe the extended endothelial cell culture method (EECM) for the differentiation of human pluripotent stem cells (hPSCs) into brain microvascular endothelial cell (BMEC)-like cells. EECM-BMEC-like cells resemble primary human BMECs in morphology, molecular junctional architecture, and diffusion barrier characteristics. A mature immune phenotype with proper endothelial adhesion molecule expression makes this model distinct from any other hPSC-derived *in vitro* blood-brain barrier (BBB) model and suitable to study immune cell migration across the BBB in a disease relevant and personalized fashion.

For complete details on the use and execution of this protocol, please refer to Lian et al. (2014), Nishihara et al. (2020a).

BEFORE YOU BEGIN

Timing: 2 days to a week

For background information on the development of the protocol, please refer to (Lian et al., 2014) for the endothelial progenitor cell (EPC) differentiation and (Nishihara et al., 2020a) for the Extended Endothelial cell Culture Method (EECM)-BMEC-like cell differentiation.

1. Acquire all critical reagents mentioned in the key resources table.
2. Prepare all stock solutions listed below and make the necessary working aliquots.
3. Order the antibodies found in the key resources table for quality control.
4. Prepare media using recipes below, only when needed.
5. Acquire/thaw hPSCs and maintain as undifferentiated cultures in mTeSR based medium.

Cells need to be passaged at least twice before starting the differentiation.

Note: Both the hPSC maintenance and differentiation media are stable for up to 2 weeks at 4°C.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD31 Antibody, anti-human, FITC (clone AC128), (dilution 1:200)[b]	Miltenyi Biotec	Cat#130-117-390
APC-Cy7 Mouse Anti-Human CD31 (clone WM59), (0.5 µL/sample)[f]	BD Biosciences	Cat#563653
APC-Cy7 Mouse IgG1, κ Isotype Control (clone MOPC-21), (0.5 µL/sample)[f]	BD Biosciences	Cat#557873
APC Mouse Anti-Human CD62E (clone 68-5H11), (2 µL/sample)[f]	BD Biosciences	Cat#551144
APC Mouse IgG1, κ Isotype Control (clone MOPC-21), (2 µL/sample)[f]	BD Biosciences	Cat#555751
BV421 Mouse Anti-Human CD54 (clone HA58), (0.5 µL/sample)[f]	BD Biosciences	Cat#564077
BV421 Mouse IgG1, k Isotype Control (clone X40), (0.5 µL/sample)[f]	BD Biosciences	Cat#562438
BV510 Mouse Anti-Human CD62P (clone AC1.2), (2.5 µL/sample)[f]	BD Biosciences	Cat#743756
BV510 Mouse IgG1, k Isotype Control (clone X40), (2.5 µL/sample)[f]	BD Biosciences	Cat#562946
FITC Mouse Anti-human CD106 (clone 51-10C9), (20 µL/sample)[f]	BD Biosciences	Cat#551146
FITC Mouse IgG1, κ Isotype Ctrl Antibody (clone MOPC-21), (5 µL/sample)[f]	BioLegend	Cat# 400108
PE Mouse Anti-Human CD102 (clone CBR-IC2/2), (4 µL/sample)[f]	BD Biosciences	Cat#558080
PE Mouse IgG2a kappa Isotype Control (clone eBM2a), (1 µL/sample)[f]	Invitrogen	Cat#12-4724-42
PE/Cyanine7 anti-human CD99 Antibody (clone 3B2/TA8), (0.5 µL/sample)[f]	BioLegend	Cat#371314
PE/Cyanine7 Mouse IgG2a, κ Isotype Ctrl Antibody (clone MOPC-173), (0.5 µL/sample)[f]	BioLegend	Cat#400232
Anti-VE-cadherin antibody (clone F-8), (dilution 1:200), (fixative MeOH)[e]	Santa Cruz Biotechnology	Cat#sc-9989
Claudin 5 Monoclonal Antibody (clone 4C3C2), (dilution 1:200), (fixative MeOH)[e]	Invitrogen	Cat# 35-2500
Occludin Monoclonal Antibody (clone OC-3F10), (dilution 1:100), (fixative MeOH)[e]	Invitrogen	Cat#33-1500
Purified anti-human CD54 Antibody (clone HA58), (dilution 1:100), (fixative n/a, live cell staining)[e]	BioLegend	Cat#353102
Purified Mouse Anti-Human CD106 (clone 51-10C9), (dilution 1:100), (fixative n/a, live cell staining)[e]	BD Biosciences	Cat#555645
Actin, Smooth Muscle Ab-1, Mouse Monoclonal Antibody (clone 1A4), (dilution 1:200), (fixative 1% PFA)[e]	Fisher Scientific	Cat#MS113P
Monoclonal Anti-Calponin antibody produced in mouse (clone hCP), (dilution 1:15000), (fixative 1% PFA)[e]	Sigma-Aldrich	Cat#C2687
Anti-TAGLN/Transgelin (polyclonal), (dilution 1:1000), (fixative 1% PFA)[e]	Abcam	Cat#ab14106
Cy3 AffiniPure F(ab') Fragment Goat Anti-Mouse IgG, F(ab')2 fragment specific, (dilution 1:200)[e]	Jackson ImmunoResearch	Cat#115-166-006
Cy3 AffiniPure Donkey Anti-Rabbit IgG (H+L), (dilution 1:200)[e]	Jackson ImmunoResearch	Cat#711-165-152
Chemicals, peptides, and recombinant proteins		
AbC Total Antibody Compensation Bead Kit[f]	Invitrogen	Cat#A10497
Accutase[b,c]	Sigma-Aldrich	Cat#A6964-500ml
Acetic acid[b,c]	Sigma-Aldrich	Cat#695092
Advanced D-MEM/F-12[b]	Life Technologies	Cat#12634
B-27 Supplement (50×), serum free[b,c]	Thermo Fischer Scientific	Cat#17504044
Bovine serum albumin (BSA), 7.5% in dPBS[b,c]	Sigma-Aldrich	Cat#A8412-100ml
CellTracker Green CMFDA Dye[g]	Invitrogen	Cat#C7025
CHIR99021[b]	Selleck Chemicals	Cat#S1263
Collagen IV from human placenta[c]	Sigma-Aldrich	Cat#C5533
DAPI (4',6-Diamidino-2-Phenylindole, Dilactate), (concentration 1 µg/mL), (fixative PFA/MeOH)[e]	Invitrogen	Cat#D3571
Dimethylsulfoxide (DMSO), sterile[b,c]	Sigma-Aldrich	Cat#D2650
DMEM (1×), [+] 4.5 g/L D-glucose, [-] L-glutamine, [-] pyruvate[g]	Thermo Fischer Scientific	Cat#31053-028
Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM-F12)[a,b,c]	Thermo Fischer Scientific	Cat#11320074

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dulbecco's (d) PBS (without calcium, magnesium)[b]	Thermo Fisher	Cat#14190250
EasySepFITC Positive Selection Kit II[b]	STEMCELL Technologies	Cat#18558
Fetal Bovine Serum, qualified[b,c]	Thermo Fischer Scientific	Cat#10270106
Fibronectin from bovine plasma[c]	Sigma-Aldrich	Cat#F1141
Ficoll-Paque PLUS[g]	Sigma-Aldrich	Cat#GE17144002
Fluorescein sodium salt[d]	Sigma-Aldrich	Cat#F6377
GlutaMAX Supplement[b]	Thermo Fischer Scientific	Cat#35050-061
Glutaraldehyde solution[g]	Sigma-Aldrich	Cat#G6257
HCL[e]	Sigma-Aldrich	Cat#H1758
HEPES buffer solution[g]	Thermo Fischer Scientific	Cat#15630-056
Hoechst 33342 Solution, (concentration 4 μM)[e]	Fisher Scientific	Cat#PI62249
Human Endothelial SFM (hESFM)[b,c]	Thermo Fischer Scientific	Cat#11111-044
Human fibroblast growth factor 2[b,c]	Tocris	Cat#233-FB-500
Kanamycin Sulfate (100×)[g]	Thermo Fischer Scientific	Cat#15160-047
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate[b]	Sigma-Aldrich	Cat#A8960-5G
L-Glutamine 200 mM (100×)[g]	Thermo Fischer Scientific	Cat#25030-024
Matrigel, growth factor reduced[a,b]	Corning	Cat#354230
MEM NEAA (100×)[g]	Thermo Fischer Scientific	Cat#11140-035
Methanol[e]	Sigma-Aldrich	Cat#32213
Mowiol[e]	Sigma-Aldrich	Cat#81381
mTeSR1 complete kit (basal medium plus 5× supplement) [a,b]	STEMCELL Technologies	Cat#85850
NaCl[e]	Sigma-Aldrich	Cat#71376
Paraformaldehyde[e]	Millipore	Cat#104005
Pen Strep[g]	Thermo Fischer Scientific	Cat#15140-122
Recombinant Human IFN-gamma Protein[e,f,g]	R&D Systems	Cat#285-IF-100
Recombinant Human IL-2[g]	BD Biosciences	Cat#554603
Recombinant Human TNF-alpha Protein[e,f,g]	R&D Systems	Cat#210-TA-020
Recombinant Human VEGF165[c]	PeproTech	Cat#100-20
ROCK inhibitor Y-27632[a,b,c]	Tocris	Cat#1254
RPMI medium 1640[g]	Thermo Fischer Scientific	Cat#21875-034
Skim milk[e]	BD Biosciences	Cat#232100
Sodium azide (NaN_3)[e]	Sigma-Aldrich	Cat#71290
Sodium pyruvate[g]	Thermo Fischer Scientific	Cat#11360-039
Tris base[e]	Sigma-Aldrich	Cat#93362
Triton X-100[e]	Sigma-Aldrich	Cat#X100
Versene[a]	Thermo Fischer Scientific	Cat#15040-066
Water, sterile, cell culture[c]	Sigma-Aldrich	Cat#W3500
Experimental models: Cell lines		
iPS(IMR90)-4 human induced pluripotent stem cells	WiCell	RRID:CVCL_C437
H9 (WA09) human embryonic stem cells	WiCell	RRID:CVCL_9773
Other		
0.22 μm Syringe filter	TPP	Cat#99722
40 μm Falcon cell strainer[b]	Falcon	Cat#352340
5 mL Round-bottom tube[b]	SPL	Cat#40005
15 mL Centrifuge tube	Biofil	Cat#CFT011500
50 mL Centrifuge tube	Biofil	Cat#CFT011150
50 mL Centrifuge tube (fit for 40 μm Falcon cell strainer)[b]	nerbe plus	Cat#02-572-8001
96-Well plate, round bottom[f]	SPL	Cat#34096
Chamber slides[e,g]	Thermo Fischer Scientific	Cat#178599
Corning tissue culture plates (6-well)	Corning	Cat#3506
Corning tissue culture plates (12-well)	Corning	Cat#3512
Corning tissue culture plates (24-well)	Corning	Cat#3526
Corning tissue culture plates (96-well)	Corning	Cat#3596
Cryo Tube 20[c]	TPP	Cat#89020

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
EasySepMagnet[b]	STEMCELL Technologies	Cat#18000
PLAQUE EN VERRE 12 CAVITES D 16MM[d,e]	MMS	Cat#10489011
Scalpel[d,e]	FEATHER	Cat#2975-11
Transwells, PC Membrane, 0.4 μm, 12 mm, TC-Treated[d,e]	Corning	Cat#3401

[a]indicates reagents use for hPSCs thawing and maintenance (section 1–3 of protocol).
[b]indicates reagents use for EPC differentiation (section 4–6 of protocol).
[c]indicates reagents use for EECM-BMEC like cell differentiation (section 7–12 of protocol).
[d]indicates reagents use for measuring sodium fluorescein permeability (section 13 of protocol).
[e]indicates reagents use for immunofluorescence staining (section 14 of protocol).
[f]indicates reagents use for flow cytometry analysis (section 15 of protocol).
[g]indicates reagents use for T-cell adhesion assay (section 16 of protocol).

MATERIALS AND EQUIPMENT

We recommend against the use of antibiotics in culture and differentiation of hPSCs. Pay close attention to working at sterile conditions. Buy sterile centrifuge tubes, microcentrifuge tubes, and pipet tips or autoclave before use. Prepare medium under sterile and endotoxin-free conditions. If some materials are not sterile, use 0.22 μm syringe filters for sterile filtration.

mTeSR1 medium (commercially available mTeSR1 complete kit)	Final concentration	Amount
Basal medium	n/a	400 mL
5 × supplement provided from mTeSR1 complete kit	1 ×	100 mL
Total	n/a	500 mL

⚠ CRITICAL: Store mTeSR1 medium at 2°C–8°C for up to 2 weeks or −20°C for up to 6 months. Avoid warming the medium for extended periods of time in the 37°C water bath, because bFGF is unstable at this temperature.

Matrigel stock solution aliquot	Final concentration	Amount
Matrigel, growth factor reduced	2.5 mg	depends on lot
Total	n/a	depends on lot, approximately 250 μL

⚠ CRITICAL: Matrigel is sensitive to temperature. Place Matrigel at least 8 h at 4°C for thawing. Pre-chill pipet tips that fit a 1000 microliter pipette and microcentrifuge tubes at −20°C. When aliquoting Matrigel, place Matrigel and microcentrifuge tubes on ice within the biosafety cabinet. Store aliquots at −20°C up to 6 months. The Matrigel concentration varies from lot to lot. Consult the manufacturer's datasheet to calculate the precise volume of the aliquot containing 2.5 mg Matrigel.

