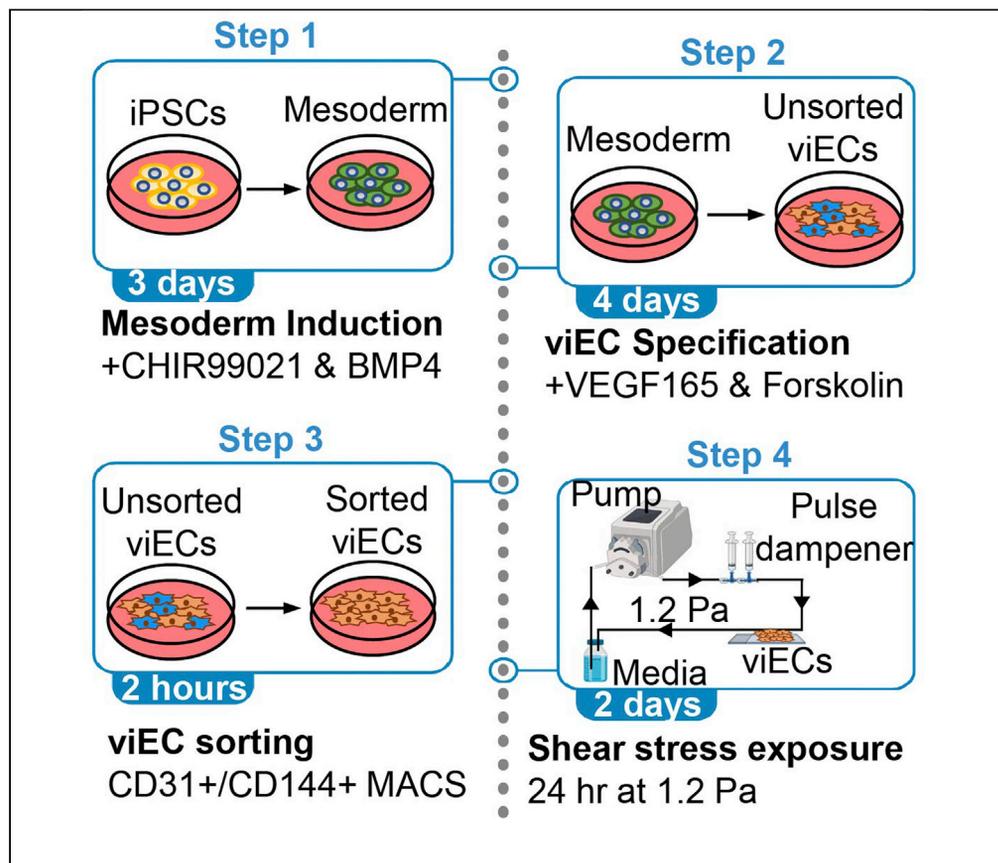


Protocol

Differentiation and characterization of human iPSC-derived vascular endothelial cells under physiological shear stress



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Highlights

A protocol for differentiating vascular endothelial cells (viECs) from human iPSCs

Generation of up to 55 million viECs from 3.5 million iPSCs within 7 days

Design and use of parallel-plate flow chamber to study EC behavior under flow

viECs express EC markers, upregulate flow-sensitive genes, and align to flow direction

Induced pluripotent stem cells (iPSCs) offer a potentially unlimited source to generate endothelial cells (ECs) for numerous applications. Here, we describe a 7-day protocol to differentiate up to 55 million vascular endothelial cells (viECs) from 3.5 million human iPSCs using small molecules to regulate specific transcription factors. We also describe a parallel-plate flow chamber system to study EC behavior under physiological shear stress.

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Protocol

Differentiation and characterization of human iPSC-derived vascular endothelial cells under physiological shear stress

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SUMMARY

Induced pluripotent stem cells (iPSCs) offer a potentially unlimited source to generate endothelial cells (ECs) for numerous applications. Here, we describe a 7-day protocol to differentiate up to 55 million vascular endothelial cells (viECs) from 3.5 million human iPSCs using small molecules to regulate specific transcription factors. We also describe a parallel-plate flow chamber system to study EC behavior under physiological shear stress.

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

BEFORE YOU BEGIN

Note: All sterile procedures are performed in a Class II biological safety cabinet with standard aseptic technique. Cells are cultured in a humidified incubator at 37°C with 5% CO₂.

Prepare buffers, media, and growth factors

⌚ Timing: 2 h

1. Prepare iPSC media, N2B27 media, StemPro-34 SFM complete media, viEC media, and MACS buffer as outlined in the respective tables in the [materials and equipment](#) section.
2. Prepare Y-27632 (ROCK inhibitor), CHIR99021, BMP4, VEGF164, forskolin, heparin, and SB431542 stock solutions as outlined in the [materials and equipment](#) section.

Aliquot matrigel

⌚ Timing: 30 min

To avoid multiple freeze-thaws, aliquot Matrigel into small single-use aliquots.

3. Thaw a 5 mL bottle of hESC-qualified Matrigel on ice in the refrigerator (4°C) for 16–20 hours.
4. In a sterile cell culture hood, place the thawed Matrigel bottle on ice and divide the Matrigel into 120 µL aliquots in 0.5 mL pre-chilled microcentrifuge tubes.
5. Place each tube on ice immediately after filling with Matrigel.



△ **CRITICAL:** Matrigel gels very quickly at temperatures above 22°C. It must be kept on ice when outside of the freezer or refrigerator.

- When finished aliquoting the contents of the bottle, store the aliquots at –20°C until the lot expiration date.

Coat plates with matrigel

⌚ **Timing:** 35 min

Human induced pluripotent stem cells (iPSCs) are cultured in 6-well plates coated with human embryonic stem cell (hESC)-qualified Matrigel for maintenance culture, and Matrigel-coated T-75 flasks for differentiation. Matrigel is used at 1:100 dilution in DMEM/F-12 media.

Note: Before starting, aliquot Matrigel into 120 µL single-use aliquots as described in the [before you begin](#) section.

- One 120 µL aliquot of Matrigel can coat two 6-well plates or 3 T-75 flasks. To begin coating, thaw the desired number of aliquots of Matrigel on ice for 1 hour.
- For each 120 µL Matrigel aliquot, prepare 12 mL cold DMEM/F-12 in a conical tube.
- Use a 200 µL pipette to transfer thawed Matrigel to the cold DMEM/F-12.
- Mix the solution and add 1 mL per well for 6-well plates or 4 mL per T-75.

Note: Since this is a small volume of coating solution, gently shake the flask or well to ensure that the entire surface is covered. Do not plate cells on Matrigel that has dried up. See [troubleshooting 1](#).

- Incubate the plates for 30 minutes at 37°C in a humidified cell culture incubator.

⏸ **Pause point:** If they are not being used on the same day as coating, Matrigel-coated plates can be wrapped in Parafilm and stored at 2–8°C for up to 1 week. Keep the plates on a level surface to prevent uneven coating. When ready to use, remove Parafilm and warm the plate in a humidified incubator at 37°C for 30 min. Do not use the plates if the Matrigel solution on all or part of the culture surface has evaporated.

- When ready to use the Matrigel-coated plate, aspirate the DMEM/F-12 and add cell solution. Refer to the [step-by-step method details](#) section for specific cell densities for each step of the protocol.

Make acetic acid stock

⌚ **Timing:** 10 min

Collagen I is diluted in 0.02 M acetic acid to coat flasks for culturing viECs.

Note: Collagen is stable in acidic solution and low ionic strength ([Cooper, 1970](#)).

- Make a sterile solution of 0.02 M acetic acid by diluting glacial acetic acid (17.4 M) in distilled H₂O (dH₂O) and filtering through a 0.22 µm filter.
 - For 500 mL 0.02M acetic acid, add 575 µL glacial acetic acid to 500 mL dH₂O and filter.
 - Diluted acetic acid can be stored at 20°C–25°C for up to 2 years, but we routinely use all 500 mL within 2 months.

Coat plates with collagen

⌚ Timing: 1 h

Differentiated viECs are cultured on plates coated with 0.05 mg/mL collagen I. Plates can be coated with collagen I between 1 hour and 1 week in advance of plating viECs.

Note: Collagen I promotes normal EC phenotype as opposed to fibronectin, which is deposited preceding atherosclerotic lesion development *in vivo* and promotes EC inflammation (Al-Yafeai et al., 2018). Additionally, we have found in our experiments that viEC adhesion during parallel plate flow experiments is improved when they are plated on collagen I rather than fibronectin.

Note: Corning Collagen I stock solution concentration varies based on lot but is always between 3–4 mg/mL and is dissolved in 0.02 M acetic acid. Before starting, make a solution of sterile 0.02 M acetic acid as described in the [materials and equipment](#) section to use for further diluting the Collagen I to 0.05 mg/mL per the manufacturer's instructions.

14. Calculate the volume of collagen I solution needed based on the following volume relations and the number of flasks desired: 5 mL collagen I solution per T-75 flask, 10 mL collagen I solution per T-175 flask, and 2 mL collagen I solution per SlideFlask.
15. Dilute collagen I to 0.05 mg/ml in 0.02 M acetic acid of the appropriate volume for the number of plates being coated.
16. Add the diluted collagen I solution to the flasks and incubate at 20°C–25°C for 1 hour on a flat surface such that coating solution is evenly distributed across the culture area.
17. Aspirate the collagen I solution and wash plates with PBS once.
18. Aspirate PBS and use the plates immediately or store at 4°C for up to one week.

⚠ **CRITICAL:** Do not forget to wash collagen-coated plates with PBS to remove the toxic acetic acid. See [troubleshooting 2](#).

Fabricate, assemble, and sterilize parallel plate perfusion circuit components

⌚ Timing: 2 weeks

19. Design and fabricate the parallel plate flow chamber
 - a. Fabricate a parallel plate flow chamber from acrylic or polycarbonate as detailed in the engineering designs and descriptions provided in the [materials and equipment](#) section. The exact dimensions can be customized to enable the desired shear stress and flow rate based on the pump being used.

Note: Our flow chamber which is pictured throughout the protocol has a metal bottom with a glass window in the center to enable visualization of the slide, and an acrylic top. Alternatively, for ease we recommend that the entire flow chamber be made of acrylic, polycarbonate, or another cell-compatible material.

- b. Screw twelve 10–24 threaded 1" Phillips flat head screws into the threaded holes of the bottom half of the chamber.
- c. Reserve twelve 10–24 wingnuts that fit the screws to be used to assemble the chamber during flow experiment set-up.
- d. Screw in a 5/32" barbed tube fitting + 10–32 male threaded pipe to each side of the top piece of the chamber to serve as inlet/outlet ports.
- e. A silicone gasket separates the two pieces of the flow chamber and provides a flow path. Cut a gasket of desired thickness such that it fits onto the bottom half of the chamber and frames

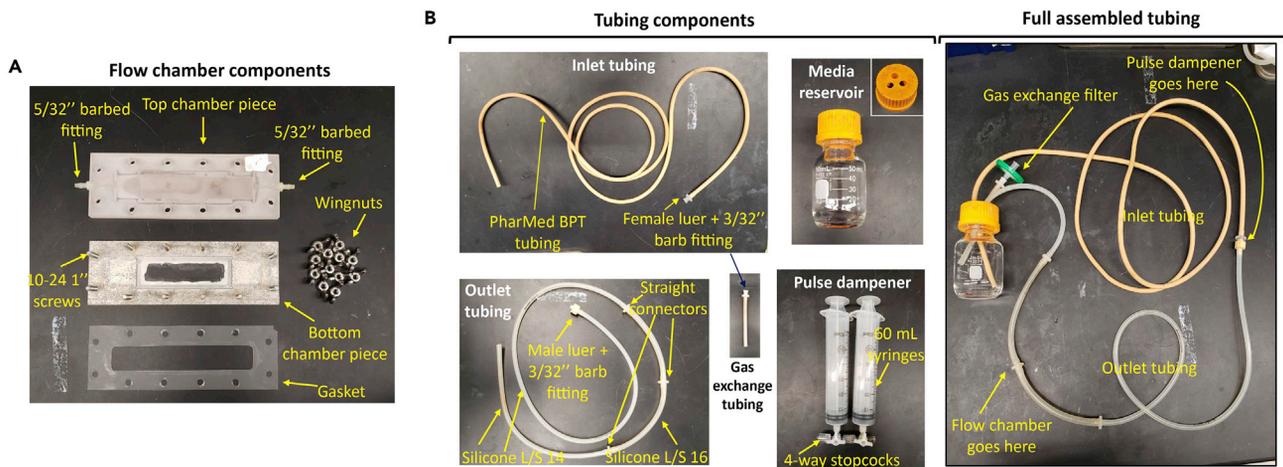


Figure 1. Flow circuit components

(A) Flow chamber components, including top piece, bottom piece, gasket, screws, and wingnuts.