Human fibroblast growth factor 2 (bFGF/FGF2) stock solution	Final concentration	Amount
Human fibroblast growth factor 2	100 μg/mL	500 μg
Dulbecco's (d) PBS	n/a	5 mL
Bovine serum albumin (BSA), 7.5% in dPBS	0.1%	66.7 μL
Total	n/a	5 mL

⚠ CRITICAL: Make 20–200 μL aliquots (depending on the amount you anticipate to be using within 1 month) and store aliquots at −20°C for up to 3 months. Keep a single working aliquot of stock solution at 4°C up to 1 month. Avoid freezing-thawing cycles.

ROCK inhibitor (Y-27632) stock solution	Final concentration	Amount
ROCK inhibitor Y-27632	10 mM	10 mg
Water, sterile, cell culture	n/a	2.9561 mL
Total	**n/a**	**2.9561 mL**

⚠ CRITICAL: Make 100–200 μL aliquots (depending on the amount you anticipate using within 2 months) and store aliquots at −80°C for up to 1 year. Keep a single working aliquot of stock solution at 4°C up to 2 months. Avoid freezing-thawing cycles.

L-ascorbic acid stock solution	Final concentration	Amount
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	100 mg/mL	5 g
Water, sterile, cell culture	n/a	50 mL
Total	**n/a**	**50 mL**

⚠ CRITICAL: Make 1 mL aliquots and store at −20°C for up to 1 year. If L-ascorbic acid dose not easily dissolve, place 50 mL tube containing L-ascorbic acid and sterile water on the rotator at 4°C until completely dissolved before making aliquots.

CHIR99021 stock solution	Final concentration	Amount
CHIR99021	10 mM	10 mg
Dimethylsulfoxide (DMSO), sterile	n/a	2.1490 mL
Total	**n/a**	**2.1490 mL**

⚠ CRITICAL: Make 100–200 μL aliquots (depending on the amount you anticipate using within 1 month) and store aliquots at −20°C up to 1 year. Keep a single working aliquot of stock solution at 4°C up to 1 month. Avoid freezing-thawing cycles.

Acetic acid, 0.5 mg/mL solution	Final concentration	Amount
Acetic acid	0.5 mg/mL	119 μL
Water, sterile, cell culture	n/a	250 mL
Total	**n/a**	**~250 mL**

⚠ CRITICAL: Filter sterilize and store at 4°C

Collagen IV for filter coating	Final concentration	Amount
Collagen IV from human placenta	1 mg/mL	5 mg
Acetic acid, 0.5 mg/mL solution	n/a	5 mL
Total	**n/a**	**5 mL**

⚠ CRITICAL: Allow to dissolve at least 4 h at 4°C before using and storing at 4°C. Collagen IV dissolved in acetic acid is used for Transwell filter coating.

Collagen IV for tissue culture plate coating	Final concentration	Amount
Collagen IV from human placenta	1 mg/mL	5 mg
Water, sterile, cell culture	n/a	5 mL
Total	n/a	5 mL

⚠ CRITICAL: Allow to dissolve at least 4 h at 4°C. Make 300 μL aliquots and store at −20°C. Collagen IV dissolved in water is used for tissue culture plate coating.

Alternatives: Collagen IV dissolved in acetic acid can be used for tissue culture plate coating.

4:1:5 collagen IV/fibronectin/water coating solution	Final concentration	Amount (per Transwell filter)
Collagen IV for filter coating	400 μg/mL	80 μL
Fibronectin from bovine plasma	100 μg/mL	20 μL
Water, sterile, cell culture	n/a	100 μL
Total	n/a	200 μL

⚠ CRITICAL: Prepare 4:1:5 collagen IV/fibronectin/water coating solution just before use. Coat Transwell filters (PC Membrane, 0.4 μm, 12 mm, TC-Treated, Costar 3401) at least 4 h at 37°C before using. We recommend incubating coated filters more than 8 h.

LaSR basal medium	Final concentration	Amount
Advanced D-MEM/F-12	n/a	500 mL
GlutaMAX Supplement	n/a	6.25 mL
L-ascorbic acid stock solution (100 mg/mL)	n/a	305 μL
Total	n/a	~500 mL

⚠ CRITICAL: Store LaSR basal medium at 2°C–8°C for up to 2 weeks.

DMEM/F-12-10 medium	Final concentration	Amount
Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM-F12)	n/a	450 mL
Fetal Bovine Serum, Qualified	10%	50 mL
Total	n/a	500 mL

⚠ CRITICAL: Store DMEM/F-12-10 medium at 2°C–8°C up to 1 month.

Flow buffer-1	Final concentration	Amount
Dulbecco's (d) PBS	n/a	467 mL
Bovine serum albumin (BSA), 7.5% in dPBS	5 mg/mL	33.3 mL
Total	n/a	500 mL

⚠ CRITICAL: Store Flow buffer-1 at 2°C–8°C up to 6 months.

hECSR medium	Final concentration	Amount
Human Endothelial SFM (hESFM)	n/a	98 mL
B-27 Supplement (50×), serum free	1 ×	2 mL
Human fibroblast growth factor 2 (bFGF/FGF2) stock solution (100 μg/mL)	20 ng/mL	20 μL
Total	n/a	~100 mL

⚠ CRITICAL: Store hECSR medium at 2°C–8°C up to 2 weeks.

Freezing medium for EPCs, EECM-BMEC-like cells and SMLCs	Final concentration	Amount
hECSR medium	n/a	30 mL
Fetal Bovine Serum, Qualified	30%	15 mL
Dimethylsulfoxide (DMSO), sterile	10%	5 mL
ROCK inhibitor (Y-27632) stock solution	5 μM	25 μL
Total	n/a	~50 mL

⚠ CRITICAL: Store Freezing medium at 2°C–8°C up to 2 weeks.

VEGF stock solution	Final concentration	Amount
Recombinant Human $VEGF_{165}$	100 μg/mL	10 μg
Water, sterile, cell culture	n/a	100 μL
Total	n/a	100 μL

⚠ CRITICAL: Make 5 μL aliquots and store at −20°C up to 1 year. Keep a single working aliquot of stock solution at 4°C up to 1 week. Avoid freezing-thawing cycles.

TNFα stock solution	Final concentration	Amount
Recombinant Human TNF-alpha Protein	100 μg/mL	20 μg
Dulbecco's (d) PBS	n/a	200 μL
Bovine serum albumin (BSA), 7.5% in dPBS	0.1%	2.667 μL
Total	n/a	~203 μL

⚠ CRITICAL: Make 5 μL aliquots and store at −80°C up to 3 months. Keep a single working aliquot of stock solution at 4°C up to 1 week. Avoid freezing-thawing cycles.

IFNγ stock solution	Final concentration	Amount
Recombinant Human IFN-gamma Protein	200 μg/mL	100 μg
Dulbecco's (d) PBS	n/a	500 μL
Bovine serum albumin (BSA), 7.5% in dPBS	0.1%	6.667 μL
Total	n/a	~507 μL

⚠ CRITICAL: Make 5–10 μL aliquots and store at −80°C up to 3 months. Keep a single working aliquot of stock solution at 4°C up to 1 week. Avoid freezing-thawing cycles.

Tris-Buffered Saline (TBS) 1×	Final concentration	Amount
Tris Base	50 mM	6.05 g
NaCl	150 mM	8.76 g
HCl	n/a	
distilled water	n/a	~1000 mL
Total	**n/a**	**1000 mL**

⚠ CRITICAL: To prepare, dissolve Tris Base and NaCl in 800 mL of distilled water. Adjust pH to 7.4 with HCl and make volume up to 1000 mL with distilled water. TBS is stable at 4°C up to 3 months.

Blocking buffer	Final concentration	Amount
Skim milk	5% (w/v)	5 g
Tris-Buffered Saline (TBS) 1×	n/a	100 mL
Triton X-100	0.3% (v/v)	300 µL
NaN_3, 10% solution in water	0.04% (v/v)	400 µL
Total	**n/a**	**~100 mL**

⚠ CRITICAL: Make 10 mL aliquots and store at −20°C up to 6 months. Keep a single working aliquot of stock solution at 4°C up to 2 weeks.

FACS buffer	Final concentration	Amount
Dulbecco's (d) PBS	n/a	500 mL
Fetal Bovine Serum, Qualified	2.5%	10 mL
NaN_3, 10% solution in water	0.1%	5 mL
Total	**n/a**	**515 mL**

⚠ CRITICAL: Store buffer at 2°C–8°C up to 6 months.

Sodium fluorescein stock solution	Final concentration	Amount
Dulbecco's (d) PBS	n/a	2.6577 mL
Fluorescein sodium salt	10 mM	10 mg
Total	**n/a**	**2.6577 mL**

⚠ CRITICAL: Make 50 µL aliquots and store at −20°C up to 2 years. Store at 2°C–8°C in the dark up to 6 months. To make working concentration, dilute 1:1000 in hECSR medium (final concentration 10 µM). Sodium fluorescein is light sensitive. Avoid exposure to light.

T-cell medium	Final concentration	Amount
RPMI Medium 1640	n/a	82.8 mL
Sodium Pyruvate	1%	1 mL
MEM NEAA (100×)	1%	1 mL
Kanamycin Sulfate (100×)	1%	1 mL
L-Glutamine 200 mM (100×)	1%	1 mL
Pen Strep	1%	1 mL
Fetal Bovine Serum, Qualified	10%	10 mL
Recombinant Human IL-2	500 units/mL	depends on lot
Total	**n/a**	**~100 mL**

⚠ CRITICAL: Store T-cell medium at 2°C–8°C up to 1 week. Recombinant human IL-2 manufacturer instructions (https://www.bdbiosciences.com/ds/pm/tds/554603.pdf) report an activity range of 0.06–1.0 × 10^9 units/mg. The concentration of recombinant human IL-2 in the culture medium must be optimized depending on the T cell subset (e.g., naïve T cell, activated T cell) of your interest.

T-cell wash buffer	Final concentration	Amount
T-cell medium	10%	10 mL
RPMI Medium 1640	n/a	87.5 mL
HEPES Buffer Solution	25 mM	2.5 mL
Total	n/a	100 mL

⚠ CRITICAL: Store T-cell wash buffer at 2°C–8°C up to 1 week.

migration assay medium	Final concentration	Amount
DMEM (1×), [+] 4.5 g/L D-Glucose, [-] L-Glutamine, [-] Pyruvate	n/a	90.5 mL
HEPES Buffer Solution	25 mM	2.5 mL
Fetal Bovine Serum, Qualified	5%	5 mL
L-Glutamine 200 mM (100×)	2%	2 mL
Total	n/a	100 mL

⚠ CRITICAL: Store migration assay medium at 2°C–8°C up to 1 week.

CellTracker Green CMFDA Dye stock solution	Final concentration	Amount
CellTracker Green CMFDA Dye	3 mM	50 µg
Dimethylsulfoxide (DMSO), sterile	n/a	36 µL
Total	n/a	36 µL

⚠ CRITICAL: Make 3 µL aliquots and store at −20°C up to 1 year. To make a working solution, dilute 1:3000 in migration assay medium (final concentration 1 µM). CMFDA is light sensitive. Avoid exposure to light.

STEP-BY-STEP METHOD DETAILS

Preparing hESC/hiPSC cultures for endothelial progenitor cell (EPC) differentiation

⏲ Timing: 10–14 days

This step describes the expansion of hPSC lines for producing healthy colonies of human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs) suitable for endothelial progenitor cell (EPC) differentiation. hPSCs are cultured under feeder-free conditions and passaged when reaching 80% confluency. hPSCs are passaged at least twice or cultured 2 weeks before starting the EPC differentiation.

⏲ Timing: 1–1.5 h

This step describes the preparation of Matrigel-coated 6-well plates for hPSC culture. This Matrigel coating procedure is also used to prepare plates for use in hPSC differentiation.

1. Prepare Matrigel-coated 6-well plates before starting the hPSC culture.

a. Add 30 mL cold DMEM/F-12 to a 50 mL tube. Using a micropipette, remove 1 mL of DMEM/F-12.
b. Using 1 mL of DMEM/F-12, pipet up and down to thaw and dissolve a Matrigel aliquot (containing 2.5 mg Matrigel).
c. Add thawed Matrigel to the 50 mL tube, containing 29 mL cold DMEM/F-12.
d. Add 1 mL of the resulting Matrigel solution to each well of the 6-well plates.
e. Agitate each plate to ensure coverage of the complete bottom of each well with Matrigel solution and place in incubator (37°C, 5% CO_2) for at least 1 h.

Note: If all Matrigel-coated wells will not be needed immediately for culture, add 1 mL DMEM/F12 instead to the wells not needed and store in incubator (37°C, 5% CO_2) up to 1 week.

Timing: 30–60 min

This step describes the method of thawing hPSC lines. If hPSCs are in continuous culture in your laboratory, skip this step and proceed directly to step 3.

2. Thaw hESC/hiPSC lines.
   a. Prepare a 15 mL centrifuge tube containing 4 mL mTeSR1 medium at 20°C–25°C.
   b. Thaw 1 vial of cryopreserved hESCs/hiPSCs by rapidly rubbing the tube between your hands or swirling it in a 37°C water bath until there is only a small piece of ice remaining.
   c. Slowly add 500 μL of mTeSR1 medium to the cryotube and gently pipet up and down once to completely thaw hPSCs, then slowly transfer hPSCs to the centrifuge tube containing mTeSR1 medium.

Note: At the first signs of thawing, hPSCs should immediately be transferred into 15 mL tube containing 4 mL of mTesR1 medium to reduce cryopreservation-mediated damage to the cell membranes.

⚠ CRITICAL: Avoid singularization of the hPSCs as hPSCs will attach and survive better if maintained as cell clumps.

   d. Centrifuge hPSCs at 200 *g*, 5 min, at 20°C–25°C. While hPSCs are centrifuging, label a Matrigel-coated plate with the name of the cell line, passage number, and passage date.
   e. Remove the supernatant and carefully resuspend the pellet in 2 mL of mTeSR1 medium + 10 μM ROCK inhibitor Y-27632 (add ROCK inhibitor stock solution at 1:1000) per well of a 6-well plate. Aspirate Matrigel coating solution from the 6-well plate and seed the hPSCs suspension onto Matrigel-coated wells.

Note: Consult documentation from the source of hESCs/hiPSCs for the recommended number of wells one vial should be thawed into.

⚠ CRITICAL: Avoid too intense resuspension as hPSCs will better attach and survive if maintained as cell clumps.

   f. Transfer 6-well plate into the incubator (37°C, 5% CO_2) and gently move the plate back and forth and from side to side on the incubator shelf to evenly distribute the cells.

Note: Avoid circular motions to prevent cells from pooling in the center of the well. It is important to place the plate onto the incubator shelf first and move the plate using the shelf as support inside the incubator.

   g. One day later, remove ROCK inhibitor Y-27632 by replacing medium in each well with 2 mL of mTeSR1 medium.

⏲ Timing: 3–7 days (when you use hPSCs already thawed previously)

⏲ Timing: 2–3 weeks (when you use freshly thawed hPSCs)

This step describes the maintenance of hPSCs, which is essential before starting EPC differentiation.

3. Maintenance of hPSCs (passage before start of differentiation)
   a. Change mTeSR1 medium every day to reduce probability of spontaneous differentiation.

   Note: Because the proliferation rate is hPSC line-dependent, hPSCs will be ready for the first passage in 4–7 days.

   b. Once hPSCs reach approximately 80% confluency, typically when individual colonies begin to touch, prepare a Matrigel-coated 6-well plate for passaging.
   c. Aspirate medium from one well of hPSCs, wash hPSCs with 1 mL Versene, and subsequently aspirate Versene from well.
   d. Add 1 mL of Versene to the well of hPSCs. Incubate at 37°C, 5% CO_2 in the incubator for 4–7 min, depending on cell line.
   e. While hPSCs are dissociating, label a new Matrigel-coated plate with the name of the cell line, passage number, passage date, and split ratio (usually 1:6 ratio). Aspirate Matrigel coating solution from 3 wells and add 1 mL of mTeSR1 medium per well of the 6-well plate.
   f. Prepare a 15 mL centrifuge tube containing 2 mL of mTeSR1 medium at 20°C–25 °C.
   g. Observe the detachment process of the hPSCs under the microscope. Once hPSCs start to detach (Methods video S1), remove Versene and dissociate hPSCs by vigorously pipetting 2 mL of mTeSR1 medium.

   Note: If hPSCs do not detach well, gently scrape hPSCs using the tip of a 5 mL pipet and pipetting mTeSR1 over the hPSCs again. Gentle scraping yields larger clumps of hPSCs than prolonged Versene treatment, which may improve hPSC maintenance.

   h. Collect 1 mL of the resulting hPSCs suspension in mTeSR1 medium and transfer to the 15 mL conical tube containing 2 mL of mTeSR1 medium.
   i. Distribute 1 mL of hPSC suspension into each well of the plate prepared in step "e" (for a total volume 2 mL per well). In this way, hPSCs are passaged at a 1:6 ratio.
      Note: Split ratio may need to be optimized for individual hPSCs lines or based on the hPSC number needed for EPC differentiation.
   j. Transfer 6-well plate into the incubator (37°C, 5% CO_2) and gently move the plate back and forth and from side to side on the incubator shelf to evenly distribute the cells.

   Note: Avoid circular motions to prevent cells from pooling in the center of the well. It is important to place the plate onto the incubator shelf first and move the plate using the shelf as support inside the incubator.

   k. If using a newly thawed hPSC line, maintain hPSC in culture and passage at least once more before initiating an EPC differentiation. This allows hPSCs recover from freezing.

⚠ CRITICAL: Usually the newly passaged hPSCs reach 80% confluency in 4–7 days. If it takes longer, the risk of spontaneous differentiation increases. In this case, we recommend increasing split ratio.

⚠ CRITICAL: Make sure that the hPSC cultures used for EPC differentiation (section "differentiation hPSCs into endothelial progenitor cells (EPCs)") lack spontaneous cell differentiation events. To eliminate cells that have undergone spontaneous differentiation, either (i) passage undifferentiated hPSC colonies by picking colonies using a sterile pipette tip or (ii)

treat cultures with Versene for a reduced time (2–3 min) as explained in step "d," which often facilitates selective detachment of hPSC colonies while spontaneously differentiated cells remain attached to the plate. For the details on the maintenance of hPSC colonies, please refer to manufacturer's instructions (https://cdn.stemcell.com/media/files/manual/10000005505-Maintenance_of_Human_Pluripotent_Stem_Cells_mTeSR1.pdf?_ga=2.138301808.1976489461.1615541621-432292338.1615280857).

Differentiation of hPSCs into EPCs

Timing: 9 days

This step describes the differentiation of hPSC lines into endothelial progenitor cells (EPCs). For background information on the development of the protocol, please refer to Nishihara et al. (2020a), Baoet al. (2016), and Lian et al. (2014).

Timing: 3 days

This step describes the first phase of the EPC differentiation protocol: seeding and expansion of hPSCs prior to EPC differentiation.

4. Singularized hPSC seeding and expansion for $CD31^+CD34^+$ EPC differentiation (Day -3 – Day -1)
   a. Initiate differentiation once hPSC colonies reach 80% confluency (Figure 1).
   b. Prepare Matrigel-coated 12-well plates using the method in step 1, except use 0.5 mL of Matrigel solution per well of each 12-well plate. Incubate at least 1 h at 37°C.
   c. Aspirate mTeSR1 medium from one well of hPSCs in a 6-well plate.

   Note: Typically, we collect hPSCs from one well of 6-well plate (2.5 – 3.5 $\times 10^6$ cells) for differentiation.
   d. Add 1 mL Accutase to the well and incubate at 37°C until all the hPSCs have completely detached (approximately 7 min).
   e. Using a micropipette, collect hPSCs, and pipette gently over surface 2–3 times to dislodge any remaining hPSCs and obtain a single cell suspension. Transfer hPSCs into a 15 mL centrifuge tube containing 4 mL of mTeSR1 medium.
   f. Resuspend well and take 10 μL of the hPSC suspension and pipet into a Neubauer Chamber.
   g. Centrifuge hPSCs for 5 min at 200 *g*, at 20°C–25 °C.
   h. Count hPSCs on Neubauer Chamber and calculate appropriate volume of cells to add to each 12-well plate (range 75–400 $\times 10^3$ hPSCs per well of a 12-well plate).

   Note: We recommend seeding at least 6 wells of a 12-well plate for EPC differentiation.

   CRITICAL: The seeding density must be optimized for each individual hPSC line and for each passage number depending on the growth rate of the hPSCs. When using a hPSC line for the first time, we recommend seeding at several different seeding densities (e.g., 75 $\times 10^3$, 100 $\times 10^3$, 200 $\times 10^3$, 400 $\times 10^3$ hPSCs per well of a 12-well plate) and evaluating the resulting yield of $CD31^+$ EPCs.
   i. After centrifuging down the hPSCs, remove the supernatant and resuspend the pellet in 1 mL of mTeSR1.
   j. Add appropriate volume of hPSCs calculated in step "h" to each well of a 12-well plate containing 1 mL mTeSR1 + 10 μM ROCK inhibitor Y-27632 (add ROCK inhibitor stock solution at 1:1000).
   k. Transfer 6-well plate into the incubator (37°C, 5% CO_2) and gently move the plate back and forth and from side to side on the incubator shelf to evenly distribute the cells.

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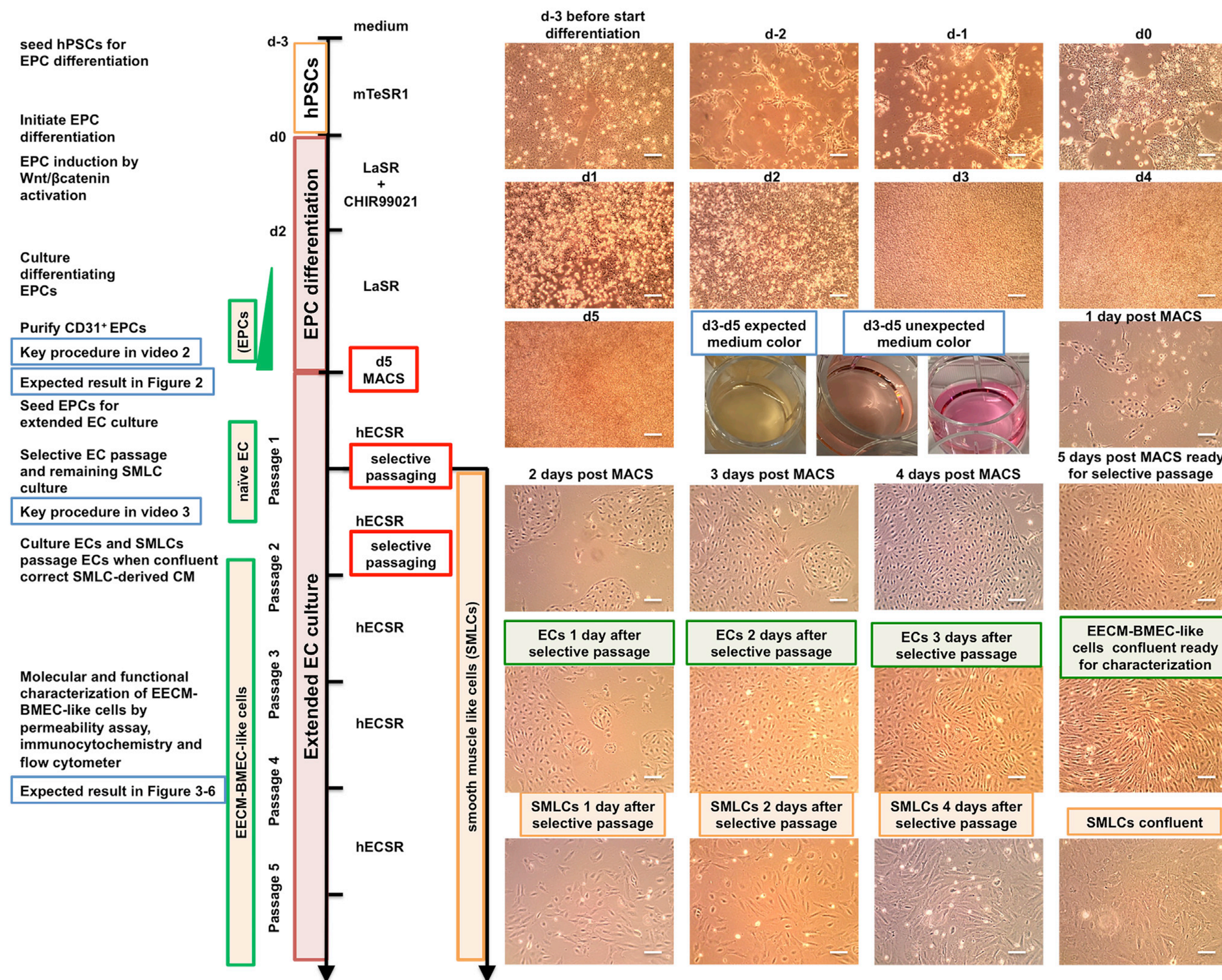

Figure 1. Protocol overview

Schematic of protocol steps, typical brightfield image of cells, and snapshot of medium color during differentiation is shown. Scale bar = 100 μm.

Note: Avoid circular motions to prevent cells from pooling in the center of the well. It is important to place the plate onto the incubator shelf first and move the plate using the shelf as support inside the incubator.

l. Approximately 24 h later (i.e., Day -2), remove mTeSR1 + ROCK inhibitor and replace medium with 2 mL of mTeSR1 medium per well.

Note: Stem cells treated with the ROCK inhibitor should have a mesenchymal morphology (Figure 1).

m. Approximately 24 h later (i.e., Day -1), replace medium with 2 mL of mTeSR1 medium per well.

Note: After withdrawal of the ROCK inhibitor, hPSCs should regain their typical hPSC morphology of tightly packed cells with well-defined edges (Figure 1).

⚠ CRITICAL: Seeding density of hPSCs at Day -3 is critical for successful EPC differentiation. Seeding density needs to be optimized for each hPSC line, as well as for each passage. For example, seeding 75–100 $\times 10^3$ hPSCs per well of a 12-well plate works well for the

commercially available IMR90-4 line. The range of seeding density varies from 75–400 $\times 10^3$ hPSCs per well of a 12-well plate (20–100 $\times 10^3$/cm^2).

Timing: 6 days

This step describes the second phase of the EPC differentiation protocol. Briefly, mesoderm differentiation is initiated by adding the GSK3 inhibitor for 2 days, and the cells are subsequently expanded for 3 days.