(B) Tubing components, including inlet tubing, outlet tubing, gas exchange tubing, media reservoir, and pulse dampener.

a culture area around the slide (Figure 1A). We use 0.02" thick gaskets with 3' × 1' slides of 0.04" (1 mm) thickness, which allows imaging with a 10× microscope objective. To view at 20× or 50×, an extra-long working distance objective is needed.

Note: Thicker slides may be used as long as the depth of groove for the slide in the bottom piece of the flow chamber is modified to fit the thicker slide and the thickness of the bottom piece of the chamber and the slide combined is smaller than the working distance of the microscope objective being used. 1 mm slides are suitable for 10× normal working distance objectives. Slides thicker than 1 mm may require extra-long working distance objectives to visualize the cells. Slides thinner than 1 mm are not recommended as they may break too easily.

20. Measure the height of the flow path

- Use a slide of known thickness to calibrate the focus knob of a microscope such that the height in mm corresponding to moving one tick mark of the focus knob is known. Mark an "A" on the bottom of a 1 mm thick slide and a "B" on the top of the slide. Use the microscope to focus on the A, then on the B, and count the number of tick marks and focus knob rotations required to move between the two points. Divide 1 mm by the number of tick marks between A and B to get the distance that the focus knob moves in mm per tick mark.
- Mark a spot on the top of another slide with an "A" and place it in the flow chamber.
- Lay the gasket flush on the bottom of the chamber.
- Mark a spot on the bottom surface of the top half of the flow chamber (the side that will come into contact with the slide) with a "B".
- Assemble the chamber and screw all twelve wingnuts tightly.
- Fill the chamber with media or PBS from one of the side ports using a syringe.
- Use the calibrated microscope to focus on the "A" and note the tick number on the focus knob.
- Focus on "B" and note the tick number on the focus knob.
- Determine the number of rotations & tick marks between the two focal planes and multiply by the mm per tick mark calibration calculated in step 20a to determine the height above the slide in the flow chamber.

Note: The measured height should be similar to the thickness of the gasket if the slide is flush with the bottom half of the chamber when sitting in its groove. As an example, our gasket is 0.05 mm and our measured flow path height is 0.0495 mm. The small decrease in the

measured height of the flow path reflects the nominal gasket thickness and gasket compression.

21. Sterilize the parallel plate flow chamber.

Note: Acrylic can be sterilized by ethylene oxide (EtO) or with bleach followed by UV light exposure for 1 h. It cannot withstand autoclaving. Polycarbonate can be autoclaved, although its dimensions will alter slightly over time with repeated autoclaving.

22. Assemble the pulse dampener

- Connect two 4-way stopcocks to form the bottom of the pulse dampener (Figure 1B). These will be used control media flow in and out of the pulse dampener during parallel plate flow experiment set-up.
- Do not connect the syringes from the figure now; these will be added later when setting up the flow chamber.
- Sterilize the stopcocks using ethylene oxide sterilization (they are not autoclavable).

23. Assemble the tubing

- Inlet tubing:** cut a long stretch of Masterflex L/S Precision Pump PharMed BPT L/S 14 tubing (Cole Parmer EW-06508-14) that can reach from inside a cell culture incubator to above the incubator where the pump sits. The length depends on the incubator dimensions but may be around 36 inches. This tubing will connect the reservoir with the left side of the pulse dampener and must reach out of the cell culture incubator to connect to the pump.

Note: Since the inlet tubing reaches outside the incubator and must withstand the pressure of the closed incubator door without deforming and forming bubbles in the flow circuit, PharMed BPT tubing is used which performs well under high pressure.

- Insert a female luer + 3/32" barb fitting on one end of the inlet tubing (Figure 1B). This will connect to one end of the pulse dampener later.
- Outlet tubing:** cut two 10 inch stretches of Masterflex L/S Precision Pump Peroxide-Cured Silicone L/S 14 tubing (Cole Parmer EW-96400-14) and two 4 inch stretches of Masterflex L/S Precision Pump Peroxide-Cured Silicone L/S 16 tubing (Cole Parmer EW-96400-16). This tubing will connect the pulse dampener to the flow chamber on one side and connect the other side of the flow chamber back to the reservoir.

Note: Since the outlet tubing is completely contained inside the cell culture incubator and does not have to withstand the pressure of the closed incubator door, Peroxide-Cured Silicone tubing is used which has advantages in biocompatibility and transparency to allow visualization of the media flow.

Optional: The short stretches of L/S 16 tubing are needed to fit the 5/32" inlet/outlet ports included on the flow chamber. We choose to use short stretches of L/S 16 tubing and connect them with longer stretches of L/S 14 tubing which forms the bulk of the outlet tubing to reduce media usage, as L/S 14 tubing has a smaller inner diameter than L/S 16 and therefore requires less media to fill. If media cost is not an issue, the L/S 14 tubing can be omitted in favor of only two 14-inch stretches of L/S 16 tubing to form the outlet tubing. This will not affect the results of the parallel plate flow chamber experiment, but will double the amount of media needed during experiment set-up.

- Use straight tubing connectors and reducers to connect the tubing in the following order: L/S 14 – L/S 16 – L/S 16 – L/S 14 (Figure 1B).
- Insert a male luer + 3/32" barb fitting on one end of the outlet tubing. This will connect to the right side of the pulse dampener later.

- f. **Gas exchange tubing:** cut a 3-inch stretch of Masterflex L/S Precision Pump Peroxide-Cured Silicone L/S 14 tubing (Cole Parmer EW-96400-14) and insert a female luer + 3/32" barb fitting on one end (Figure 1B). This will be connected to a syringe filter during flow experiment set-up and inserted through the media reservoir cap to allow sterile gas exchange.
- g. **Media reservoir:** Obtain a 50 mL media bottle with a screw cap and drill 3 holes in the cap large enough to fit the L/S 14 size tubing (Figure 1B).

Note: The L/S 14 and L/S 16 size tubing of the lengths we report require 30 mL of media to fill the tubing and leave enough media left in the reservoir to flow through the circuit. For other sizes of tubing, the media volume and reservoir size should be optimized.

- h. **Assembly:** Screw the media reservoir closed and insert the empty ends of the inlet tubing, outlet tubing, and gas exchange tubing into the holes in the cap (Figure 1B). This is the complete flow circuit tubing into which the pulse dampener and flow chamber will be inserted during flow experiment set-up.
- i. Use autoclave tape on the cap to secure the tubing.
- j. Sterilize the tubing and reservoir by autoclaving or EtO sterilization.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
mTeSR Plus	STEMCELL Technologies	5825
StemPro-34 SFM	Thermo Fisher Scientific	10639011
Neurobasal medium	Gibco	21103049
DMEM/F-12, HEPES	Gibco	11330057
N-2 supplement	Gibco	17502048
B-27 supplement, minus vitamin A	Gibco	12587010
Matrigel hESC-qualified matrix	Corning	354277
Glacial acetic acid	Fisher Scientific	A385-500
Collagen I, rat tail	Corning	354236
PBS without calcium or magnesium	Gibco	10010049
2-Mercaptoethanol	VWR	97064-590
Penicillin-streptomycin (10,000 U/mL)	Gibco	15140122
Fetal bovine serum, heat inactivated	Gibco	10082147
GlutaMAX supplement	Gibco	35050061
Heparin	Sigma-Aldrich	H3149
Human VEGF165	GenScript	Z02689
Bovine serum albumin	Sigma-Aldrich	A7906
0.5M EDTA, pH 8.0	Invitrogen	15575020
Cryo-SFM freezing medium	PromoCell	C-29912
Y-27632 (ROCK inhibitor)	BioVision	1596
Accutase cell detachment solution	Innovative Cell Technologies	AT104
Forskolin	Abcam	ab120058
Human BMP4	PeproTech	120-05ET
CHIR99021	Cayman Chemical	13122
SB431542 (TGF- β inhibitor)	Stem Cell Technologies	72234
4% paraformaldehyde in PBS	Alfa Aesar	J61899-AP
Critical commercial assays		
CD31 MicroBead Kit, human	Miltenyi Biotec	130-091-935
CD144 (VE-Cadherin) MicroBeads, human	Miltenyi Biotec	130-097-857
LS columns	Miltenyi Biotec	130-042-401

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Method videos S1, S2, and S3	This paper; Mendeley Data	https://doi.org/10.17632/mbh572kw.1
Supplemental SolidWorks part files (1 & 2)	This paper; Mendeley Data	https://doi.org/10.17632/mbh572kw.1
Experimental models: cell lines		
Human induced pluripotent stem cells (iPSCs)	Progeria Research Foundation	iPSC lines: donor 168 clones 2, 3, 6; donor du11; donor 167 clones 2, 6; donor 003 clones 1A, 1B, 1C
Other		
Polystyrene Nunc Lab-Tek SlideFlasks	Thermo Scientific	170920
Parallel-plate flow chamber	Custom	n/a
Masterflex L/S Precision Pump Tubing, PharMed BPT, L/S 14	Cole-Parmer	EW-06508-14
Masterflex L/S Precision Pump Tubing, Peroxide-Cured Silicone, L/S 14	Cole-Parmer	EW-96400-14
Masterflex L/S Precision Pump Tubing, Peroxide-Cured Silicone, L/S 16	Cole-Parmer	EW-96400-16
Male luer lock to hose barb adaptor, 3/32"	Cole-Parmer	EW-45504-02
Female luer to hose barb adaptor, 3/32"	Cole-Parmer	EW-45501-02
Straight tube connector, 5/32"	McMaster-Carr	5117K44
Straight tube reducer, 5/32" to 3/32"	McMaster-Carr	5117K56
5/32" barbed tube fitting × 10–32 male threaded pipe	McMaster-Carr	5463K53
Matte non-reinforced silicone sheet 0.02" thickness (gasket)	Specialty Manufacturing Inc	https://www.smimfg.com/off-the-shelf/
10–24 1" stainless steel Phillips flat head screws	McMaster-Carr	91771A247
10–24 zinc-plated steel wing nuts	McMaster-Carr	90866A011
Masterflex fitting, polycarbonate, four-way stopcock with male luer lock	Cole-Parmer	EW-30600-04

MATERIALS AND EQUIPMENT

iPSC media (mTeSR Plus)		
Reagent	Final Concentration	Amount
mTeSR Plus Basal Media	n/a	400 mL
mTeSR Plus supplement	1×	100 mL
Total	n/a	500 mL

- Thaw mTeSR Plus supplement at 20°C–25°C for 1 hour (or until thawed completely) or at 4°C for 16–20 hours.
- Add the full contents of one bottle of thawed mTeSR Plus supplement to one bottle of mTeSR Plus basal media.
- Filter the media through a 0.22 μm filter.
- Aliquot the complete mTeSR Plus media (basal + supplement) into 40 mL aliquots in conical tubes and store at –20°C for up to 6 months. When ready to use an aliquot, thaw at 20°C–25°C until thawed or 4°C for 16–20 hours. Store thawed media at 4°C for up to 2 weeks. Do not refreeze thawed media aliquots.

N2B27 media

Reagent	Final Concentration	Amount
Neurobasal media	n/a	25 mL
DMEM-F12 media	n/a	25 mL
N-2 supplement	1×	0.5 mL
B-27 supplement without vitamin A	1×	1 mL
2-Mercaptoethanol	50 μM	0.05 mL
Penicillin/Streptomycin	1×	0.5 mL
Total	n/a	~50 mL

- Thaw N-2 and B-27 without vitamin A supplements in a 37°C water bath until just thawed.
- Aliquot the supplements into single-use aliquots of 1 mL B-27 and 0.5 mL N-2. Store at –20°C for up to 12 months.
- When ready to make N2B27 media, thaw one single-use aliquot of B-27 (1 mL) and one single-use aliquot of N-2 (0.5 mL) in a 37°C water bath until just thawed.
- Combine all the components listed in Table 2 and filter through a 0.22 μm filter.
- Store complete N2B27 media at 4°C protected from light for up to 1 week.

StemPro-34 SFM complete media

Reagent	Final Concentration	Amount
StemPro-34 basal media	n/a	500 mL
StemPro-34 supplement	1×	13 mL
GlutaMAX	1×	5 mL
Penicillin/Streptomycin	1×	5 mL
Total	n/a	~500 mL

- Thaw one bottle of StemPro-34 supplement at 4°C for 16–20 hours.
- Add complete contents of thawed supplement to one bottle of StemPro-34 basal media, along with 5 mL GlutaMax and 5 mL Penicillin/Streptomycin as listed in Table 3.
- Store complete StemPro-34 SFM media at 4°C protected from light for up to 1 month.

viEC media

Reagent	Final Concentration	Amount
StemPro-34 complete media	n/a	450 mL
Heat inactivated fetal bovine serum (HI FBS)	10%	50 mL
Heparin	2 μg/mL	500 μL
VEGF165	50 ng/mL	250 μL
Total	n/a	500 mL

- Thaw heat inactivated FBS at 4°C for 16–20 hours and aliquot into 50 mL aliquots. Store at –20°C until the expiration date noted on the original bottle.
- When ready to make viEC media, thaw an aliquot of heat inactivated FBS in a 37°C water bath until just thawed or at 4°C for 16–20 hours.
- Combine the reagents listed in Table 4 and filter through a 0.22 μm filter.
- Store viEC media at 4°C for up to 1 month.

Note: If less than 500 mL of viEC media is needed, scale down each ingredient listed in Table 4 to maintain the same ratios of the reagents to make the desired volume of viEC media.

Note: We have found that adding 10% serum to the viEC media improves cell attachment during parallel plate flow experiments when the cells are exposed to high shear stress. However, the serum can be omitted from the media without affecting cell survival if serum-free conditions after differentiation are desired.

MACS buffer		
Reagent	Final Concentration	Amount
PBS without calcium or magnesium	n/a	50 mL
Bovine serum albumin (BSA)	0.5%	250 mg
0.5M EDTA	2 mM	200 μ L
Total	n/a	50 mL

- Combine the reagents listed in Table 5 to make 50 mL MACS buffer. If necessary, the solution can be heated in a 37°C water bath for a few minutes to allow BSA to dissolve.
- Filter MACS buffer solution through 0.22 μ m filter and store at 4°C for up to 1 month.

Growth factor stock solutions

- Y-27632 (ROCK inhibitor): 10 mM (1000 \times) solution in DMSO, store at –20°C for up to 6 months.
- CHIR99021: 8 mM (1000 \times) solution in DMSO, store in 50 μ L single-use aliquots at –20°C for up to 1 year.
- Human BMP4: 25 μ g/mL (1000 \times) solution in 5 mM citric acid, store in 50 μ L single-use aliquots at –20°C for up to 1 year.
- Human VEGF165: 100 μ g/mL solution in sterile water, store in 80 μ L single-use aliquots at –20°C for up to 6 months.
- Forskolin: 10 mM (5000 \times) solution in DMSO, store in single-use 8 μ L aliquots at –20°C for up to 1 month.
- Heparin: 2 mg/ml (1000 \times) solution in PBS without calcium or magnesium, store at 4°C for up to 2 years.
- SB431542: 20 mM (2000 \times) solution in DMSO, store at –20°C for up to 6 months.

Parallel plate flow chamber design

The parallel plate flow chamber consists of 5 components:

- The top half of the chamber (Figures 2A and 2B) includes a 10–32 threaded inlet port and outlet port on either side that fit 10–32 threaded pipe + 5/32" barbed fittings. From each side port, a 0.74" long and 0.16" diameter hole extends into the chamber component to connect with a rectangular 0.98" wide by 0.20" high by 0.06" thick hole that extends from the bottom of the piece upwards, forming a path for media to flow in through the side port, through the cylindrical hole, and down to the bottom of the chamber. This chamber part also has twelve 0.23" diameter holes that loosely fit 10–24 screws.

Note: The 12 holes in the top half of the chamber are larger than the diameter of the screws and are not threaded such that the whole top piece can be easily dropped onto all 12 screws simultaneously during assembly without having to tighten each screw individually.

- The bottom half of the chamber includes a groove to fit a 3 \times 1" (75 \times 25mm), 0.04" (1 mm) thick slide and 12 threaded holes to tightly fit 10–24 thread 1" flat head Phillips screws (Figures 2C and 2D).
- A silicone gasket that sets the height of the flow path is cut to 1.89 cm width and 11.5 cm length, with 12 holes to fit around the screws (Figure 1A).

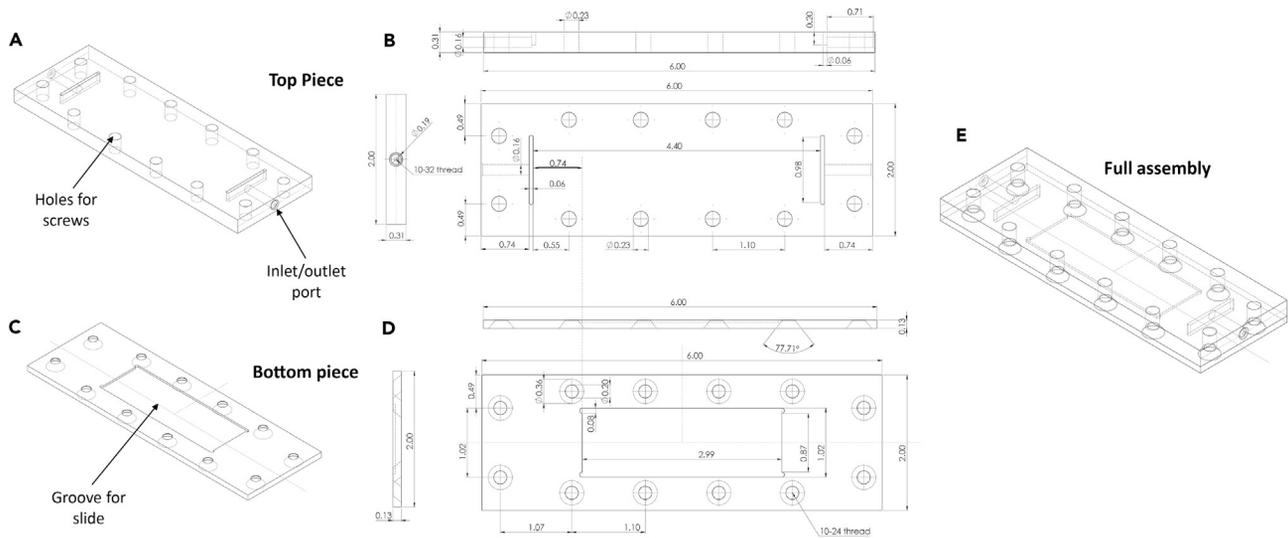


Figure 2. Engineering drawings of the parallel plate flow chamber

(A) Top piece, projected view.

(B) Top piece, overhead and side views.

(C) Bottom piece, projected view.

(D) Bottom piece, overhead and side views.

(E) Full assembly of top and bottom pieces fit together, projected view. Dimensions: inches. See Supplemental Item 1, Flow Chamber Top Piece.

SolidWorks part file for the top component of the parallel plate flow chamber, and Supplemental Item 2, Flow Chamber Bottom Piece. SolidWorks part file for the bottom component of the parallel plate flow chamber.

- Twelve 10–24 thread 1" screws and accompanying 10–24 wingnuts.
- Two 5/32" barb fittings with 10–32 male threaded pipe to fit the inlet and outlet ports of the chamber top piece (Figure 1A).

Figure 2E shows the assembly of the top and bottom components of the chamber, and the SolidWorks part files for both components are included as a Mendeley Data file. The flow chamber can be made of any cell compatible material. Ideally, the chamber should be transparent to allow visualization of the slide. Our chamber bottom is made of metal with a glass window in the slide area, and the top is made of acrylic. For ease of fabrication and use, the chamber can be made entirely of acrylic or of polycarbonate instead. We use a 0.02" (~0.05 cm) thick gasket and the exact height of our flow path is 0.019" (0.0495 cm). Gaskets purchased from Specialty Manufacturing Inc range from 0.002" to 0.06" in thickness but typical gasket thickness for parallel plate flow chambers ranges from 0.007" to 0.02" to keep flow rates reasonable. The width to height ratio of the flow path should be equal to or greater than 20:1 to ensure that the shear stress is uniform on the cell surface.

Note: The SolidWorks files can be viewed with eDrawings software (<https://www.edrawingsviewer.com/download-edrawings>).

STEP-BY-STEP METHOD DETAILS

iPSC plating and culture

⌚ Timing: ≥ 1 week (30 min/day)

1. Plate iPSCs

- Coat one 6-well plate with hESC-qualified Matrigel diluted 1:100 in DMEM/F-12 at 37°C for 30 minutes (refer to before you begin steps 7–12 for full details).

- b. Remove a vial of iPSCs from liquid nitrogen and place in a water bath set to 37°C. Swirl the vial in the water to heat evenly until only a small ice chunk remains (~1 minute).

Note: We freeze our iPSCs at a density of 1 million cells in 1 mL of Cryo-SFM freezing medium and thaw this amount into 2 wells of a 6-well plate.

- c. In a biosafety cabinet, use a 1000 μ l pipette to transfer thawed cells to a 15 mL conical tube.
- d. Using a serological pipette, slowly add 9 mL warm mTeSR Plus to the cells.
- e. Centrifuge the cells at 170 \times g for 5 minutes.
- f. Aspirate the supernatant and resuspend the cells in 4 mL warm mTeSR Plus with 10 μ M ROCK inhibitor (Y-27632).

Note: ROCK inhibitor has been shown to significantly increase hESC and iPSC survival after cryopreservation and is critical for sufficient iPSC survival at this stage (Watanabe et al., 2007; Claassen, Desler and Rizzino, 2009). Without ROCK inhibitor treatment on the first day after thawing, the survival of the plated iPSCs will significantly decrease.

- g. Plate the cells in 2 wells of the Matrigel-coated 6-well plate by aspirating the Matrigel solution in those 2 wells and adding 2 mL cell solution per well.
 - h. Shake the plate gently to evenly disperse cells and incubate in a humidified incubator at 37°C with 5% CO₂ for 24 hours.
2. The next day, replace the media with 2 mL fresh mTeSR Plus without ROCK inhibitor per well.
 3. Feed the cells with 2 mL warm mTeSR Plus per well every day for 2–3 more days until iPSC colonies are large enough to passage.
 4. For the first passage after thawing iPSCs, if the cells are not 60%–80% confluent within 4 days after thawing, passage the cells at a 1:1 ratio using EDTA.
 - a. Aspirate Matrigel from the required number of Matrigel-coated wells and add 2 mL mTeSR Plus to each well. For a 1:1 passage from 2 wells, 2 new Matrigel-coated wells are needed.
 - b. Wash cells once with 1 mL PBS without calcium or magnesium per well.

Note: EDTA chelates calcium and magnesium to allow cell dissociation, so PBS without calcium or magnesium is used so that the EDTA can chelate any residual calcium and magnesium surrounding the cells.

- c. Add 1 mL warm 0.5 mM EDTA to each well and leave at 20°C–25°C for 3 minutes.
- d. Carefully aspirate the EDTA from the cells. Do not aspirate the cells.
- e. Add 1 mL warm mTeSR Plus to each well of dissociated cells. The calcium and magnesium in the media will neutralize any remaining EDTA.
- f. Scrape cells using a cell scraper and gently triturate using a 1000 μ L pipette 4–6 times.

Note: Do not over triturate the cells or else the colonies will become too small, causing low cell survival. Colonies of ~10 cells are ideal.

- g. Combine the dissociated cells from both original wells and split among the new wells that were filled with mTeSR Plus in step 4a.
 - h. Incubate at in a humidified incubator at 37°C for 24 hours.
5. Continue to feed the cells with 2 mL warm mTeSR Plus daily and passage at low ratios (1:1 or 1:2) every 3 days until cells recover from cryopreservation and achieve 60%–80% confluence within 3 days. The cells should recover and grow at normal rate in one or two passages.
 6. For continued culture after recovery from thawing, repeat step 4 to passage cells at a 1:6 ratio in 6-well plates every 3 days. Note that to split 1 well of cells at a 1:6 ratio, 6 new wells should be filled with media in step 4a and 160 μ L dissociated cell solution will be added to each of the new wells in step 4g.