5. Endothelial progenitor differentiation with GSK3 inhibitor CHIR99021 (Day 0–Day 5)
   a. At Day 0, prepare LaSR basal medium supplemented with 8 μM CHIR99021 and allow to warm to 20°C–25 °C. Remove mTeSR1 and add 2 mL of LaSR basal medium supplemented with 8 μM CHIR99021 to each well of 12-well plate.
   b. At Day 1, aspirate the medium and replace with 2 mL of LaSR basal medium supplemented with 8 μM CHIR99021 at 20°C–25°C.
   c. At Day 2, 3, and 4, aspirate the medium and replace with 2 mL of LaSR basal medium (without CHIR99021) prewarmed to 20°C–25°C.

CRITICAL: hPSCs should be confluent by Day 1, or at the latest Day 2 (Figure 1). If the hPSCs do not reach confluency by Day 2, the differentiation usually fails. In this case, we recommend increasing the seeding density of hPSCs.

CRITICAL: If many cells are detached from the surface at Day 3–Day 5, we recommend decreasing the initial hPSCs seeding density. See troubleshooting problem 1.

CRITICAL: Though we identified 7–8 μM CHIR99021 is optimal for the hPSC lines that we tested, other hPSC lines may respond to CHIR99021 treatment differently. Therefore optimization of CHIR99021 concentration may be required.

CRITICAL: The effect of CHIR99021 may change between different lots, therefore the CHIR99021 concentration should be validated when switching the CHIR99021 lot.

Timing: 3–4 h

This step describes the final phase of the EPC differentiation protocol: purification of EPCs by MACS.

6. Purification of EPCs by magnetic activated cell sorting (MACS) using positive selection for FITC-labeled CD31$^+$ cells.
   a. At Day 5, aspirate the medium, add 1 mL Accutase to each well, and incubate at 37°C, 5% CO_2 in the incubator for 6–8 min.
   b. Pipette 5–10 times with a micropipette to obtain a single-cell suspension and then filter the cell suspensions through a 40 μm Falcon cell strainer into a 50 mL Centrifuge tube containing 12 mL DMEM/F-12-10 medium.

   Note: We use 50 mL Centrifuge tube from nerbe plus because the diameter fits well to the 40 μm Falcon cell strainer.
   c. Fill DMEM/F-12-10 medium up to 50 mL to stop Accutase reaction, resuspend cells and take 10 μL of the cell suspension and place onto Neubauer Chamber.

   Note: If you use more than two 12-well plates (more than 24 mL of Accutase), transfer cell suspension into two 50 mL tubes.
   d. Centrifuge the cells at 200 *g* for 5 min at 20°C–25°C.
   e. Count cell number.

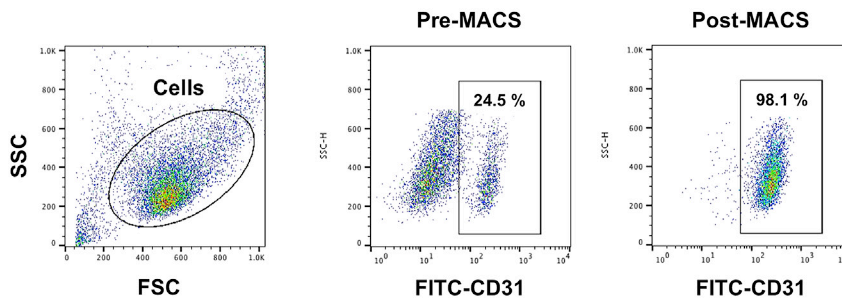

Figure 2. Flow cytometry analysis of pre-MACS and post-MACS sorted cells at day 5 of EPC differentiation
Samples were examined for the fraction of CD31+ cells using a flow cytometer.

f. After centrifugation, aspirate the supernatant and add 15 mL DMEM/F-12-10 medium to resuspend the pellet. Transfer the resulting cell suspension into a fresh 15 mL centrifuge tubes.
g. Centrifuge the cells at 200 *g* for 5 min at 20°C–25°C.
h. Aspirate the supernatant and resuspend the cell pellets at a concentration of 1×10^7 cells per 100 μL Flow Buffer-1.
i. Add 1:100 of FcR blocking reagent (provided from EasySep FITC Positive Selection Kit II) and incubate for 5 min.
j. Add 1:200 of FITC-conjugated CD31 antibody.

Note: Manufacturer recommends 1:50 dilution for FITC-conjugated CD31 antibody without providing antibody concentration, however we have found that 1:200 dilution works as well. You may need to adjust appropriate dilution of FITC-conjugated anti-CD31 antibody depending on lot number (range from 1:50 -1:400).

k. Incubate the mixture in the dark at 20°C–25°C for 30 min.
l. After incubation, add 10 mL FlowBuffer-1 and take a 10 μL sample to perform flow cytometry analysis (Figure 2) and centrifuge the remaining cell suspension at 200 *g* for 5 min at 20°C–25°C.

Note: If you cannot perform flow cytometry analysis immediately, store the sample for flow cytometry at 4°C until finished with the MACS.

⚠ CRITICAL: It is recommended that the pre-sort cell mixture contain more than 10% of $CD31^+$ EPCs before proceeding to MACS. If the EPC percentage is less than 6%, optimize initial seeding density and/or CHIR99021 concentration.

m. Aspirate the supernatant, resuspend the cell pellet at a concentration of 1×10^7 cells per 100 μL Flow Buffer-1, and add EasySep FITC Selection Cocktail at 5 μL per 100 μL antibody labeled cell suspension. Resuspend well and incubate at 20°C–25°C for 15 min.
n. Vortex Magnetic Nanoparticles and add Magnetic Nanoparticles at 5 μL per 100 μL cell suspension, resuspend well by pipetting and incubate at 20°C–25°C for 10 min.
o. Bring the cell suspension to a total volume of 2.5 mL Flow Buffer-1, resuspend the cells, and then transfer into a 5 mL round-bottom flow cytometry tube.
p. Place the flow cytometry tube (without cap) into the magnet and incubate for 5 min.
q. Pick up the magnet, and in one continuous motion invert the magnet and flow tube, pouring off the supernatant fraction, containing FITC-CD31 antibody unlabeled cells. Leave the magnet and tube inverted for 2–3 s allowing supernatant drop off, remove the drop at the edge of the tube by careful aspiration with a Pasteur pipet attached to a vacuum pump, then return tube containing the $CD31^+$ cells to an upright position (Methods video S2).

⚠ CRITICAL: The EasySep manufacturer's instructions (https://cdn.stemcell.com/media/files/pis/DX22365-PIS_1_0_0.pdf?_ga=2.180300580.1448779528.1614351881-737646090.1614351881) do not recommend shaking or blotting off any drops that may remain hanging from the mouth of the tube, however we have found that careful aspiration of these last drops increases EPC purity after MACS.

r. Remove the flow cytometry tube, containing the $CD31^+$ cells from the magnet, add 2.5 mL FlowBuffer-1, and resuspend the cell suspension by gently pipetting up and down 2–3 times.
s. Place the flow tube back into the magnet and incubate for 5 min.
t. Repeat steps "q–s" three times and then step "q" once more (for a total of 4 washes).
u. Remove the flow cytometry tube from the magnet and resuspend the purified $CD31^+$ cells in an appropriate amount of desired medium (hECSR for extended EC culture or Freezing medium for freezing).
v. Resuspend $CD31^+$ cells and take a 10 μL sample of cell suspension for counting.
w. Take an additional 10 μL sample of cell suspension for flow cytometry analysis. Use a flow cytometer to assess the percentage of $CD31^+$ cells in the pre-MACS (step "l") and post-MACS samples (Figure 2).

⚠ CRITICAL: For successful selective EC passaging described below, we recommended post-MACS purity of > 95%. If the purity is low, see troubleshooting problem 2.

Extended endothelial cell culture method to differentiate brain microvascular endothelial cell-like cells (EECM-BMEC-like cells) and smooth muscle-like cells (SMLCs)

⏲ Timing: 10–15 days

This step describes the culture of EPCs and induction of BMEC-like cells by the extended endothelial cell culture method, as well as SMLC culture and preparation of SMLC-derived conditioned medium (CM). SMLCs are a biproduct of EECM-BMEC-like cell differentiation and their co-culture or the CM derived from SMLCs is essential to achieve optimal VCAM-1 expression on EECM-BMEC-like cells.

⏲ Timing: 30–60 min

This step describes the preparation of collagen IV coated plates for the EECM-BMEC-like cell culture.

7. Prepare 10 μg/ml collagen IV (Collagen IV for tissue culture plate coating)-coated 6-well plate
   a. Dilute a 300 μL aliquot of collagen IV (1 mg/mL) in 30 mL sterile water (Water, sterile, cell culture from Sigma) and add 1 mL of the colloidal solution to each well of the 6-well plates. Incubate plates with collagen IV for at least 30 min at 37°C and for up to 1 week.

⏲ Timing: 3–5 days

This step describes the culture of EECM-BMEC-like cells on collagen IV-coated plates before selective EC passage.

8. Seeding hPSC-derived EPCs for extended endothelial cell culture
   a. Aspirate collagen IV coating solution from the 6-well plate. Add 1–2 × 10^5 purified $CD31^+$ EPCs in 2 mL hECSR supplemented with 5 μM ROCK inhibitor Y-27632 (add ROCK inhibitor stock solution at 1:2000) per well of the 6-well plate.

Note: Seeding density must be optimized for individual hPSC lines. We recommend seeding $CD31^+$ EPCs at a density such that $CD31^+$ cells reach 100% confluency in 3–7 days.

b. Transfer 6-well plate into the incubator (37°C, 5% CO_2) and gently move the plate back and forth and from side to side on the incubator shelf to evenly distribute the cells.

Note: Avoid circular motions to prevent cells from pooling in the center of the well. It is important to place the plate onto the incubator shelf first and move the plate using the shelf as support inside the incubator.

c. Approximately 24 h later, remove ROCK inhibitor by aspirating hECSR medium and replacing with 2 mL fresh hECSR medium pre-warmed to 20°C–25°C.
d. Thereafter, change hECSR medium every second day until CD31$^+$ cells reach 100% confluency (Figure 1)

Note: If the regular cell-feeding schedule cannot be accommodated over the weekend, we recommend changing medium on Friday evening and again Monday early morning.

e. Once CD31$^+$ cells reach 100% confluency, perform selective passaging as described below (step 9).

⚠ CRITICAL: In general, waiting until CD31$^+$ cells reach 100% confluency will lead to successful selective passage. However, for some hPSC lines, smooth muscle-like cells (SMLCs) also detach if cultures are 100% confluent. In this case, we recommend attempting selective passage with a less confluent (80% or less) culture.

Timing: 3–5 days

After initial expansion of EPCs, resulting cultures typically contain both ECs and SMLCs. This step describes a method to separate the two cell types and generate pure cultures of ECs (to establish EECM-BMEC-like cells) and SMLCs (for preparation of SMLC-derived CM).

9. Selective EC passage to establish EECM-BMEC-like cells and SMLCs (Methods video S3)

a. Prepare a collagen IV-coated 6-well plate as described in step 7.
b. Aspirate hECSR medium from 6-well plate containing CD31$^+$ cells and add 1 mL of Accutase to each well of the 6-well plate.
c. Carefully monitor cell morphology under a microscope. Once ECs (but not non-ECs) appear round (Figure 9, Methods video S3), tap the edge of the 6-well plate to detach ECs. After tapping, many ECs should detach and non-ECs remain attached to the plate (Figure 9).

Note: The procedure normally takes 2–5 min.

d. Collect detached ECs by avoiding resuspending non-ECs and transfer to a 15mL centrifuge tube containing 4 mL of hESFM.
e. Add 2 mL hECSR medium to each well containing remaining attached non-ECs to obtain smooth muscle-like cells (SMLCs). Return the plate to the incubator.
f. Resuspend the EC suspension in the 15 mL tube well and collect 10 μL of EC suspension for counting.
g. Centrifuge ECs at 200 *g*, 5 min, at 20°C–25°C.
h. Aspirate supernatant and resuspend the pellet in 2 mL of hECSR medium per 1–2 × 10^5 ECs.
i. Aspirate collagen IV coating solution from the new 6-well plate. Add 2 mL EC suspension per well.
j. Transfer 6-well plate into the incubator (37°C, 5% CO_2) and gently move the plate back and forth and from side to side on the incubator shelf to evenly distribute the cells.

Note: Avoid circular motions to prevent cells from pooling in the center of the well. It is important to place the plate onto the incubator shelf first and move the plate using the shelf as support inside the incubator.

k. Thereafter, change hECSR medium every second day until ECs reach 100% confluency (typical EC growth is shown in Figure 1).

Note: If the regular cell-feeding schedule cannot be accommodated over the weekend, we recommend changing medium on Friday evening and again Monday early morning.

Note: Usually, 2–3 selective passages will yield nearly pure cultures of ECs (Figure 1), which are ready for functional assays (steps 13–16). In general, we consider ECs at passages 3–5 as EECM-BMEC-like cells. Proliferation typically slows after further passaging, however this is variable between different hPSC lines.

Note: If selective passaging is unsuccessful, see troubleshooting problem 3.

l. For SMLC culture, change hECSR medium every other day (typical SMLC growth is shown in Figure 1). To produce SMLC-conditioned medium (CM), instead of aspirating hECSR medium when changing medium, collect hECSR medium and filter using a 0.22 μm filter. This CM can be used for enhancing VCAM-1 expression on EECM-BMEC-like cells.