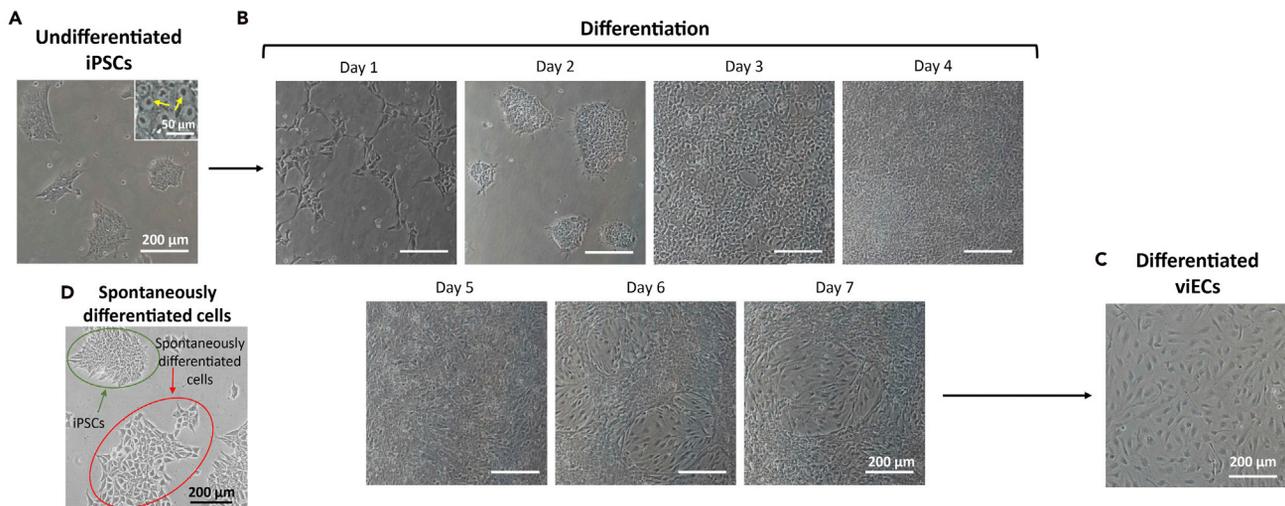


Figure 3. Representative images of iPSC-derived endothelial cell differentiation stages

- (A) Typical morphology of undifferentiated iPSCs. Inset shows prominent iPSC nucleoli (yellow arrows).
 (B) Images on days 1–7 of differentiation (mesoderm through endothelial induction).
 (C) Typical morphology of differentiated viECs 3 days after sorting.
 (D) Example of spontaneously differentiated cells which have different morphology than undifferentiated iPSCs.

△ **CRITICAL:** Poor survival immediately after thawing is expected, but iPSCs still must be passaged every 3–4 days to prevent spontaneous differentiation. If 60%–80% confluence is not reached after 3 days, passage iPSCs at a lower ratio (even 1:1) until they recover from cryopreservation. After one or two passages, they should reach 60%–80% confluence after 3 days and can be passaged 1:6 routinely. See [troubleshooting 3 and 4](#).

Note: iPSCs can be passaged dozens of times without losing pluripotency or proliferative activity. Karyotype should be checked every 20 passages to rule out karyotypic abnormalities which may occur. iPSC karyotyping can be done by a number of services, such as WiCell.

Note: iPSCs should be passaged at least 2–3 times after thawing before starting a differentiation. Culture and passage iPSCs until a sufficient number of cells for differentiation is attained (3.5 million).

viEC differentiation

⌚ **Timing:** 7 days (15–30 min/day, 2 h on day 7)

This protocol to differentiate vascular endothelial cells (viECs) from human iPSCs was adapted from [Patsch et al., \(2015\)](#). Other methods to produce viECs from iPSCs are reviewed by [Williams and Wu \(2019\)](#). Full characterization of viECs is reported in [Atchison et al., \(2020\)](#). Representative images of each step of differentiation are shown in [Figure 3](#).

7. Day 0: Plate iPSCs

- Coat one T-75 flask with hESC-qualified Matrigel diluted 1:100 in DMEM/F-12 at 37°C for 30 minutes.
- Wash 60%–80% confluent iPSCs once with 1 mL PBS per well.
- Aspirate PBS and add 1 mL Accutase to each well. Incubate at 37°C for 3 minutes.
- Lightly tap the plate to lift the iPSCs. If they have not all detached, incubate the plate at 37°C for additional time in increments of 2 minutes until all cells have detached.

Note: Total time in Accutase should not exceed 7 min. Accutase is a gentler alternative to trypsin and is used for iPSCs and iPSC-derived cells to decrease cell damage and increase cell viability after single-cell dissociation.

- e. Dilute Accutase 1:1 using warm mTeSR Plus. Collect the cells from all wells into a 15 mL conical tube and count cells.

Note: Typical iPSC yield from one 6-well plate is 8–10 million cells.

- f. Centrifuge 3.5 million cells for one T-75 differentiation (or 47,000 cells/cm² if using a different size plate for differentiation) at 170 × g for 5 minutes.

△ CRITICAL: This seeding density should be optimized for different cell lines for best results. We recommend testing a range between 37,000 and 47,000 cells/cm². The cells should look similar in density to the Day 1 image shown in [Figure 3B](#). Cell density that is too low will reduce differentiation efficiency while cell density that is too high will cause a large amount of cell death due to cells overcrowding in the flask.

Note: The differentiation can be scaled up or down by using a different size flask and adjusting the media volumes accordingly while using the same seeding density (37,000–47,000 cells/cm²).

- g. Aspirate the supernatant and resuspend the cells in 15 mL warm mTeSR Plus + 10 μM ROCK-Inhibitor.
 - h. Aspirate Matrigel and add cell suspension to the Matrigel-coated T-75 flask.
8. Day 1–3: Lateral Mesoderm Induction
- a. Prepare mesoderm induction media: 50 mL warm N2B27 medium supplemented with 8 μM (50 μL) CHIR-99021 and 25 ng/mL (50 μL) hBMP4.
 - b. Aspirate mTeSR Plus and add mesoderm induction media to cells. Leave for 3 days in the incubator without media change.
9. Day 4–6: Endothelial Cell Induction
- a. Prepare endothelial cell induction media: 40 mL warm complete StemPro-34 medium supplemented with 200 ng/mL (80 μL) VEGF165 and 2 μM (8 μL) forskolin.
 - b. Change media to fresh 40 mL endothelial cell induction media every day on days 4, 5, and 6.
 - c. Collect conditioned media from differentiating endothelial cells on days 5, 6, and 7 for later use.

Note: Some cell death on days 4–6 is expected but excessive cell death indicates that the iPSC seeding density on day 0 was too high. See [troubleshooting 5](#).

△ CRITICAL: Do not accidentally aspirate the old media while changing media on days 5–7 as it will be needed later to make conditioned media for culturing the viECs after sorting.

10. Day 7: Endothelial Cell Sorting
- a. Prepare 50 mL chilled MACS buffer and keep on ice in the cell culture hood.
 - b. Prepare 20 collagen-coated T-75 flasks by coating with 0.05 mg/mL Collagen I in 0.02 N Acetic Acid at 20°C–25°C for 1 hour, then rinsing with PBS (refer to [before you begin](#) steps 14–18 for full details).
 - c. Collect day 7 conditioned media from cells and wash with 10 mL PBS.
 - d. Add 5 mL Accutase and incubate at 37°C for 5 minutes.
 - e. Add 5 mL cold StemPro-34 and transfer cells to a 50 mL conical tube.
 - f. Count cells.
 - g. Centrifuge at 170 × g for 5 minutes.
 - h. Aspirate supernatant and wash cells with 10 mL cold MACS buffer.

- i. Centrifuge at $170 \times g$ for 5 minutes. Meanwhile, retrieve MACS beads and place in cell culture hood.
- j. Resuspend cells in 8 μL cold MACS buffer per million cells.
- k. Add 2 μl per million cells each of Fc Blocking Buffer, anti-CD31 MACS beads, and anti-CD144 MACS beads. Mix well by pipetting after adding each component.
- l. Incubate cells on ice for 15 minutes.
- m. While the cells are incubating, prepare complete conditioned media by combining conditioned media collected from days 5, 6, and 7 (120 mL total) and adding the equal volume (120 mL) of fresh complete StemPro-34 medium supplemented with 240 μL heparin (2 $\mu\text{g}/\text{ml}$) and 1.2 mL Pen/Strep. Filter through 0.22 μm vacuum filter.

Note: As this media is difficult to filter, we recommend using Nalgene Rapid-Flow filters for this step (VWR 73520-984).

- n. Warm conditioned media to 37°C in a water bath.
- o. After the 15-minute incubation, wash labeled cells by add 1 mL cold MACS buffer per million cells (or a maximum of 30 mL MACS buffer if cell yield is greater than 30 million).
- p. Centrifuge at $170 \times g$ for 5 minutes.
- q. Aspirate supernatant and resuspend cells in 1 mL cold MACS buffer.
- r. Prepare LS column and magnet on a MACS stand in the cell culture hood. Obtain three 50 mL conical tubes and one 15 mL conical tube for sorting and place in the hood.
- s. Add 1 mL cold MACS buffer to LS column. Collect the flow-through in the first 50 mL conical tube and discard.

△ CRITICAL: Do not allow the column to dry! When flowing is complete, move immediately to step 10t.

- t. Add the cell suspension to the LS column. Collect the flow-through in the second 50 mL conical tube.
- u. Collect the flow-through from the previous step and reapply to the top of the column. Collect the flow-through in the third 50 mL conical tube.
- v. Add 500 μL MACS buffer to the LS column. Continue to collect the flow-through in the third 50 mL conical tube.
- w. Repeat step 10v two more times to wash out any remaining negative cells from the column.

Optional: This is the negative fraction. It can be discarded or kept for further analysis if desired.

- x. Remove LS column from the magnet and place in the prepared 15 mL conical tube. Add 1 mL PBS and use the plunger that came with the column to push the cells out of the column into the conical tube. This is the positive fraction.
- y. Add 4 mL PBS to the retrieved positive cells.
- z. Count cells and centrifuge at $170 \times g$ for 5 minutes.

Note: On average, ~50% CD31/CD144 positive cells are recovered after sorting. Cell count after sorting ranges from 10 to 55 million cells from one T-75.

- aa. Plate 26,000 cells/ cm^2 in collagen coated T-75 flasks (~2 million cells per T-75) with 20 mL warm complete conditioned media described in step 10m. Replace with 10 mL media the next day, then every other day until conditioned media is depleted or until first passage.

Note: We report the optimal seeding density after sorting for our iPSC lines; this should be optimized for each cell line. The iPSC lines for which we have optimized this differentiation protocol are listed in in the [key resources table](#) and were obtained from the Progeria Research Foundation.

viEC culture and maintenance

⌚ Timing: 15–30 min per day

11. Feed viECs with 10 mL warm viEC media every other day.

Note: viECs should be cultured in viEC media starting when any of the following conditions are met: (1) when conditioned media is depleted, OR (2) following the first passage, OR (3) the day before cryopreserving p0 viECs.

▣▣ **Pause point:** After reaching 90% confluence, viECs can be cryopreserved (1 million cells per vial in 1 mL CryoSFM freezing medium) and stored in liquid nitrogen. If cryopreserving cells at p0, culture in viEC media for 24 h before freezing cells even if not all of the conditioned media has been depleted and discard the extra conditioned media.

12. Passage viECs when 90% confluent.
 - a. viECs can be cultured in well plates, flasks, or SlideFlasks depending on the experiment. Coat the culture vessel with 0.05 mg/mL Collagen I diluted in 0.02 N Acetic Acid at 20°C–25°C for 1 hour, then wash once with PBS (refer to [before you begin](#) steps 14–18 for full details on collagen coating).

Note: Volumes of reagents presented in this protocol are for a T-75.

- b. Add 5 mL Accutase to viECs and incubate at 37°C for 2–3 minutes.
- c. Lightly tap the plate to detach the cells.
- d. Dilute Accutase with 5 mL warm viEC media.
- e. Collect the cells in a conical tube and count cells.
- f. Centrifuge cells at $170 \times g$ for 5 minutes.
- g. Aspirate supernatant and resuspend cells in viEC media. Plate 15,000–25,000 viECs/cm².

Note: One T-75 will usually yield ~2 million cells, which can be passaged 1:2 into two T-75 s.

- h. Feed 24 hours after passaging, then every other day until the next passage.

▣▣ **Pause point:** viECs can be cryopreserved at any passage.

13. To cryopreserve viECs, passage using Accutase and freeze 1 million cells per cryovial in 1 mL CryoSFM freezing medium.
14. To thaw cryopreserved viECs, thaw into warm viEC media then plate on collagen coated plates (1 million cells per T-75). Feed the day after thawing, then every other day.

Optional: Supplementation with 10 μ M TGF β inhibitor SB431542 when feeding viECs significantly improves viEC proliferation ([Figure 5F](#)). Without it, viECs become senescent by passage 3 ([Figure 5G](#)). With 10 μ M SB431542 supplementation, we have been able to maintain viECs for at least 5 passages without inducing senescence and while maintaining confluent monolayers. See [troubleshooting 6](#).

Prepare viECs for parallel plate flow experiment

⌚ Timing: 3 days

Parallel plate flow chamber experiments can be used to study viEC behavior under physiological shear stress. viECs are cultured in polystyrene 9 cm² SlideFlasks and placed in a custom parallel plate flow chamber that allows media to be pumped over the cells at a specified flow rate. After 24–48 hours, cells can be harvested for gene or protein expression analysis.

15. Coat Nunc polystyrene SlideFlasks with 0.05 mg/mL Collagen I in 0.02 M Acetic Acid at 20°C–25°C for 1 hour, then wash once with PBS. 2 mL of coating solution is needed to coat each flask (refer to before you begin steps 14–18 for full details on collagen coating).
16. As described in step 12, use Accutase to passage viECs to slides at a density of 27,000 viECs/cm² (~250,000 viECs per 9 cm² SlideFlask) in 4 mL of viEC media per SlideFlask. Feed the cells the day after passaging, then every other day.