Note: We usually pool SMLC-CM until SMLCs reach 100% confluency.

Cryopreservation and thawing of EPCs, EECM-BMEC-like cells, or SMLCs

Timing: 30 min–1 h

After MACS, resulting EPCs can be cryopreserved for future use. Frozen EPCs can be used for extended endothelial cell culture (step 8) after thawing (step 12). This step describes the method of freezing EPCs.

10. Freezing EPCs
    a. Immediately after the last MACS wash (step 6 ''q–s''), resuspend the EPCs in freezing medium at a concentration of 1–2 $\times$ 10^6 cells per mL. Transfer 1 mL of EPCs suspension to each cryotube.
    b. Transfer cryotubes to a controlled-rate freezing device and immediately place at -80°C.
    c. 24 to 48 h later transfer to liquid nitrogen tank for long-term storage.

Note: EPCs can be stored in liquid nitrogen for at least 2 years.

Timing: 30 min–1 h

After selective passage, resulting EECM-BMEC-like cells or SMLCs can be cryopreserved for future use. Frozen EECM-BMEC-like cells and SMLCs are ready to use for quality control analyses (steps 13–16) after thawing (step 12). This step describes the method of freezing of EECM-BMEC-like cells and SMLCs.

11. Freezing EECM-BMEC-like cells and SMLCs
    a. Aspirate hECSR medium from 6-well plate containing EECM-BMEC-like cells or SMLCs and add 1 mL of Accutase per well of a 6-well plate.
    b. Incubate at 37°C, 5% CO_2 in the incubator until cells detach (approximately 7 min for EECM-BMEC-like cells and 20–30 min for SMLCs).
    c. Collect cells in a 15 mL centrifuge tube containing 4 mL of hESFM or DMEM/F-12-10 per 1 mL Accutase.
    d. Resuspend EECM-BMEC-like cells or SMLCs suspension well and collect 10 μL of cell suspension for counting.

   e. Centrifuge EECM-BMEC-like cells or SMLCs at 200 *g*, 5 min, at 20°C–25°C.
   f. Aspirate supernatant and resuspend the EECM-BMEC-like cells or SMLCs pellet in freezing medium at a cell number of 1–2 × 10^6 cells per mL. Transfer 1 mL of cell suspension to each cryotube.
   g. Transfer cryotubes to a controlled-rate freezing device and immediately place at −80°C.
   h. 24 to 48 h later transfer cryotubes to liquid nitrogen tank for long-term storage.

Note: EECM-BMEC-like cells and SMLCs can be stored in liquid nitrogen for at least 2 years.

Note: Fresh cultured EECM-BMEC-like cells and thawed EECM-BMEC-like cells show comparable permeability values for sodium fluorescein and adhesion molecule phenotype.

⚠ CRITICAL: If EECM-BMEC-like cell proliferation is slow before freezing, EECM-BMEC-like cells may not form good confluent monolayers when cultivated on Transwell filters.

⏲ Timing: 30 min–1 h

This step describes the method for thawing EPCs, EECM-BMEC-like cells and SMLCs.

12. Thawing EPCs, EECM-BMEC-like cells and SMLCs
   a. Prepare a collagen IV-coated 6-well plate as described in step 7.
   b. Prepare a 15 mL Centrifuge tube containing 4 mL of hESFM or DMEM/F-12-10.
   c. Thaw a cryotube containing EPCs, EECM-BMEC-like cells, or SMLCs rapidly rubbing the tube between your hands it in your hand or swirling in a 37°C water bath until there is only a small piece of ice remaining.
   d. Slowly add 500 μL of hESFM or DMEM/F-12-10 medium to the cryotube, and then transfer the cell suspension to the centrifuge tube and resuspend.
   e. Rinse the cryotube once with 1 mL hESFM or DMEM/F-12-10 and transfer to the centrifuge tube.
   f. Centrifuge cells at 200 *g*, 5 min, at 20°C–25°C.
   g. Remove supernatant and resuspend the pellet in 2 mL hECSR medium supplemented with 5 μM ROCK inhibitor Y-27632 at a cell count of 2–3 × 10^5 cells. Add 2 mL cell suspension to each well of a 6-well plate.
   h. Transfer 6-well plate into the incubator (37°C, 5% CO_2) and gently move the plate back and forth and from side to side on the incubator shelf to evenly distribute the cells.

   Note: Avoid circular motions to prevent cells from pooling in the center of the well. It is important to place the plate onto the incubator shelf first and move the plate using the shelf as support inside the incubator.

   i. One day later, remove ROCK inhibitor Y-27632 by replacing medium in each well with hECSR medium.

Note: We recommend waiting until EECM-BMEC-like cells become 100% confluent before starting functional assays and molecular characterization. EECM-BMEC-like cells typically become 100% confluent at 2-3 days after thawing.

Quality control analyses of EECM-BMEC-like cells

Below, we provide protocols for quality control analyses of EECM-BMEC-like cells, including functional assays for small molecule permeability and immune cell adhesion, and molecular assays for key endothelial junctional and immune cell adhesion molecule expression. These quality control analyses are critical to ensure that the differentiation has proceeded successfully and that EECM-BMEC-like cells derived from different hPSC lines and in different laboratories are substantially similar and have expected phenotypes.

Table 1. Example of raw sodium fluorescein fluorescence intensity data measured on a plate reader and permeability coefficient calculation

min	filter_1	filter_2	filter_3	blank filter
15	79	88	93	1641
30	131	184	182	3246
45	212	232	202	3668
60	266	303	284	4529
60 (apical)	38564	37897	39005	32926
background	16	n/a	n/a	n/a
Subtract background (raw value - background)	n/a	n/a	n/a	n/a
15	63	72	77	1625
30	115	168	166	3230
45	196	216	186	3652
60	250	287	268	4513
60 (apical)	38548	37881	38989	32910
Correct signal (accounting for signal loss from sampling bottom well)	n/a	n/a	n/a	n/a
15	63	72	77	1625
30	121.3	175.2	173.7	3392.5
45	208.13	233.52	203.37	3991.25
60	270.813	310.352	288.337	4912.125
60 (apical)	38548	37881	38989	32910
Clearance volume at each time point (μL)	n/a	n/a	n/a	n/a
15	2.451	2.851	2.962	74.066
30	4.720	6.938	6.683	154.626
45	8.099	9.247	7.824	181.917
60	10.538	12.289	11.093	223.889
slope (μL/min)	0.184	0.204	0.170	3.178
Pe (μL/min)	0.196	0.218	0.180	n/a
Pe (10^{-3}cm/min)	0.175	0.195	0.161	n/a

Timing: 6 days for EECM-BMEC-like cell culture and 1.5–2 h for permeability assay

This step describes methodology for quality control of the diffusion barrier properties of EECM-BMEC-like cells by performing a permeability assay for a small molecular tracer.

13. Measuring sodium fluorescein permeability (Methods video S4) (sample raw data and calculations are shown in Table 1 and expected results are shown in Figure 11)
    a. Prepare 200 μL of 4:1:5 collagen IV/fibronectin/water coating solution (see materials and equipment) per Transwell filter (PC Membrane, 0.4 μm, 12 mm, TC-Treated from Costar). Add 200 μL to each Transwell filter and incubate in the incubator (37°C, CO_2 5%) for at least 4 h. We recommend incubating coated filters more than 8 h.
    b. Aspirate hECSR medium from EECM-BMEC-like cells at passage 3–5 and add 1 mL of Accutase per well of a 6-well plate.
    c. Incubate at 37°C, 5% CO_2 in the incubator until EECM-BMEC-like cells detach (approximately 7 min).
    d. Collect EECM-BMEC-like cells in a 15 mL centrifuge tube containing 4 mL of hESFM or DMEM/F-12-10.
    e. Resuspend EECM-BMEC-like cell suspension well and take 10 μL of cell suspension for counting.
    f. Centrifuge EECM-BMEC-like cells at 200 *g*, 5 min, at 20°C–25°C.
    g. Aspirate supernatant and resuspend the EECM-BMEC-like cell pellet in 500 μL hECSR medium with a cell count of 1.12×10^5 cells. Aspirate the collagen IV/fibronectin/water coating

solution from Transwell filters and add 500 μL of the EECM-BMEC-like cell suspension to each Transwell filter. Add 1.5 mL of hECSR medium to the bottom chamber.

Note: We recommend preparing triplicate Transwell filters for measurement and one blank filter without cells.

h. Transfer 6-well plate into the incubator (37°C, 5% CO_2) and gently move the plate back and forth and from side to side on the incubator shelf to evenly distribute the cells.

Note: Avoid circular motions to prevent cells from pooling in the center of the well. It is important to place the plate onto the incubator shelf first and move the plate using the shelf as support inside the incubator.

i. Thereafter change hECSR medium every second day until day 6.
j. At day 6, prepare a new 12-well plate containing 1.5 mL warm hECSR medium per well. Use forceps to carefully transfer the Transwell filters intended for permeability assay (including the blank Transwell filter) to this new 12-well plate.
k. In a 15 mL centrifuge tube, prepare 500 μL of 10 uM sodium fluorescein solution in hECSR medium (add sodium fluorescein stock solution at 1:1000) per Transwell filter (including the blank Transwell filters).
l. Remove hECSR medium from the top chamber and replace with 500 μL hECSR medium containing 10 μM sodium fluorescein in all samples (including the blank Transwell filters).
m. At 15, 30, 45, and 60 min, collect 150 μL of medium from the bottom chamber of each Transwell and place into a 96-well plate (flat bottom). Go into the bottom chamber at the side of the filter and pipette gently up and down 2–3 times with micropipette before extracting 150 μL. Replace the missing volume with 150 μL pre-warmed hECSR medium into the bottom chamber.
n. At the 60-minute time point, also collect 150 μL of medium from the top chamber of each Transwell and transfer to a 96-well plate.
o. Place an additional 150 μL of hECSR medium without sodium fluorescein in the 96-well plate for background subtraction.

Note: Samples from the top chambers may need to be diluted to prevent an oversaturated signal.

p. Read the fluorescence intensity using a fluorescent plate reader (485 nm excitation/530 nm emission) (example of raw data measured on a plate reader and example of Pe calculation is found in Table 1).
q. Correct the signal by removing background and accounting for signal loss from sampling the bottom chamber. For example, after subtracting the background, the 15-minute sample exhibits a signal of 1000 relative fluorescence units (RFU) and the 30-minute sample exhibits a signal of 1500 RFU. The corrected signal at 30 min is (1500 RFU + (1000 RFU × 150 μL/1500 μL)) = 1500 RFU + 100 RFU = 1600 RFU. When calculating the 45 minute corrected value, use 1600 RFU as the 30-minute sample value.
r. Calculate the clearance volume at each time point (Coisne et al., 2005; Cecchelli et al., 2014).
   Clearance volume = (V_B × ($S_{B,t}$)) / ($S_{T,\ 60\ min}$), where V_B is the volume of bottom chamber (1500 μL); $S_{B,t}$ is the corrected signal of the bottom chamber at time, t; $S_{T,60\ min}$ is the signal of top chamber at 60 min.
s. Calculate the linear slope of clearance volume vs. time using linear regression for each of the triplicate culture Transwell filters (m_c) and the blank filter (m_f).
t. Calculate sodium fluorescein permeability for each of the triplicate culture Transwell filters by subtracting the "resistance" of the blank Transwell filter (reciprocal slope of clearance volume vs. time) from the "resistance" of the cell-containing Transwell filter using the following formula: $1/Pe = 1/m_c - 1/m_f$.

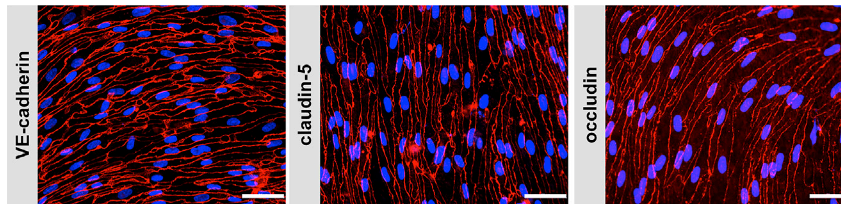

Figure 3. Immunofluorescence staining of EECM-BMEC-like cells grown on 0.4 μm pore Transwell filters for 6 days
VE-cadherin (red), claudin-5 (red), occludin (red), and nuclei (DAPI; blue) are shown. Staining was performed after permeability assays. Scale bar = 50 μm.

u. The slope of reciprocal clearance volume vs. time should have units min/μL; to convert to typical units, use the area of the Transwell filter (1.12 cm^2): Pe [cm/min] = $(1/Pe)^{-1}$ [μL/min] / (1000 μL/cm^3 × 1.12 cm^2).

Note: After the permeability assay, Transwell filters can be fixed with −20°C precooled methanol (150 μL top chamber/1 mL bottom chamber) for 20 s. After rehydrating and washing, cut filters out using a scalpel (Methods video S5), transfer to a 24-well plate and add 150 μL of blocking buffer for blocking and cell membrane permeabilization. Then stain for junctional molecules to evaluate monolayer confluency as described below. After staining, transfer filters to a glass slide, mount with Mowiol, and coverslip.

Timing: 2 days (24-well plate) or 6 days (Transwell filter) for cell culture and 2–3 h for immunofluorescence staining

This step describes immunofluorescence staining methods for quality control of EECM-BMEC-like cells and SMLCs. Specifically, EECM-BMEC-like cells are stained for junctional proteins to confirm maturation of cell-cell junctions, and for key endothelial adhesion molecules. SMLCs are stained for key components of smooth muscle cell contractile machinery.