Note: The viEC seeding density should be optimized for each cell line to form a confluent monolayer on the SlideFlask.

Optional: viEC response to treatments can be tested by supplementing the media with therapeutics of interest at this time.

Set up parallel plate flow experiment

⌚ Timing: 3 h

With a confluent monolayer of viECs in SlideFlasks, parallel plate flow experiments can be set up to study viEC behavior under physiological shear stress. Based on counting adhered cells in images of slides taken before beginning flow experiments, viEC are confluent at a cell density of $53,028 \pm 11,690$ cells/cm² (mean \pm SD). If testing a treatment on the viECs for a specified number of days, a non-treated control that was cultured for the same duration should be used to account for differences in endothelial behavior based on cell density or duration in culture. For example, if testing a 7-day treatment of a particular therapeutic followed by a 24-hour parallel plate flow experiment, we would also culture a non-treated SlideFlask of viECs for 7 days and perform a 24-hour parallel plate flow experiment as a control.

17. Assemble and sterilize the flow circuit components as described in the [before you begin](#) section.
18. Obtain and autoclave two fine-point tweezers.
19. Warm 37 mL viEC media in a 37°C water bath.
20. In the biosafety cabinet, assemble the pulse dampener by obtaining the two sterile 4-way stopcocks linked in series and screwing two sterile 60 mL Luer-Lock syringes into the top outlet of each stopcock ([Figure 1B](#)).

Note: There are also commercial pulse dampeners available that can be used (e.g., Cole Parmer 0759620), but these use large media volumes. We use this custom pulse dampener to reduce the required media volume.

21. Pull the plunger for each syringe up to the 25 mL mark.
22. Assemble the rest of the tubing by screwing the female luer + 3/32" barb fitting on the inlet tubing to the left side of the pulse dampener and the male luer + 3/32" barb fitting on outlet tubing to the right side ([Figure 1A](#)).
23. Attach a 0.22 μ m syringe filter to the female luer + 3/32" barbed fitting of the gas exchange tubing and use parafilm to secure the connection ([Figure 1B](#)).
24. Stabilize the pulse dampener by taping the syringes to a conical tube holder such that they are perpendicular to the flat surface of the biosafety cabinet.
25. Fill the reservoir bottle with 30 mL warm viEC media and screw the cap closed.
26. If the autoclave tape securing the tubing on the cap has come loose, reinforce it with lab tape.
27. Secure the media reservoir bottle and the pulse dampener conical rack to a tray using lab tape ([Figure 4A](#)).

⚠ CRITICAL: If the flow circuit components are not appropriately secured to the tray, they may be knocked over in the incubator which will introduce air bubbles into the flow circuit.

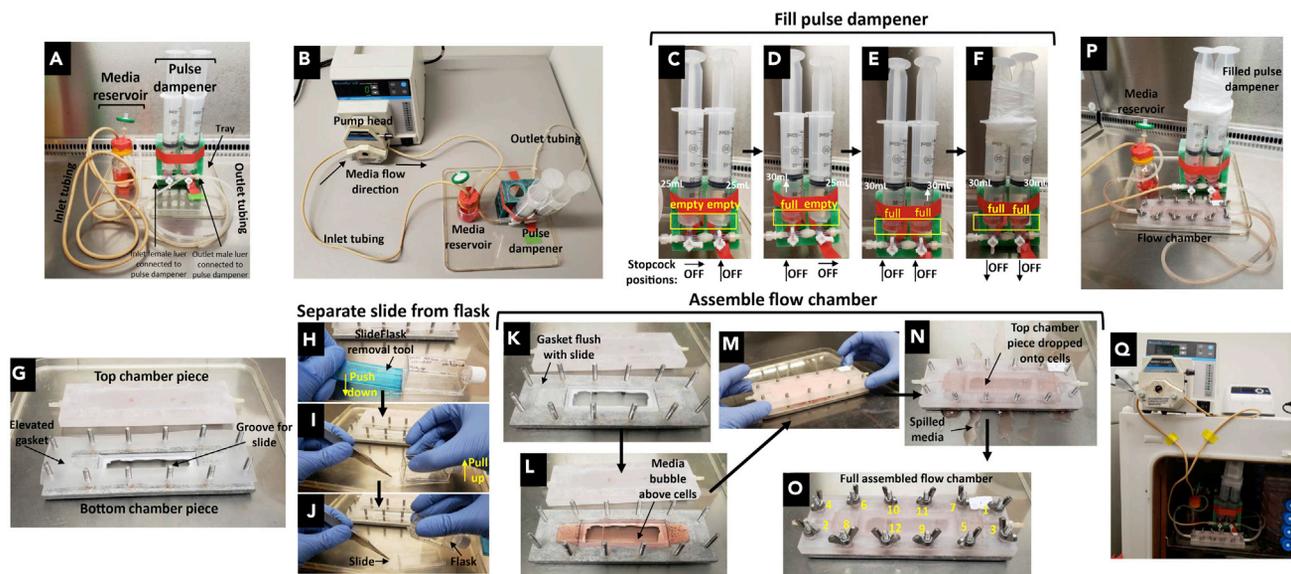


Figure 4. Parallel plate flow experiment set-up

- (A) Pulse dampener and filled media reservoir assembled and secured to tray.
 (B) Flow circuit without flow chamber, attached to the pump.
 (C–F) Stopcock settings to fill both syringes of the pulse dampener.
 (G) Flow chamber before inserting the slide (gasket is elevated to allow room for the slide to be inserted).
 (H–J) Process to separate the slide from the flask component of the SlideFlask.
 (K–O) Flow chamber assembly: (K) slide placed in the flow chamber flush with the gasket; (L) chamber after 7 mL viEC media is pipetted onto the slide; (M) chamber with top piece held in place over bottom piece before being released; (N) chamber after releasing the top piece in place onto the bottom piece; (O) full assembled flow chamber after screwing in wing nuts.
 (P) Full assembled flow circuit with flow chamber inserted.
 (Q) Flow chamber in cell culture incubator attached to the pump sitting on top of the incubator.

28. Attach the inlet tubing to the pump (Figure 4B).
29. Fill the inlet tubing by turning on the pump at a speed of 9 mL/min. Stop the pump when media can be seen flowing through the stopcocks of the pulse dampener.
30. Fill the first syringe in the pulse dampener with 5 mL of media by using the stopcocks to allow flow up into the first syringe and to block flow to the tubing past the syringe. Turn the left stopcock so that the OFF switch is pointed to the right blocking flow past the first syringe, and the second stopcock so that the OFF switch is pointed up, blocking flow into the second syringe (Figure 4C). Turn on the pump until the media level reaches 5 mL inside the syringe, then turn it off (Methods video S1).

Note: The syringe will build some air pressure inside at first, then the pressure will reach a level such that the plunger will start to move up. Allow it to lift while the media flows into the syringe until the appropriate level. The plunger should now be at 30 mL after filling the syringe.

31. Fill the second syringe in the pulse dampener with 5 mL of media by using the stopcocks to block media flow into the first syringe, allow flow into the second syringe, and block flow to the rest of the tubing past the second syringe. Turn the left stopcock so that the OFF switch is pointed up to block flow into the first syringe and turn the right stopcock so that the OFF switch is pointed right to block flow past the second syringe (Figure 4D). Turn on the pump until the media level reaches 5 mL inside the second syringe, then turn it off (Methods video S2). The plunger for both syringes should now be at the 30 mL mark.
32. Use the stopcocks to block flow to each syringe (Figure 4E) and use parafilm to secure the syringe plungers at their current position such that they will not move up when air pressure builds

in the syringes due to higher flow rate. Both stopcocks should be turned so that their OFF switches are pointed up.

△ CRITICAL: If the plungers are not properly secured, they will rise when the flow rate is increased and may burst open in the incubator, at which point the experiment is not recoverable. Make sure to secure them well with plenty of parafilm. See [troubleshooting 7](#).

33. Mark the location of each plunger's end on each syringe (around the 30 mL mark) with a lab marker. This will allow you to track the plungers as you raise the flow rate to make sure they are not moving with increased pressure.
34. Turn both stopcocks so that the OFF switches are pointed down, allowing the media to flow in all directions through the tubing and syringes ([Figure 4F](#)). See [troubleshooting 8](#).
35. Fill the rest of the tubing until media drips out of the outlet tubing back into the reservoir bottle ([Methods video S3](#)).

Note: The media should now flow continuously without pulsing.

36. Shake the tubing to move air bubbles out through the outlet. Ensure that no air bubbles remain in the tubing.
37. Slowly turn up the pump to 52 mL/min in increments of ~15 mL/min. Ensure that the pulse dampener plungers do not move and the flow circuit is stable. Allow the pump to continue running at this speed as a test while you assemble the flow chamber.

Note: The media level inside the pulse dampener syringes will rise when the flow rate is turned up, but the plunger level should not rise since they are secured in place by parafilm. See [troubleshooting 7](#).

Note: A flow rate of 52 mL/min achieves a physiological shear stress of 1.2 Pa based on the dimensions of our chamber (see step 61 for the calculation).

38. Assemble the bottom of the flow chamber by laying it flat on the biosafety cabinet surface or a sterile tray and using sterile tweezers to secure the gasket onto the screws at a slightly elevated position from the bottom of the chamber ([Figure 4G](#)). The top of the flow chamber should lie nearby with the surface that will eventually touch the cells facing upwards.
39. Retrieve the SlideFlask of confluent viECs from the incubator.
40. Spray the SlideFlask with 70% ethanol, dry with a Kim wipe, and place it on a sterile surface in the cell culture hood.
41. Aspirate the media from the flask and use a SlideFlask removal tool to remove the culture chamber from the slide ([Figures 4H–4J](#)). While using the forceps to keep the slide flat on the tray, wedge the tool underneath the lip of the flask and use it as a lever to crack the flask off the slide ([Figure 4H](#)). Keep pressure on the forceps to keep the slide flat and use your other hand to pull the flask completely off the slide ([Figures 4I and 4J](#)).
42. Use sterile tweezers to transfer the slide to its designated groove in the bottom piece of flow chamber.
43. Use both pairs of sterile tweezers to bring the gasket down, making it flush with the bottom of the chamber ([Figure 4K](#)).
44. Use a 5 mL serological pipette to add 7 mL warm viEC media on top of the slide and allow it to fill the interior area created by the gasket ([Figure 4L](#)). The media should bubble above the culture area, slightly over-filling it.
45. To assemble the top, line it up with the screws of the bottom then bring it halfway down to the surface of the cells ([Figure 4M](#)). Release the top chamber piece from your fingers quickly to allow it to fall into place ([Figure 4N](#)).

Note: Dropping the top of the chamber rather than lowering it slowly onto the cells allows fewer bubbles to be created on top of the slide. Some media will spill in the process; this is to be expected and is not concerning.

Optional: Bubble creation should be minimized as they can shear the cells off as they move across the culture area and bubbles that get stuck in a particular location create obstructions that distort the media flow in that area. Avoiding creating bubbles at this step can be tricky so to minimize bubble formation, practice this step without cells using only water or media before performing the full flow experiment. See [troubleshooting 9](#).

46. Secure the two halves of the flow chamber by screwing in the wingnuts ([Figure 4O](#)). Start from the corners and move to the middle. As you screw in each wingnut, screw in nuts diagonally opposite each other such that the pressure remains even on both sides of the chamber. The wingnuts are numbered in [Figure 4O](#) to show the correct order.
47. Use pliers to tighten the wingnuts as much as possible.

△ CRITICAL: The wingnuts must be screwed tightly (without breaking the acrylic) such that media does not leak out of the sides of the flow chamber. See [troubleshooting 10](#).

48. Wipe off any spilled media from the flow chamber using Kim wipes soaked in 70% ethanol.
49. Turn the pump off. The flow circuit should have been running stably at 52 mL/min for some time now.
50. Connect the outlet tubing to the flow chamber first. Disconnect it from the 5/32" straight tubing connector between the two short stretches of L/S 16 tubing and connect one end of the L/S 16 tubing to one of the flow chamber ports.

Note: A small drop of media may drop out of the opposite flow chamber port when the outlet is connected.

Note: Either side of the flow chamber can serve as the inlet or outlet. Just be sure to track which direction the flow runs on the slide such that future cell imaging can be calibrated to flow direction.

51. Connect the stretch of L/S 16 tubing to the other side of the flow chamber.

Optional: Wrap a small piece of parafilm around the connection site between the tubing and the flow chamber to prevent leaking in this area. While optional, we have found this advantageous to prevent leaking in our experiments. See [troubleshooting 10](#).

52. Place the flow chamber on the tray with the pulse dampener and the media reservoir ([Figure 4P](#)).
53. Turn the pump down to 9 mL/min and turn it on. Allow the media to flow through the flow chamber and drip into the reservoir for a few seconds.

△ CRITICAL: Do not forget to turn the pump down to 9 mL/min before flowing over the cells as exposing the cells to 52 mL/min immediately without any flow conditioning will cause them to detach from the slide. See [troubleshooting 11](#).

54. Turn the pump off. Disconnect the tubing from the pump and transfer the tray with the flow circuit to a cell culture incubator.

Note: Make sure to set the rack inside the incubator such that the pulse dampener can fit. It measures ~10 inches high.

55. Place the pump on top of the cell culture incubator.
56. Connect the inlet tubing to the pump and tape to the incubator such that the incubator door can be closed (Figure 4Q).
57. Turn on the pump and close the incubator.
58. To condition the cells to shear stress exposure, slowly ramp up the flow rate over a period of two hours. First, maintain the flow at 9 mL/min flow rate for 15 minutes.
59. Turn the pump flow rate up to 18 mL/min and maintain this rate for 45 minutes.
60. Turn the pump flow rate up to 36 mL/min and maintain this rate for 1 hour.
61. Turn the pump flow rate up to 52 mL/min and maintain this rate for 24 hours.

Note: Pump flow rate can be set to achieve desired shear stress based on flow chamber dimensions. The relationship between shear stress $\left(\tau, \frac{\text{dynes}}{\text{cm}^2}\right)$ and flow rate $\left(Q, \frac{\text{mL}}{\text{s}}\right)$ for a chamber of width w (cm) and height h (cm) for a Newtonian fluid of viscosity μ $\left(\frac{\text{g}}{\text{s cm}}\right)$ is as follows:

$$\tau = \frac{6Q\mu}{wh^2}$$

To convert the shear stress from cgs units (dyne/cm²) to SI units (Pa=N/m²), divide the value in cgs units by 10.

This relationship is valid for laminar flow in a rectangular channel and when the fluid velocity only depends on the vertical direction (Truskey, Yuan and Katz, 2009). For laminar flow in a rectangular channel, the Reynolds number must be < 2000. The Reynolds number for a rectangular channel is calculated by the following equation:

$$Re = \frac{\rho v \left(\frac{2wh}{w+h}\right)}{\mu}$$

where v $\left(\frac{\text{cm}}{\text{s}}\right)$ is the fluid velocity and ρ $\left(\frac{\text{g}}{\text{cm}^3}\right)$ is the fluid density (Truskey, Yuan and Katz, 2009; Kandlikar, 2013). For the flow to depend only on the vertical direction, the following conditions must be met:

Entrance length $L_e > 0.05 Re \left(\frac{2wh}{w+h}\right)$. This establishes that the velocity does not vary with the direction of flow (i.e., it is fully developed) (Kandlikar, 2013).

$w \gg h$ (Typically, $\frac{w}{h} > 20$). This establishes that the velocity does not vary along the width of the channel, except at the edges.

For our chamber and media: $w = 1.89 \text{ cm}$, $h = 0.0495 \text{ cm}$, $\mu = 0.0107 \frac{\text{g}}{\text{s cm}}$, and $\rho = 1.02 \frac{\text{g}}{\text{cm}^3}$. Based on the shear stress equation, to achieve a shear stress of $\tau = 1.2 \text{ Pa} = 12 \frac{\text{dynes}}{\text{cm}^2}$ which is the physiological average away from branches, we need a flow rate of:

$$Q = \frac{12 * 1.89 * 0.0495^2}{6 * 0.0107} = 0.87 \frac{\text{cm}^3}{\text{s}} = 52 \frac{\text{mL}}{\text{min}}$$

Based on this flow rate, the fluid velocity is as follows:

$$v = \frac{Q}{A} = \frac{Q}{wh} = \frac{0.87}{1.89 * 0.0495} = 9.3 \frac{\text{cm}}{\text{s}}$$

The Reynolds number equals:

$$Re = \frac{1.02 * 9.3 * \frac{2 * 1.89 + 0.0495}{1.89 + 0.0495}}{0.0107} = 85 < 2000$$

This satisfies the condition for laminar flow.

The entrance length $L_e = 0.05 * 85 * 0.096 = 0.41$ cm. Note that the slide is placed 1.9 cm beyond the entrance, so the flow is fully developed before it reaches the slide.

Finally, the width to height ratio $\frac{w}{h} = \frac{1.89}{0.0495} = 38 > 20$, satisfying the second condition for flow to depend only on the vertical direction.

Optional: We have seen viEC alignment and altered gene expression after 24 h of flow. If studying longer-term phenotypes or if alignment is not seen after 24 h, viEC time under flow can be extended to 48 h. See [troubleshooting 12](#).

Take down flow chamber experiment

⌚ Timing: 1 h

62. After 24 hours, turn the pump off and disconnect the tubing from the pump.
63. Move the flow circuit tray to the biosafety cabinet.
64. Disconnect the tubing from the flow chamber and use a 5/32" straight tubing connector to link the open ends of the tubing. Move the flow chamber to a clean surface to disassemble it.
65. Use pliers to loosen each wingnut, again moving from the corners to the middle and unscrewing the diagonally opposite pairs together. Remove all the wingnuts.
66. Lift the top half of the flow chamber off of the bottom half to expose the cells.
67. Use tweezers to remove the gasket such that the slide of cells can be reached.

Note: Move quickly during this time such that the media on the cells does not evaporate. They will detach from the slide quickly if not moved into warm media immediately.

68. Use tweezers to transfer the slide into a petri dish filled with warm viEC media. Make note of the flow direction for future reference if planning on imaging the slide.
69. Fix the cells for immunohistochemistry, prepare for DAF-FM DA assay, or isolate RNA as desired.
70. When finished preparing the cells as desired, rinse the flow chamber with water and scrub with Alconox. Dry completely then EtO sterilize. Wingnuts can be autoclaved.
71. Fill the media reservoir with bleach and leave for 15 minutes, then rinse with dH₂O. Allow to dry on a drying rack.
72. Flush the tubing with 70% ethanol, then with dH₂O, then with air. Hang and allow to air-dry completely.
73. Reassemble the tubing and reservoir and sterilize by autoclaving or EtO sterilization.
74. Discard the 60 mL syringes and flush the 4-way stopcocks with ethanol.
75. Allow the stopcocks to dry, then EtO sterilize.

Note: The tubing and chamber components can be reused indefinitely as long as they do not leak. If the chamber endures damage or cracks that cause leaking, then it must be replaced. We have been using our chambers for several years without any issues with cracking. The only component of the system that needs to be replaced regularly is the 4-way stopcocks, which will crack over time due to the high pressure of the media flow. The stopcocks should be replaced when they crack and cause leaking, which may occur every 2–3 months if they are being used frequently.

Note: Contamination problems are very rare and indicate either a mistake in the flow experiment set-up process or in sterilizing the components. If contamination problems arise, filter all media and repeat steps 70–75 to sterilize all the components and use them again. If the contamination persists despite re-sterilization, replace all the components except the flow chamber (tubing, tubing connectors, and stopcocks).

Fix slide for immunohistochemistry

⌚ Timing: 40 min

Upon completion of a flow experiment, viECs can be fixed on the slide for immunohistochemistry. This protocol begins following step 68, with the slide of cells placed in a petri dish with warm viEC media.

76. Aspirate viEC media and wash the slide with PBS twice.
77. In the fume hood, add 4% paraformaldehyde (PFA) to cover the slide.
78. Leave cells with PFA in the fume hood at 20°C–25°C for 15 minutes.
79. Aspirate PFA and wash cells with PBS 3 times for 5 minutes each.

⏸ **Pause point:** Fixed cells can be stained immediately or stored at 4°C covered with PBS in a petri dish and wrapped in parafilm.

Retrieve RNA after flow experiment

⌚ Timing: 1 h

Upon completion of a flow experiment, RNA can be isolated from the viECs for RT-PCR or RNAseq. This protocol begins following step 68, with the slide of cells placed in a petri dish with warm viEC media.

80. Aspirate viEC media and wash the slide with PBS once.
81. Use tweezers to pick up the slide and dab the bottom of the slide (NOT the culture area) lightly with a Kim wipe to remove excess PBS.
82. Place the slide on a dry, flat, clean surface.
83. Add 220 μ l lysis buffer to the culture area such that it fills the area with cells.

Note: The specific lysis buffer will depend on the RNA extraction kit being used. We use the Qiagen RNeasy mini kit (Qiagen 74104) with lysis buffer RLT + β -mercaptoethanol.

84. Scrape the lysis buffer and cells off the slide into 1 well of a 6-well plate.
85. Repeat steps 80–84 for all the slides if processing several at once. Include a static control for each flow slide.
86. Proceed with the RNA extraction according to the protocol for the specific RNA extraction kit being used.

⏸ **Pause point:** Isolated RNA can be stored at -80°C for 1 year.

DAF-FM DA assay to detect nitric oxide

⌚ Timing: 1 h

Upon completion of a flow experiment, viECs can be stained for nitric oxide using the DAF-FM diacetate (DAF-FM DA) assay. This protocol begins following step 68, with the slide of cells placed in a petri dish with warm viEC media.

87. Prepare 5 mM DAF-FM DA by dissolving 50 μg DAF-FM DA in 20 μL DMSO. Dissolved DAF-FM DA stock can be stored at -20°C for 6 months.
88. Prepare enough serum-free viEC media with complete StemPro-64, 50 $\mu\text{g}/\text{ml}$ VEGF, and 2 $\mu\text{g}/\text{ml}$ heparin for all samples. Warm to 37°C .
89. Add 1 μM DAF-FM DA to warm serum-free viEC media.
90. Aspirate media from slides and add serum-free viEC media with DAF-FM DA.
91. Incubate viECs with DAF-FM DA for 30 minutes at 37°C .
92. Aspirate media with DAF-FM DA and rinse cells once with PBS.
93. Aspirate PBS and add fresh warm serum-free viEC media.
94. Incubate viECs for 15 minutes at 37°C .
95. Image viECs immediately at $10\times$ magnification. DAF-FM DA appears in the FITC channel.
96. Quantify DAF-FM DA-positive viECs per unit area using image analysis software (e.g., FIJI).

Note: viECs should be imaged within 30 min after completing the labeling process to avoid losing the DAF-FM DA signal as it is free-floating in the cytoplasm and not bound. We have not attempted fixing viECs after the DAF-FM DA assay and performing immunohistochemistry with other antibodies. If this is necessary, it may be possible to optimize but the fixation and washing steps may remove the DAF-FM DA signal.

EXPECTED OUTCOMES

Endothelial cells (ECs) line the inner walls of blood vessels and regulate vascular tone, structure, and inflammation. ECs are crucial to maintaining vascular homeostasis and their dysfunction plays a critical role in driving prevalent vascular pathologies such as atherosclerosis and hypertension (Williams and Wu, 2019). Primary human ECs are difficult to obtain and have limited plasticity and proliferative capacity, making them unsuitable for *in vitro* applications requiring large numbers of cells. In contrast, iPSCs offer a potentially unlimited source to generate ECs for a variety of applications including disease modeling, cell therapies, tissue graft vascularization, and microphysiological systems. Here we have described a simple, rapid protocol to differentiate vascular endothelial cells (viECs) from human iPSCs under serum-free conditions using small molecules to regulate specific transcription factors. Since endothelial cell behavior is highly dependent on exposure to physiological shear stress, we also describe the design and use of a parallel plate flow chamber system to assay for viEC alignment, gene expression, and protein expression after exposure to flow. Once the appropriate materials and procedures are established, the success rate of differentiation and parallel plate flow experiments is very high.

Undifferentiated iPSCs form colonies of tightly packed cells with prominent nucleoli (Figure 3A). Figure 3B shows typical morphology observed on days 1–7 of differentiation and Figure 3C shows typical viEC morphology 3 days after sorting. On day 1, cells form a network of single cells due to the presence of ROCK inhibitor. On day 2 after ROCK inhibitor is removed, cells should aggregate into colonies. On days 3–4, cells proliferate and form mesoderm and the media may turn yellow due to low pH. On day 5 after 24 hours of culture in endothelial induction media, cell morphology will begin to change as the cells differentiate. On days 6 and 7, two cell layers should form, with non-endothelial cells forming the top layer and endothelial cells forming the bottom layer. Differentiating ECs can be seen through holes in the top cell layer which typically forms a patchy network as shown in Figure 3B. Some cell death on days 3–7 is normal due to the high cell density in the flask, though excessive cell death may indicate that the iPSC seeding density on day 0 was too high. MACS sorting for CD31+ and CD144+ cells recovers ~50% of the cells on average based on cell count before and after sorting, with yields up to 55 million cells from one T-75 immediately after sorting.