14. Immunofluorescence staining of EECM-BMEC-like cell monolayers and SMLCs (expected results are shown in Figures 3, 4, and 5)

Note: Immunofluorescence staining can be performed on chamber slides, 96-well plates, or Transwell filters. We here describe the method for EECM-BMEC-like cells or SMLCs cultured on 16-well chamber slides or 96-well plates. For cultivation of EECM-BMEC-like cells on Transwell filters, see step "13".

a. Prepare collagen IV-coated chamber slides or 96-well plates as described in step 7, except use a volume of 60 μL of coating solution per well of a 16-well chamber slide or 96-well plate.
b. Aspirate hECSR medium from the 6-well plate containing EECM-BMEC-like cells or SMLCs and add 1 mL of Accutase per well of the 6-well plate.
c. Incubate at 37°C, 5% CO_2 in the incubator until cells detach (approximately 7 min for EECM-BMEC-like cells and 10–20 min for SMLCs).
d. Collect EECM-BMEC-like cells or SMLCs in a 15 mL centrifuge tube containing 4 mL of hESFM or DMEM/F-12-10 per 1 mL of Accutase.
e. Resuspend EECM-BMEC-like cells or SMLCs suspension well and take 10 μL of cell suspension for counting.
f. Centrifuge EECM-BMEC-like cells or SMLCs at 200 *g*, 5 min, at 20°C–25°C.
g. Aspirate supernatant and resuspend at 2.5 × 10^5 EECM-BMEC-like cells or SMLCs per mL of hECSR medium. Aspirate the collagen IV coating solution and add 100 μL of EECM-BMEC-like cells or SMLCs suspension to each well of the 16-well chamberslide or 96-well plate (2.5 × 10^4/well).

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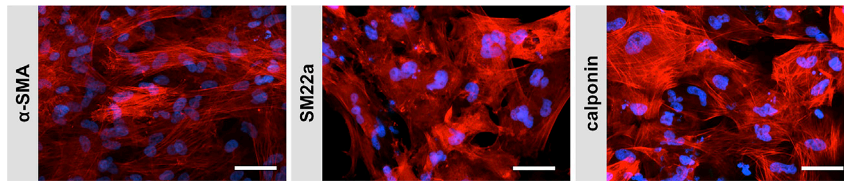

Figure 4. Immunofluorescence staining of SMLCs grown on chamberslide
α-SMA (red), SM22a (red), calponin (red), and nuclei (DAPI; blue) are shown. Scale bar = 50 μm.

h. Transfer 16-well chamber slide or 96-well plate into the incubator (37°C, 5% CO_2) and gently move the plate back and forth and from side to side on the incubator shelf to evenly distribute the cells.

Note: Avoid circular motions to prevent cells from pooling in the center of the well. It is important to place the plate onto the incubator shelf first and move the plate using the shelf as support inside the incubator.

i. Once EECM-BMEC-like cells or SMLCs attach to the slide or plate (1–2 h later), replace hECSR medium with 100 μL hECSR medium and remove nonadherent EECM-BMEC-like cells or SMLCs.
j. Approximately 24 h later, change hECSR medium again for feeding. To investigate adhesion molecule expression on EECM-BMEC-like cells, change hECSR medium to CM derived from SMLCs (for preparation of CM, see step ''9l'') in the presence or absence of 0.1 ng/mL TNF-α + 2 IU/mL IFN-γ for 16–24 h.
k. The primary antibody staining procedure depends on the target antigen (see key resources table: Antibodies used for immunofluorescence staining, for suggested antibody dilutions).
   i. To stain for junctional molecules (e.g., claudin-5, occludin, VE-cadherin), fix EECM-BMEC-like cells with 100 μL of −20°C precooled methanol for 20 s and rehydrate by washing 3 times with 100 μL of dPBS. Block and permeabilize cell membrane with 100 μL of blocking buffer for 10 min at 20°C–25°C, and then stain with 100 μL of primary antibodies diluted in blocking buffer for 1 h at 20°C–25°C.

Note: Although paraformaldehyde fixation permits detection of some junctional molecules (e.g., VE-cadherin), methanol fixation is superior in maintaining epitopes of tight junction molecules (e.g., claudin-5 and occludin).

   ii. To stain for adhesion molecules ICAM-1 and VCAM-1, dilute primary antibodies in 100 μL of hECSR medium, add to live EECM-BMEC-like cells, and incubate at 37°C, 5% CO_2 in the incubator for 15 min. Live cell-labeling allows for selective detection of cell surface-expressed adhesion molecules, which are functionally available for immune cell interaction. After washing with dPBS, fix EECM-BMEC-like cells with 100 μL of 1% (w/v) paraformaldehyde in dPBS for 10 min at 20°C–25°C. Wash cells with dPBS and block EECM-BMEC-like cells with 100 μL of blocking buffer for 10 min at 20°C–25°C.
   iii. To stain for α−smooth muscle actin, calponin, and SM22a, fix SMLCs with 100 μL of 1% (w/v) paraformaldehyde in dPBS for 10 min at 20°C–25°C. After washing with 100 μL of dPBS, block and permeabilize SMLCs with 100 μL of blocking buffer for 10 min at 20°C–25°C. Then incubate SMLCs with 100 μL of primary antibodies diluted in blocking buffer for 1 h at 20°C–25°C.

l. Wash EECM-BMEC-like cells or SMLCs three times with 100 μL of dPBS and incubate with 100 μL of secondary antibodies diluted in blocking buffer (see key resources table: Antibodies used for immunofluorescence staining, for appropriate secondary antibodies and suggested concentrations) for 1 h at 20°C–25°C. Add DAPI at 1 μg/mL or Hoechst 33342 at 4 μM to secondary antibody solutions to stain the cell nuclei.

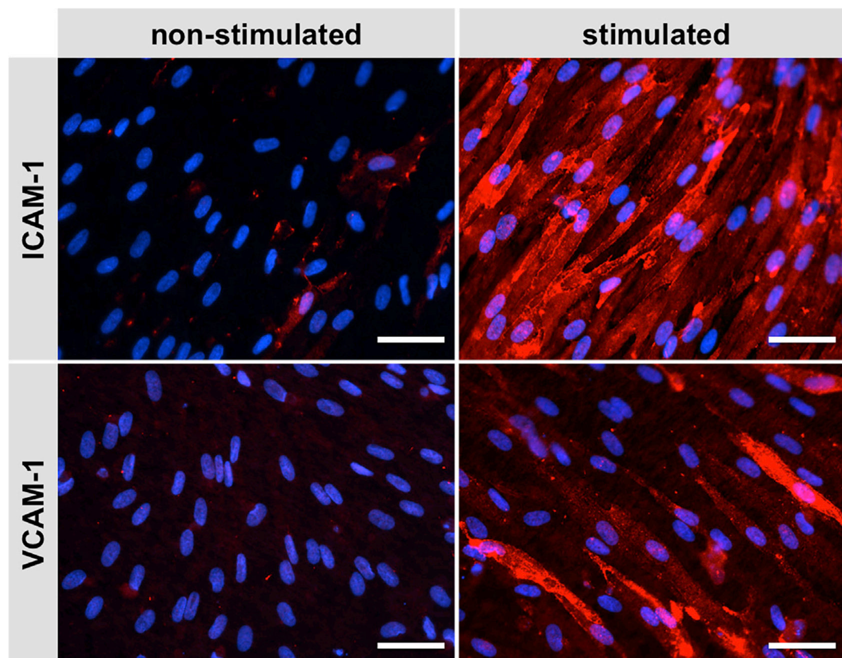

Figure 5. Immunofluorescence staining of EECM-BMEC-like cells grown on 0.4 μm pore Transwell filters in the presence of SMLC-derived CM

ICAM-1 (red), VCAM-1 (red), and nuclei (DAPI; blue) are shown for non-stimulated and inflammatory cytokine-stimulated (1 ng/mL TNF-α + 20 IU/mL IFN-γ) conditions. Scale bar = 50 μm.

m. Wash EECM-BMEC-like cells or SMLCs three times with 100 μL of dPBS. If using a chamber slide, remove plastic chamber from the glass slide, mount with Mowiol, and coverslip.
n. Examine slide/plate under a fluorescence microscope.

Timing: 2 days (24-well plate) or 6 days (Transwell filter) for EECM-BMEC-like cell culture and 2–3 h for flow cytometry assay

This step describes a flow cytometry-based method for quality control of the adhesion molecule phenotype of EECM-BMEC-like cells. This assay is especially important to ensure expression of a panel of endothelial adhesion molecules, which is a unique and characteristic feature of EECM-BMEC-like cells when compared to other hPSC-derived blood-brain barrier (BBB) models.

15. Flow cytometry analysis of adhesion molecule expression of EECM-BMEC-like cells (Expected results are shown in Figure 6)

Note: Flow cytometry analysis can be performed on EECM-BMEC-like cells cultured in 24-well plates or Transwell filters. We describe here the method of EECM-BMEC-like cells cultured on 24-well plates. For the cultivation of EECM-BMEC-like cells on Transwell filters, see step "13".

a. Prepare a collagen IV-coated 24-well plate as described in step 7, except use a volume of 300 μL of coating solution per well of 24-well plate.
b. Aspirate hECSR medium from 6-well plate containing EECM-BMEC-like cells and add 1 mL of Accutase per well of the 6-well plate.
c. Incubate at 37°C, 5% CO_2 in the incubator until EECM-BMEC-like cells detach (approximately 7 min).
d. Collect EECM-BMEC-like cells in a 15 mL centrifuge tube containing 4 mL of hESFM or DMEM/F-12-10 per 1 mL of Accutase.

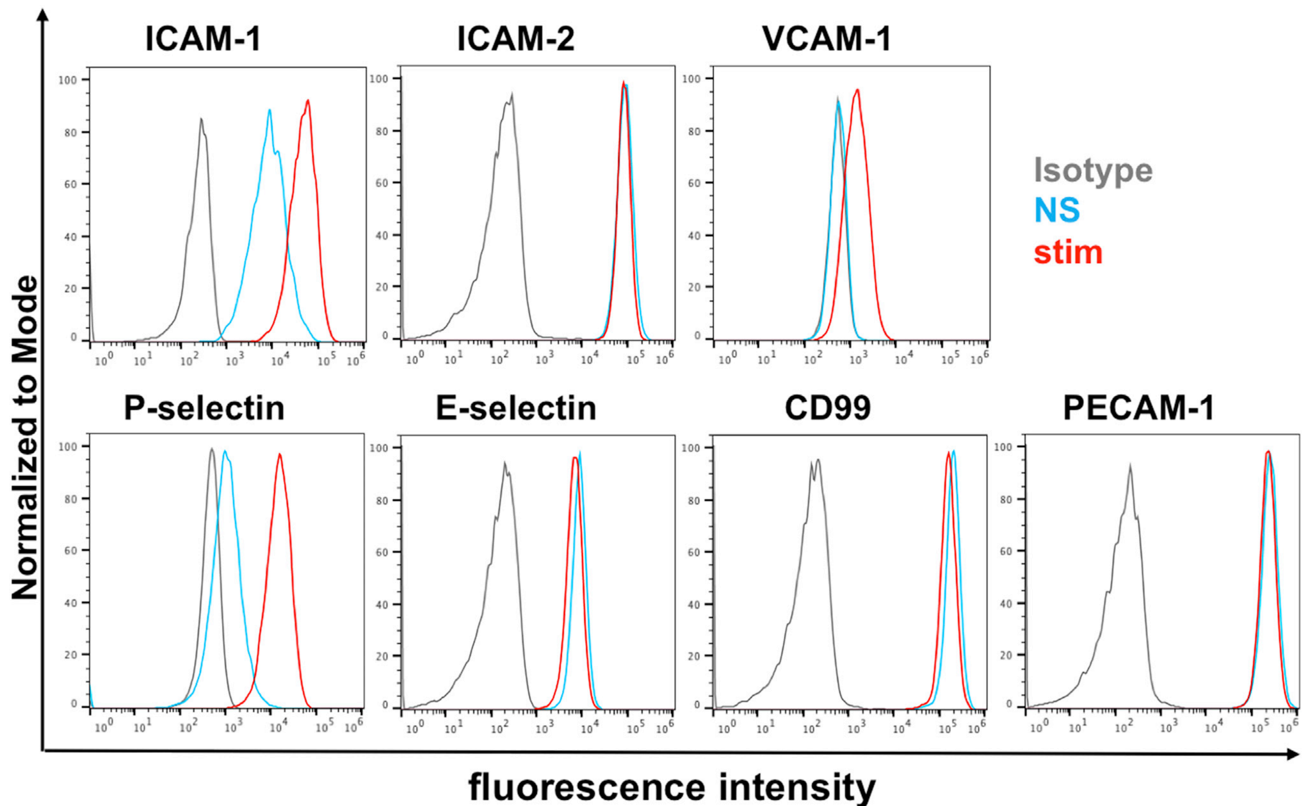

Figure 6. Flow cytometry analysis of EECM-BMEC-like cells grown on 24-well plate in the presence of SMLC-derived CM for adhesion molecules
Isotype control, non-stimulated (NS) and 16 h pro-inflammatory cytokine-stimulated condition (stim, 1 ng/mL TNF-α + 20 IU/mL IFN-γ) are represented respectively in gray, blue, and red lines in a histogram overlay.

e. Resuspend EECM-BMEC-like cells suspension well and collect 10 μL of cell suspension for counting.
f. Centrifuge EECM-BMEC-like cells at 200 *g*, 5 min, at 20°C–25°C.
g. Aspirate supernatant and resuspend at 9.5×10^4 EECM-BMEC-like cells per ml of hECSR medium. Aspirate the collagen IV coating solution and add 1 mL of EECM-BMEC-like cells suspension to each well of the collagen IV-coated 24-well plate (9.5×10^4/well).