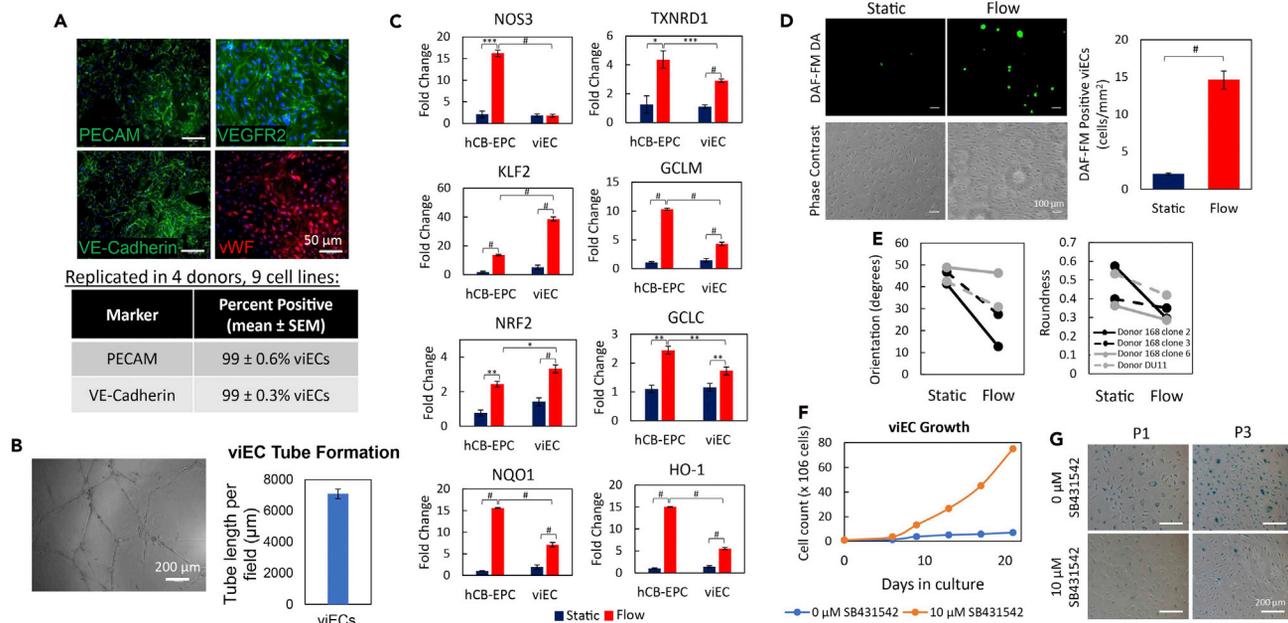


Figure 5. Characterization of viECs under static and shear stress (flow) conditions

(A) Expression of endothelial proteins platelet endothelial cell adhesion molecule (PECAM/CD31), vascular endothelial cadherin (VE-Cadherin/CD144), vascular endothelial growth factor receptor 2 (VEGFR2), and von Willebrand factor (vWF) in iPSC-derived viECs after sorting.

(B) viEC tube formation after 24 hours plated in Matrigel. N=4 cell lines, 3 replicates per cell line.

(C) Gene expression of flow-sensitive genes in human cord blood-derived endothelial progenitor cells (hCB-EPC) and viECs in Static conditions and when exposed to 24 hours of physiological shear stress ("Flow", 1.2 Pa). N=3 replicates for hCB-EPCs and N=4 viEC cell lines with 3 replicates per line.

(D) Nitric oxide production marked by DAF-FM diacetate (DA) assay in viECs exposed to 24 hours of shear stress (12 Pa). N=3.

(E) Decrease in orientation and roundness indicating alignment in 4 viEC lines. N=3 static control and 3 flow experiments per line; data taken from phase contrast images analyzed in Fiji. Orientation and roundness were averaged from measurements of 90–150 cells in each experiment.

(F) viEC growth curves with and without supplementation with 10 μ M SB431542.

(G) Staining for senescence-associated β -galactosidase in passage 1 and passage 3 viECs with and without supplementation with 10 μ M SB431542. Data in all panels is presented as mean \pm SEM. # p <0.0001. Data in panels A-E is reprinted with permission from [Atchison et al. \(2020\)](#).

After sorting, differentiated viECs will proliferate in \sim 2–4 days to form a confluent monolayer. SB431542 treatment significantly improves viEC proliferation; without treatment viECs will become senescent after 2–3 passages (Figures 5F and 5G). Sorted viECs show $99 \pm 0.6\%$ positive PECAM (CD31) expression and $99 \pm 0.3\%$ VE-Cadherin (CD144) expression (mean \pm SEM, Figure 5A). They also form tube networks when plated in Matrigel in 96-well plates for 24 hours (Figure 5B; [Atchison et al., 2020](#)).

After completing parallel plate flow experiments, we routinely fix, stain, or isolate RNA from the cells. Typical RNA yield varies between 20–100 ng/ μ L. After exposure to physiological shear stress, endothelial cells align in the direction of flow and upregulate numerous flow-sensitive genes. Endothelial nitric oxide synthase (NOS3) is upregulated in response to flow and generates nitric oxide in endothelial cells, a vasodilator with critical roles in maintaining vascular homeostasis ([Oliveira-Paula et al., 2016](#)). Nitric oxide production after exposure to shear stress is an important marker of healthy endothelial function as limited nitric oxide bioavailability is an early marker of atherosclerosis and a hallmark of EC dysfunction ([Gimbrone, Jr and Garcia-Cardena, 2016](#)). *KLF2* (Kruppel-like factor 2) is a major anti-inflammatory and anti-thrombotic flow-responsive transcription factor which regulates \sim 46% of EC flow-sensitive genes and is upregulated by EC exposure to laminar flow ([Nakajima and Mochizuki, 2017](#)). *KLF2* promotes the activation of antioxidant transcription factor *NRF2* and both of these genes promote EC quiescence ([Nakajima and Mochizuki, 2017](#)). *NRF2* regulates the expression of several targets including *NQO1*, *TXNRD1*, *GCLM*, *GCLC*, and *HO-1* ([Tonelli et al., 2018](#)). We compared the expression of these 8 genes in viECs under static and flow conditions and

compared these results with those from human cord blood-derived endothelial progenitor cells (hCB-EPCs) as a primary endothelium control (Figure 5C). Compared with hCB-EPCs under static conditions, hCB-EPCs exposed to 1.2 Pa for 24 hours upregulate all of the flow-responsive genes that we tested. viECs exposed to the same conditions upregulate all of the genes except *NOS3*, which shows no significant difference between static viECs and viECs under flow. Compared with the hCB-EPCs exposed to shear stress, the fold increase in expression of *KLF2* and *NRF2* is higher in viECs exposed the same conditions. However, while the genes are upregulated, the fold increases in expression of *NRF2* targets *NQO1*, *TXNRD1*, *GCLM*, *GCLC*, and *HO-1* in viECs exposed to shear stress are all lower than in hCB-EPCs exposed to the same conditions (Figure 5C). Importantly, despite the lack of *NOS3* upregulation detected by RT-PCR (Figure 5C), DAF-FM DA assay results show that viECs do upregulate nitric oxide production after exposure to flow for 24 hours (Figure 5D). Finally, after exposure to shear stress, viECs show alignment in the direction of flow as indicated by a significant decrease in cell orientation angle relative to the flow direction ($p=0.0371$, one-sided t-test, Figure 5E). An angle of 0° indicates complete alignment and an angle of 45° indicates no alignment. viECs exposed to shear stress also show a significant decrease in roundness ($p=0.0335$, one-sided t-test, Figure 5E) determined by the following formula: $Roundness = \frac{4A}{\pi L^2}$ where A is the cell area and L is the chord length (Atchison et al., 2020). A roundness of 1 indicates a perfect circle and a roundness of 0 indicates a straight line.

Our differentiation protocol was adapted from Patsch et al. (2015) which provides advantages in speed, yield, and efficiency compared with other protocols. We made adjustments to the Patsch et al. protocol to improve viEC attachment under shear stress conditions. Specifically, we altered the duration of endothelial induction, plate coating conditions, cell sorting process, and added a phase of culture in conditioned media. Contrary to previous protocols, this protocol uses chemically defined conditions and does not require co-culture with stromal cells or embryoid body formation (Nourse et al., 2010; Li et al., 2011). Moreover, in contrast to other chemically defined protocols whose differentiation efficiency was limited to 10%–30%, we retrieve 50% of the cells on average when we sort for CD31+ and CD144+ cells on the final day of differentiation (Orlova et al., 2014). The differentiation process is rapid, generating up to 55 million viECs from 3.5 million iPSCs within 7 days which can then be expanded for several passages. One recently published protocol reported rapid generation of iPSC-derived ECs with >90% efficiency using small molecule treatment in 2D monolayer culture (Tsujiimoto et al., 2020). However, these cells were only tested for CD31 expression, endothelial nitric oxide synthase (*NOS3*) expression, and tube formation in Matrigel. No other functional assays were reported and the behavior of these cells under shear stress is not known. The protocol we report is easily scalable, results are consistent across multiple cell lines, and it can be used to model endothelial behavior in human genetic diseases. In Atchison et al., (2020), we report the use of this protocol to produce endothelial cells from iPSCs derived from Hutchinson-Gilford Progeria Syndrome (HGPS) patients. HGPS viECs express similar levels of EC markers as healthy viECs, but exhibit lower tube formation ability in Matrigel, blunted upregulation of flow-sensitive genes, and downregulation of *NOS3* after exposure to shear stress indicating reduced nitric oxide bioavailability (Atchison et al., 2020).

Endothelial cells respond to shear stress through mechanosensitive pathways which affect phenotype and function, including antioxidant and inflammatory pathways. Moreover, different flow patterns and temporal waveforms are associated with either atheroprone or atheroprotective endothelial behavior, demonstrating the value of studying endothelial behavior under physiological shear stress and not only under static conditions. In our own experiments, we have observed shear-stress dependent changes in antioxidant and inflammatory gene expression and have demonstrated differences in nitric oxide production under shear stress between healthy and diseased endothelial cells that affect vasodilation in tissue engineered blood vessels made from these cells (Atchison et al., 2020). Parallel plate flow experiments offer the opportunity to study iPSC-derived endothelial cell behavior under physiological shear stresses *in vitro*. The protocol we report provides consistent results with a high rate of success. The transparency and dimensions of the flow chamber

allow visualization of the cells before and after flow, and enough cells can be retrieved for downstream analysis of gene and protein expression. The system has the potential to be scaled up if desired, and the shear stress can be adjusted easily by varying the flow rate. The custom pulse dampener we present decreases media volume significantly compared with commercial pulse dampeners used in other protocols (Lane et al., 2012). Further, by removing the pulse dampener or using micro-pumps with adjustable flow patterns, the system provides the potential to compare continuous and pulsatile flow or study endothelial response to atheroprone vs atheroprotective flow patterns.

QUANTIFICATION AND STATISTICAL ANALYSIS

Depending on the experimental design, we analyze the data using one- or two-way ANOVAs with post-hoc Tukey tests. Levene's test is used to test equality of variance before running the ANOVAs. If the Levene's test shows unequal variances, then nonparametric tests are used. Data is only excluded if an error is identified in the protocol (e.g., inappropriate cell seeding causes cells to detach during differentiation or bubbles in the flow chamber causes loss of cells). Immunohistochemistry images are quantified based on positive cell count per area using FIJI.

LIMITATIONS

We have reproduced this differentiation protocol numerous times using 9 different iPSC lines, including 4 healthy cell lines and 5 cell lines from donors with Hutchinson Gilford Progeria Syndrome. The rate of successful differentiation is very high, with rare exceptions when the seeding density is not optimized for the cell line being used or the input iPSCs are not of high quality (i.e., spontaneously differentiated). The viECs produced by this protocol exhibit several characteristic functions of endothelial cells including EC marker expression, alignment in the direction of flow, upregulation of flow-sensitive genes, and production of nitric oxide when exposed to flow (Atchison et al., 2020). Their main limitations are in proliferation and morphology. viECs exhibit confluent monolayers only up to passage 2, after which they come senescent. They are larger and have more rounded morphology than primary ECs, typical of a senescent EC phenotype. Treatment with 10 μ M SB431542 after sorting significantly improves viEC proliferation, allowing them to be passaged at least 5 times without becoming senescent. However, all parallel plate flow experiment results reported in Figures 5B–5E are for viECs between passages 1–3 that have not been treated with SB431542. In flow experiments with p1 viECs from one cell line, gene expression results were similar between non-treated viECs and viECs treated with 10 μ M SB431542. Further experimentation is required to compare viEC behavior under flow after treatment with SB431542 in other cell lines and at higher passages.