Note: Seed EECM-BMEC-like cells onto at least 2 wells of 24-well plate for non-stimulated and pro-inflammatory cytokine-stimulated conditions.

h. Transfer 24-well chamber slide or 96-well plate into the incubator (37°C, 5% CO_2) and gently move the plate back and forth and from side to side on the incubator shelf to evenly distribute the cells.

Note: Avoid circular motions to prevent cells from pooling in the center of the well. It is important to place the plate onto the incubator shelf first and move the plate using the shelf as support inside the incubator.

i. Approximately 24 h later, change hECSR medium to CM derived from SMLCs (for preparation of CM, see step "9l") in the presence or absence of 1 ng/mL TNF-α + 20 IU/mL IFN-γ for 16–24 h.
j. After pro-inflammatory cytokine stimulation, aspirate CM from 24-well plate containing EECM-BMEC-like cells and add 200 μL of Accutase per well of the 24-well plate. Incubate at 37°C, 5% CO_2 in the incubator until EECM-BMEC-like cells detach (approximately 7 min).

k. Collect EECM-BMEC-like cells in 15 mL centrifuge tubes containing 4 mL of hESFM or DMEM/F-12-10.
l. Resuspend EECM-BMEC-like cells suspension well and collect 10 μL of cell suspension for counting.
m. Centrifuge EECM-BMEC-like cells at 200 *g*, 5 min, at 20°C–25°C.
n. Aspirate supernatant and resuspend at 1 × 10^6 EECM-BMEC-like cells per ml of FACS buffer. Add 100 μL of EECM-BMEC-like cell suspension to each well of a 96-well plate (round bottom) (1 × 10^5/well).

Note: We recommend preparing 3 wells (unstained control, isotype control mixture, adhesion molecules staining mixture)/sample (non-stimulated and pro-inflammatory cytokine stimulated condition).

o. Centrifuge EECM-BMEC-like cells in the 96-well round bottom plate at 330 *g*, 3 min, at 4°C.

⚠ CRITICAL: From now on, maintain EECM-BMEC-like cells at 4°C to avoid antibody internalization.

p. Prepare adhesion molecule antibody staining mixture, and isotype control antibody mixture solutions in FACS buffer (total volume 100μL/sample) (see key resources table: Antibodies used for flow cytometry analysis).
q. Discard buffer by inverting the 96-well plate in one quick forceful shake of motion and then resuspend EECM-BMEC-like cells in 100 μL of prepared antibody solutions (adhesion molecules staining mixture and isotype control antibody mixture) or FACS buffer for the unstained control. Stain EECM-BMEC-like cells with fluorescent labeled antibodies at 4°C for 20 min.

Note: After centrifugation, cell pellets remain sufficiently attached to the bottom of the 96-well plate. A single quick and forceful shake of the inverted 96-well plate discards the supernatant and avoids any cell loss.

r. Prepare compensation beads for compensation. Add 1 drop of positive beads and negative beads into 2 mL of FACS buffer and aliquot compensation beads suspensions into 7 separate flow tubes, then add respective volume (see key resources table: Antibodies used for flow cytometry analysis) of each fluorophore-conjugated antibody (see key resources table: Antibodies used for flow cytometry analysis). Store at 4°C protected from light until the measurement.

Note: We recommend using antibody compensation beads for single antibody staining for compensation since EECM-BMEC-like cells do not necessarily contain both negative and positive cells for all antigens of interest.

s. After 20 min of antibody incubation, centrifuge EECM-BMEC-like cells at 330 *g*, 3 min, at 4°C.
t. Discard buffer containing antibodies by inverting the 96-well plate in one continuous motion and add 200 μL of FACS buffer and resuspend EECM-BMEC-like cells for washing.

Note: After centrifugation, cell pellets remain sufficiently attached to the bottom of the 96-well plate. A single quick and forceful shake of the inverted 96-well plate discards the supernatant and avoids any cell loss.

u. Centrifuge EECM-BMEC-like cells at 330 *g*, 3 min, at 4°C.
v. Discard buffer by inverting the 96-well plate in one continuous motion.

Note: After centrifugation, cell pellets remain sufficiently attached to the bottom of the 96-well plate. A single quick and forceful shake of the inverted 96-well plate discards the supernatant and avoids any cell loss.

w. Resuspend EECM-BMEC-like cells in 200 μL of FACS buffer and analyze on a flow cytometer.

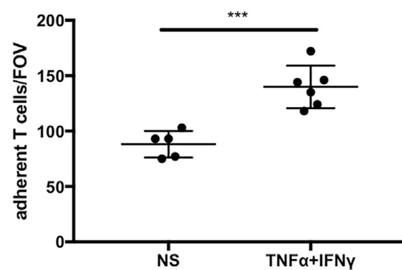

Figure 7. Adhesion assay using EECM-BMEC-like cells with T cells
The number of cell tracker-labeled T cells adherent to non-stimulated (NS) and 0.1 ng/mL TNF-α + 2 IU/mL IFN-γ stimulated EECM-BMEC-like cell monolayers were shown. Bars show the mean, and error bars show SD. Statistical analysis: unpaired t test. (P< 0.001 = ***).

Note: We use the Attune NxT Flow Cytometer (Thermofisher Scientific) and the following settings: FSC (voltage 50), SSC (voltage 280), BL1 filter (voltage 300) for FITC detection, RL1 filter (voltage 360) for APC detection, RL3 filter (voltage 340) for APC-Cy7 detection, VL1 filter (voltage 220) for BV421 detection, VL2 filter (voltage 240) for BV510 detection, YL1 filter (voltage 320) for PE detection, and YL4 filter (voltage 340) for PE-Cy7 detection.

Timing: 2 days for EECM-BMEC-like cells culture and 3–4 h for T-cell adhesion assay

This step describes a T-cell adhesion assay under static conditions to assess functional expression of endothelial adhesion molecules on EECM-BMEC-like cells. This assay is especially important since EECM-BMEC-like cells with their mature immune phenotype are the first hPSC-derived BBB model suitable to study immune cell interactions.

16. T-cell adhesion assay under static condition (expected results are shown in Figure 7)

Note: We here describe the method using human Th1 cells sorted from peripheral blood based on their chemokine receptor expression and expanded for 20 days with periodic restimulation with phytohemagglutinin and irradiated allogeneic peripheral blood mononuclear cells and human IL-2 exactly as described before (Nishihara et al., 2020a, 2020b; Sallusto et al., 1998; Engen et al., 2014). The T-cell culture medium and number of T cells added to EECM-BMEC-like cells needs to be optimized for the T cells of your interest.

a. Prepare EECM-BMEC-like cell monolayers as described in section 14 steps "a–i".

Note: We usually prepare at least 8 wells (4 wells for non-stimulated and 4 wells for pro-inflammatory cytokine-stimulated conditions).

b. Approximately 24 h later, change hECSR medium to CM derived from SMLCs (for preparation of CM, see step "9l") in the presence or absence of 0.1 ng/mL TNF-α + 2 IU/mL IFN-γ for 16–24 h.
c. Prepare a 15 mL centrifuge tube containing 10 mL T-cell wash buffer at 37°C.
d. Thaw 1 vial (8×10^6 cells) of cryopreserved T cells by rapidly rubbing the tube between your hands or swirling in a 37°C water bath until there is only a small piece of ice remaining.
e. Using a micropipette, carefully drop the cell suspension into the 15 mL centrifuge tube containing 10 mL T-cell wash buffer.

Note: At the first signs of thawing, T cells should immediately be transferred into the 15 mL tube containing 10 mL of T-cell wash buffer to reduce cryopreservation-mediated damage to the cell membranes.

f. Centrifuge T cells at 280 *g*, 5 min, at 20°C–25°C. While T cells are centrifuging, label a 24-well plate with the name of the T cell and date.
g. Remove the supernatant and carefully resuspend the pellet in 2 mL of T-cell medium and seed the T cells suspension onto 2 wells of 24-well plate.
h. Transfer 24-well plate into the incubator (37°C, 5% CO_2).

i. At the day of the experiment, label T cells with 1 μM CMFDA (add CMFDA stock solution at 1:3000) at 37°C, 5% CO_2 in the incubator for 30 min.
j. After CMFDA labeling, collect T cells into 15 mL centrifuge tube and fill up to 12 mL with T-cell wash buffer.
k. Centrifuge T cells at 280 *g*, 5 min, at 20°C–25°C.
l. Aspirate supernatant and resuspend T cells in 6 mL of T-cell wash buffer.
m. Carefully add 3 mL of Ficoll-Paque PLUS below the T cell suspension.
n. Centrifuge at 770 *g*, 20 min at 20°C–25°C with minimum brake and acceleration.
o. Using micropipette, collect live T cell interphase between the T-cell wash buffer and the Ficoll-Paque PLUS into a new 15 mL centrifuge tube and fill to 12 mL using T-cell wash buffer.

Note: For the details on usage of Ficoll-Paque PLUS, please refer to the manufacturer's instructions https://www.sigmaaldrich.com/technical-documents/protocols/biology/isolation-of-mononuclear-cells/recommended-standard-method.html

p. Centrifuge T cells at 280 *g*, 5 min, at 20°C–25°C.
q. Aspirate supernatant and resuspend T cells in 5 mL of migration assay medium.
r. Resuspend T cell suspension well and take 10 μL of cell suspension for counting.
s. Centrifuge T cells at 280 *g*, 5 min, at 20°C–25°C.
t. Aspirate supernatant and resuspend at 2×10^5 T cells per ml of migration assay medium.
u. Carefully remove CM from chamber slide containing EECM-BMEC-like cells and add 100 μL of T cell suspension (2.0×10^4 cells/well).

⚠ CRITICAL: We recommend not washing the EECM-BMEC-like cell monolayer before adding the T cells to avoid disruption of the EECM-BMEC-like cell monolayer.

v. Incubate EECM-BMEC-like cells with T cells on an orbital shaker platform for 30 min at 20°C–25°C with protection from light.
w. After 30 min of T cell-EECM-BMEC-like cell interaction, remove plastic chambers from the glass slide.
x. Wash the slide containing T cell and EECM-BMEC-like cells twice by gently dipping the slide into a jar with dPBS to remove non-adherent T cells.
y. Fix adherent T cell and EECM-BMEC-like cells with 2.5% (v/v) glutaraldehyde solution diluted in dPBS at 4°C for 2 h.

Note: We recommend using glutaraldehyde solution to fix cells after the adhesion assay since glutaraldehyde is a stronger fixative compared to paraformaldehyde and thus reduces the risk of T cells detaching from the EECM-BMEC-like cells.

z. After 2 h of fixation, wash slides containing adherent T cells and EECM-BMEC-like cells twice by dipping into a jar with dPBS and examine the slide using a fluorescence microscope.

Note: We image adherent T cells using the 10 × objective of the fluorescence microscope Nikon Eclipse E600 microscope connected to a Nikon Digital Camera DXM1200F with Nikon NIS-Elements BR3.10 software (Nikon, Egg, Switzerland). Acquire images from the center of the well. Analyze images with FIJI software (Version 2.0.0, Image J, USA) using following macro (Threshold and particle size must be manually adapted depending on signal intensity.):

```
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setAutoThreshold("Default dark");

//run("Threshold...");

setThreshold(35, 255);
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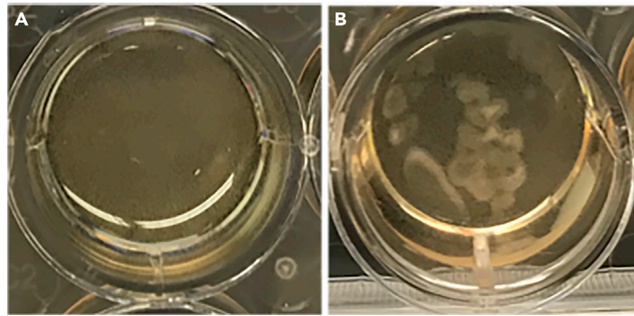


Figure 8. Image of EPC differentiation at day 4
(A) Optimal seeding density produces a confluent monolayer of differentiating cells.
(B) A seeding density that is too high results in differentiating cells detaching from plate surface.