Once optimized, the parallel plate flow protocol is consistently successful. If problems arise, they are usually due to lack of careful set-up causing bubbles in the flow circuit or leaking out of the chamber or one of the tubing connections. One limitation is that some viECs are still lost during the flow experiment, even when the shear stress ramp-up protocol is used. Enough viECs do remain adhered to the slide for downstream immunohistochemistry or RNA extraction, but multiple slides may need to be aggregated for applications requiring large cell numbers. For applications that require more cells, it may be possible to scale up the system using custom slides and larger size chambers. In this case, a new flow rate must be calculated based on the shear stress calculation provided in the [step-by-step method details](#) (step 61) using the new chamber dimensions. The new chamber width:height ratio should be at least 20:1 to make sure the flow is 1-dimensional and the shear stress is uniform (Truskey, Yuan and Katz, 2009). For applications that require imaging the cells while they are still mounted in the chamber, the thickness of the custom slides and the chamber bottom combined must be smaller than the working distance of the desired microscope objective.

TROUBLESHOOTING

Problem 1

iPSCs are growing on part of the well but not on the entire surface.

Potential solution

This is likely due to poor Matrigel coating in the part of the well without cells. Be sure to shake the plate when coating it such that the Matrigel solution covers the entire well, and never use a plate if the Matrigel solution in part of or the whole well has evaporated. If coating a T-75 and not planning on using it the same day, use 5–6 mL coating solution per flask rather than 4 mL to reduce the likelihood of the solution evaporating before the flask is used.

Problem 2

Cells grown on collagen-coated plates do not survive.

Potential solution

Make sure to wash the plate well with PBS after collagen coating to remove the acetic acid which is toxic to cells.

Problem 3

iPSCs are sparse after thawing and do not look confluent enough to passage after 3–4 days.

Potential solution

This is expected; iPSCs have poor survival after thawing. Supplementation with 10 μ M Y-27632 for the first 18–24 hours is essential to improve iPSC survival after thawing. Even if the iPSCs do not look confluent, passage them every 3–4 days anyway at low ratios (1:1 or 1:2) until they recover and proliferate normally. Do not start a differentiation until the iPSCs have been passaged enough that they proliferate normally and reach 60%–80% confluence within 3 days.

Problem 4

iPSCs do not have normal morphology and look differentiated.

Potential solution

Healthy iPSCs are small and rounded, have a high nucleus:cytoplasm ratio with prominent nucleoli, and pack into tight colonies (Figure 3A). Spontaneously differentiated cells are larger, more angular, and do not have prominent nucleoli (Figure 3D). These cells should be extremely rare or non-existent in the culture. If there are numerous spontaneously differentiated cells such as in Figure 3D, this may be due to leaving the iPSCs for too long without passaging (> 3–4 days). Start over with another vial of iPSCs and passage them on a regular 3-day schedule, varying the split ratio rather than the timetable based on confluence. Alternatively, pure iPSC colonies can be picked out of a mixed population of iPSCs and spontaneously differentiated cells using a 200 μ L pipette and transferred to a new Matrigel-coated well. However, not all transplanted colonies will survive so this technique is labor intensive with a low success rate.

Problem 5

Cell death is high during the mesoderm and endothelial induction phases of differentiation. Is this normal?

Potential solution

Some cell death is normal during days 3–7 of the differentiation process due to the high cell density in the flask and continued proliferation of the cells. This is not concerning and should not be mistaken for contamination if the media is cloudy with dead cells (unless bacteria is seen under the microscope, in which case the culture is contaminated and should be thrown out). If cell death is very high and percent positive CD31 and CD144 cells recovered after MACS sorting is lower than 50%, then the iPSC seeding density may be too high and should be optimized.

Problem 6

The viECs do not proliferate to form a confluent monolayer after p2. How can their proliferation be improved?

Potential solution

Supplementation with 10 μ M SB431542 (TGF- β inhibitor) significantly improves viEC proliferation.

Problem 7

When I increase the flow rate, the plungers of the pulse dampener syringes rise.

Potential solution

Use parafilm to tightly secure the plungers in place. This is essential to prevent them from rising. If they rise out of the barrel of the syringe while the experiment is running in the incubator, the media leakage will be catastrophic.

Problem 8

I set up the pulse dampener, but the media flowing through the tubing still looks pulsatile.

Potential solution

Ensure that both stopcocks are set to open flow to both syringes of the pulse dampener (Figure 4F). If the flow to the syringes is blocked, the pulse dampener is closed and will not affect the flow pattern.

Problem 9

The chamber is filled with bubbles.

Potential solution

There are several potential sources of bubbles and methods to avoid them. First, be sure to fill the entire tubing circuit with media and remove any bubbles before connecting the flow chamber to the circuit. Second, when the pump is turned on, the syringes of the pulse dampener will fill with more media and when the pump is turned off, they will drain slightly. If the media level in the pulse dampener is too low, the syringes will drain completely when the pump is turned off and this will introduce bubbles in the circuit. Ensure that both syringes are filled with at least 5 mL media when setting up the experiment. Third, secure the media reservoir and the pulse dampener tightly to the tray using tape such that they are not tipped over while the experiment is running, which will also introduce bubbles. Fourth, when assembling the flow chamber, drop the top of the chamber swiftly onto the bottom rather than lowering it slowly. We have found that dropping it creates more of a mess but introduces fewer bubbles in the chamber. Fifth and finally, use the PharMed BPT (polypropylene) tubing (Cole Parmer EW-06508-14) rather than silicone tubing for the inlet portion of the flow circuit that reaches outside the incubator and connects to the pump. The PharMed BPT tubing is less compliant than the silicone tubing and can withstand the pressure of the incubator door closing without generating bubbles, whereas silicone tubing will deform under the pressure and introduce bubbles in the flow circuit.

Problem 10

Media leaks from the chamber, pulse dampener, or tubing connections when the pump is turned on.

Potential solution

All connections must be secured very tightly to prevent leaking and leaking should be rectified immediately. If a large leak persists it can drain all the media from the system, and if a small leak persists it can cause contamination. If the chamber is leaking, this may be due to the gasket not being flush with the bottom of the chamber, in which case the creases of the gasket will not allow the chamber to be tightly closed and will cause leaking. Alternatively, the wingnuts may need to be secured tighter using pliers (finger strength may not be enough to secure them tightly). If the pulse dampener

or tubing connection points are leaking, they should be screwed tightly together and wrapped snugly in parafilm. We routinely wrap all tubing connections in parafilm as a preventative measure, even when leaking is not observed. If the tubing itself is leaking at a location where there is no connection, it is likely damaged and should be replaced.

Problem 11

The cells detached from the slide during the flow experiment.

Potential solution

Exposure to high shear stress after static culture can cause the cells to detach from the slide, which is why we implement a shear stress ramp-up protocol to condition them slowly to shear stress. If the cells are still detaching, try optimizing the ramp-up protocol for your specific cell line. Additionally, we have found that cell attachment during the flow experiments is improved for our cells when they are cultured in 10% serum. If using serum-free conditions, consider adding serum to the culture medium if possible.

Problem 12

The cells do not align in the direction of flow or respond to flow properly after 24 hours.

Potential solution

Check calculations and flow rate to ensure that shear stress on the cells is at least 1.0 Pa. If sufficient shear stress is being used, increase the time under flow to 48 hours.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, George Truskey (george.truskey@duke.edu).

Materials availability

We have provided engineering drawings and SolidWorks part files with the design and dimensions for the custom parallel plate flow chamber used in these studies in the [materials and equipment](#) section. We have also uploaded the supplemental method videos and the SolidWorks part files to Mendeley Data (<https://doi.org/10.17632/mbh572kw.1>).

Data and code availability

This study did not generate new datasets.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.xpro.2021.100394>.

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AUTHOR CONTRIBUTIONS

N.O.A. performed experiments, analyzed results, and wrote the original draft of the paper. G.A.T. guided project and experiment conceptualization and planning, designed flow chambers, provided funding, and reviewed and edited the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Al-Yafeai, Z., Yurdagul, A., Jr., Peretik, J.M., Alfaidi, M., Murphy, P.A., and Orr, A.W. (2018). Endothelial FN (Fibronectin) deposition by $\alpha 5\beta 1$ integrins drives atherogenic inflammation. *Arterioscler. Thromb. Vasc. Biol.* **38**, 2601–2614.
- Atchison, L., Abutaleb, N.O., Snyder-Mounts, E., Gete, Y., Ladha, A., Ribar, T., Cao, K., and Truskey, G.A. (2020). iPSC-derived endothelial cells affect vascular function in a tissue-engineered blood vessel model of hutchinson-gilford progeria syndrome. *Stem Cell Rep.* **14**, 325–337.
- Claassen, D.A., Desler, M.M., and Rizzino, A. (2009). ROCK inhibition enhances the recovery and growth of cryopreserved human embryonic stem cells and human induced pluripotent stem cells. *Mol. Reprod. Dev.* **76**, 722–732.
- Cooper, A. (1970). Thermodynamic studies of the assembly in vitro of native collagen fibrils. *Biochem. J.* **118**, 355–365.
- Gimbrone, M.A., Jr., and García-Cardena, G. (2016). Endothelial cell dysfunction and the pathobiology of atherosclerosis. *Circ. Res.* **118**, 620.
- Kandlikar, S.G. (2013). Single-phase liquid flow in minichannels and microchannels. In *Heat Transfer and Fluid Flow in Minichannels and Microchannels*, pp. 103–174.
- Lane, W.O., Jantzen, A.E., Carlon, T.A., Jamiolkowski, R.M., Grenet, J.E., Ley, M.M., Haseltine, J.M., Galinat, L.J., Lin, F.H., Allen, J.D., et al. (2012). Parallel-plate flow chamber and continuous flow circuit to evaluate endothelial progenitor cells under laminar flow shear stress. *J. Vis. Exp.* **59**, 1–11.
- Li, Z., Chu, L.F., Hou, Z., Schwartz, M.P., Hacker, T., Vickerman, V., Swanson, S., Leng, N., Nguyen, B.K., Elwell, A., et al. (2011). Functional characterization and expression profiling of human induced pluripotent stem cell- and embryonic stem cell-derived endothelial cells. *Stem Cells Dev.* **20**, 1701–1710.
- Nakajima, H., and Mochizuki, N. (2017). Flow pattern-dependent endothelial cell responses through transcriptional regulation. *Cell Cycle* **16**, 1893–1901.
- Nourse, M.B., Halpin, D.E., Scatena, M., Mortisen, D.J., Tulloch, N.L., Hauch, K.D., Torok-Storb, B., Ratner, B.D., Pabon, L., and Murry, C.E. (2010). VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering. *Arterioscler. Thromb. Vasc. Biol.* **30**, 80.
- Oliveira-Paula, G.H., Lacchini, R., and Tanus-Santos, J.E. (2016). Endothelial nitric oxide synthase: from biochemistry and gene structure to clinical implications of NOS3 polymorphisms. *Gene* **575**, 584–599.
- Orlova, V.V., Drabsch, Y., Freund, C., Petrus-Reurer, S., van den Hil, F.E., Muenthaion, S., Dijke, P.T., and Mummery, C.L. (2014). Functionality of endothelial cells and pericytes from human pluripotent stem cells demonstrated in cultured vascular plexus and zebrafish xenografts. *Arterioscler. Thromb. Vasc. Biol.* **34**, 177–186.
- Patsch, C., Challet-Meylan, L., Thoma, E.C., Urich, E., Heckel, T., O'Sullivan, J.F., Grainger, S.J., Kapp, F.G., Sun, L., Christensen, K., et al. (2015). Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells. *Nat. Cell Biol.* **17**, 994–1003.
- Tonelli, C., Chio, I.I.C., and Tuveson, D.A. (2018). Transcriptional regulation by Nr2f2. *Antioxid. Redox Signal.* **29**, 1727–1745.
- Truskey, G.A., Yuan, F., and Katz, D. (2009). *Transport Phenomena in Biological Systems*, Second Edition (Prentice Hall).
- Tsujimoto, H., Kasahara, T., Sueta, S.I., Araoka, T., Sakamoto, S., Okada, C., Mae, S.I., Nakajima, T., Okamoto, N., Taura, D., et al. (2020). A modular differentiation system maps multiple human kidney lineages from pluripotent stem cells. *Cell Rep.* **31**, 107476.
- Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J.B., Nishikawa, S., Nishikawa, S., Muguruma, K., et al. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat. Biotechnol.* **25**, 681–686.
- Williams, I.M., and Wu, J.C. (2019). Generation of endothelial cells from human pluripotent stem cells: methods, considerations, and applications. *Arterioscler. Thromb. Vasc. Biol.* **39**, 1317–1329.