```
run("Convert to Mask");

run("Watershed");

run("Analyze Particles...", "size=50-Infinity circularity=0.1-1.00 show=Overlay summarize")
```

EXPECTED OUTCOMES

The anticipated outcomes of this protocol are as follows:

EPC differentiation

EPC differentiation should yield a population of 10%–40% $CD31^{+}$ EPCs pre-MACS and ideally >95% EPCs post-MACS (Figure 2).

Permeability assay

The typical permeability coefficient of EECM-BMEC-like cell monolayers measured for sodium fluorescein is below 0.5×10^{-3} cm/min.

Immunocytochemistry staining of EECM-BMEC-like cells after permeability assay

After selective passages (EECM-BMEC-like cells at passage 3–5), EECM-BMEC-like cells should form monolayers without any remaining SMLCs and display continuous tight and adherens junctions (Figure 3).

Immunocytochemistry staining of SMLCs cultivated on a chamber slide

SMLCs should stain positive for α-SMA, SM22a, and calponin (Figure 4).

Staining of EECM-BMEC-like cells for adhesion molecules by immunofluorescence staining and flow cytometry

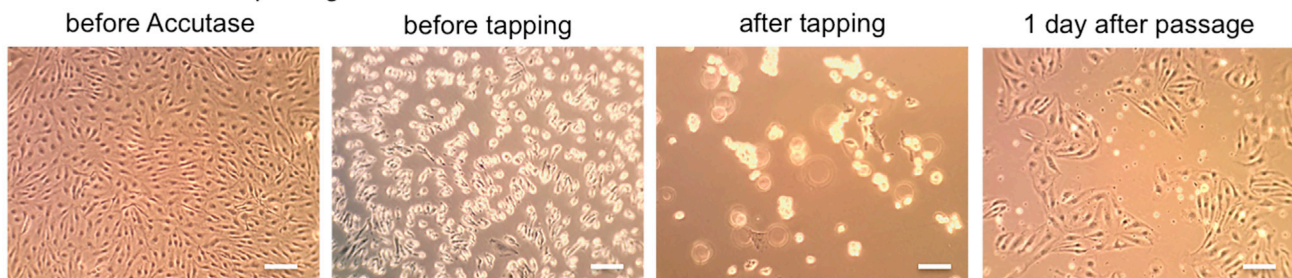
EECM-BMEC-like cells co-cultured with SMLC or cultivated using SMLC-derived CM should stain positive for ICAM-1 under both non-stimulated and proinflammatory cytokine-stimulated conditions as well as for VCAM-1 after proinflammatory cytokine stimulation (Figures 5 and 6).

T-cell adhesion on EECM-BMEC-like cells under static condition

Pro-inflammatory cytokine stimulation significantly increases the numbers of T cells adhering to EECM-BMEC-like cell monolayers (Figure 7).

LIMITATIONS

This protocol has been tested with H9 hESCs, IMR90-4 hiPSCs, six healthy control-derived hiPSC lines (Nishihara et al., 2020a) and seven patient derived hiPSC lines (Perriot et al., 2018). However,



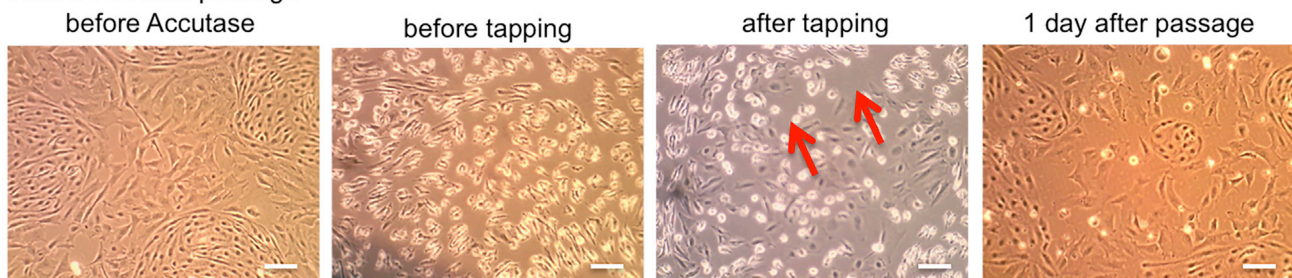


Figure 9. Bright field images of ECs during selective passage

Red arrows indicate detachment of SMLCs within SMLCs island. Scale bar = 100 μm.

the efficiency of the protocol may vary with other hPSC lines and may require adjustment of the initial seeding density and CHIR99021 concentrations.

TROUBLESHOOTING

Problem 1: Differentiating cell detachment during EPC differentiation

Differentiating cell detachment is usually observed at day 3 - day 5 of EPC differentiation (Figure 8).

Potential solution

If differentiating cells detach from the surface, decrease the initial hPSC seeding density. We recommend testing different seeding densities at day -3 (range from 20–100 $\times 10^3/cm^2$ or 75–400 $\times 10^3$ per well of a 12-well plate). Optimize seeding density for individual hPSC lines and passages. Alternatively, optimization of the CHIR99021 concentration may be required. Though we identified 7–8 μM CHIR99021 to be optimal for the hPSC lines tested by us, other lines may respond to CHIR99021 treatment differently.

Problem 2: Low EPC purity

Low EPC purity is observed when differentiating hPSCs towards EPCs.

Potential solution

If EPC purity of a pre-MACS sample is less than 10% $CD31^+$ cells, optimize the initial hPSC seeding density. Typically, increasing seeding density results in a higher EPC percentage. However, seeding densities that are too high often lead to "Problem 1" (cell detachment). We recommend testing different seeding densities at day -3 (range from 20–100 $\times 10^3/cm^2$ or 75–400 $\times 10^3$ per well of a 12-well plate) and optimizing seeding density for individual lines and passages. A successful differentiation can often be predicted by monitoring key features of the differentiating cells as follows: Differentiating cells should be confluent by day 1, or at the latest day 2. Similarly, the medium color should be nearly yellow prior to medium change on day 2 – day 5. If the color is red – orange (Figure 1), differentiation is likely to fail.

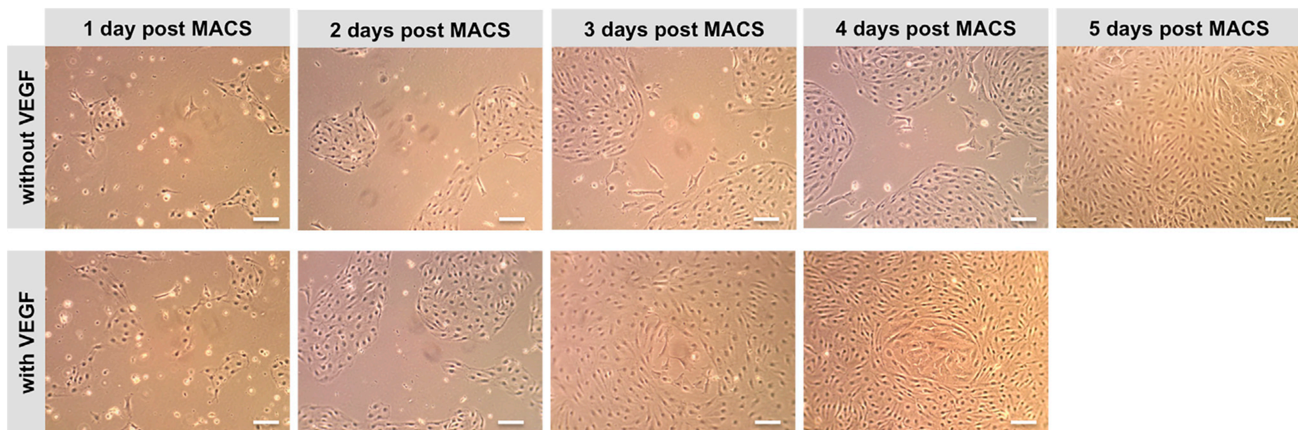


Figure 10. Bright field images of post-MACS EPCs in the presence or absence of 10 ng/mL VEGF supplementation
Scale Bars = 100 μm.

Problem 3: Failure of selective EC passage

Failure of selective passage is usually observed if the proportion of SMLCs in a culture is high (more than approximately 30%) when passaging ECs.

Potential solution

A critical parameter for a successful selective passage is the percentage of SMLCs in the culture, which should ideally be less than 10%. At more than 30% SMLCs, a successful selective passage is quite difficult (Figure 9). If EPC purity of the post-MACS sorting sample is less than 90% $CD31^+$, the population of non-ECs will increase over time. In this case, we recommend optimizing the initial hPSC seeding density to improve pre-MACS purity (see problem 2) and/or exercising additional care during MACS to remove non-bound cells (see step "6q" and Methods video S2).

A successful selective passage is typically achieved when cells are 100% confluent and SMLCs form clear islands (Figure 1). However, for some hPSC lines, SMLCs also detach if cultures are 100% confluent. In this case, we recommend attempting selective passage with a less confluent (80% or less) culture.

Alternatively, we have found that adding VEGF at 10 ng/mL to hESCR medium during the first 3–5 days of EC culture (starting immediately after MACS and until cells reach 100% confluency) facilitates rapid proliferation of ECs (Figure 10). The resulting EECM-BMEC-like cells at passage 3–5 show comparable permeability values to non-VEGF-treated cells (Figure 11).

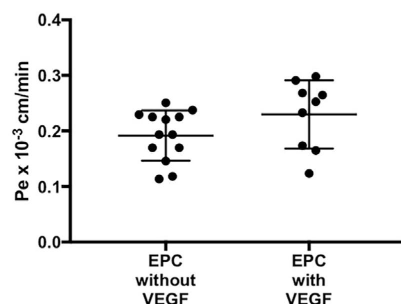


Figure 11. Permeability to sodium fluorescein of EECM-BMEC-like cells pretreated with 10 ng/mL VEGF for 3–5 days immediately after MACS compared to non-VEGF-treated cells
Bars show the mean, and error bars show SD.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Britta Engelhardt (bengel@tki.unibe.ch).

Materials availability

This study did not generate new unique reagents and the majority of materials required in this protocol are commercially available. The iPS(IMR90)-4 iPSC line and H9 (WA09) hESC line can be obtained from WiCell (https://www.wicell.org) upon completion of a material transfer agreement.

Data and code availability

This study did not generate/analyze any datasets or code that were submitted to any repositories.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100563.

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AUTHOR CONTRIBUTIONS

H.N. contributed with performing and analyzing experiments, developing methodology, funding acquisition, and writing of the original manuscript. P.K. contributed with performing and analyzing experiments. B.D.G. contributed with performing and analyzing experiments, developing methodology, and manuscript editing. S.P.P. contributed with developing methodology, funding acquisition, and editing of the manuscript. E.V.S. contributed with developing methodology, funding acquisition, and editing of the manuscript. B.E. contributed with supervision of study, funding acquisition, project administration, and writing and editing of the manuscript.

DECLARATION OF INTERESTS

B.E. received a grant from Biogen to study extended dosing of Natalizumab on T-cell migration across the blood-brain barrier and a grant from CSL Behring to investigate the molecular underpinnings of blood-brain barrier dysfunction in neurological disorders. H.N., B.D.G., S.P.P., E.V.S., and B.E. are inventors on a provisional US patent application related to the EECM-BMEC-like cells.

REFERENCES

Bao, X., Lian, X., and Palecek, S.P. (2016). Directed endothelial progenitor differentiation from human pluripotent stem cells via Wnt activation under defined conditions. Methods Mol. Biol. *1481*, 183–196.

Cecchelli, R., Aday, S., Sevin, E., Almeida, C., Culot, M., Dehouck, L., Coisne, C., Engelhardt, B., Dehouck, M.P., and Ferreira, L. (2014). A stable and reproducible human blood-brain barrier model derived from hematopoietic stem cells. PLoS One *9*, e99733.

Coisne, C., Dehouck, L., Faveeuw, C., Delplace, Y., Miller, F., Landry, C., Morissette, C., Fenart, L., Cecchelli, R., Tremblay, P., and Dehouck, B. (2005). Mouse syngenic in vitro blood-brain barrier model: a new tool to examine inflammatory events in cerebral endothelium. Lab. Invest. *85*, 734–746.

Engen, S.A., Valen Rukke, H., Becattini, S., Jarrossay, D., Blix, I.J., Petersen, F.C., Sallusto, F., and Schenck, K. (2014). 'The oral commensal Streptococcus mitis shows a mixed memory Th cell signature that is similar to and cross-reactive with Streptococcus pneumoniae. PLoS One *9*, e104306.

Lian, X., Bao, X., Al-Ahmad, A., Liu, J., Wu, Y., Dong, W., Dunn, K.K., Shusta, E.V., and Palecek, S.P. (2014). 'Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. Stem Cell Reports *3*, 804–816.

Nishihara, H., Gastfriend, B.D., Soldati, S., Perriot, S., Mathias, A., Sano, Y., Shimizu, F., Gosselet, F., Kanda, T., Palecek, S.P., Du Pasquier, R., Shusta,

E.V., and Engelhardt, B. (2020a). 'Advancing human induced pluripotent stem cell-derived blood-brain barrier models for studying immune cell interactions. FASEB J. *34*, 16693–16715.

Nishihara, H., Soldati, S., Mossu, A., Rosito, M., Rudolph, H., Muller, W.A., Latorre, D., Sallusto, F., Sospedra, M., Martin, R., Ishikawa, H., Tenenbaum, T., Schroten, H., Gosselet, F., and Engelhardt, B. (2020b). 'Human CD4(+) T cell subsets differ in their abilities to cross endothelial and epithelial brain barriers in vitro. Fluids Barriers CNS *17*, 3.

Perriot, S., Mathias, A., Perriard, G., Canales, M., Jonkmans, N., Merienne, N., Meunier, C., El Kassar, L., Perrier, A.L., Laplaud, D.A., Schluep, M., Deglon, N., and Du Pasquier, R. (2018). Human induced pluripotent stem cell-derived astrocytes are differentially activated by multiple sclerosis-associated cytokines. Stem Cell Reports *11*, 1199–1210.

Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., Mackay, C.R., Qin, S., and Lanzavecchia, A. (1998). 'Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. Eur. J. Immunol. *28*, 2760–2769.