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Efficient Differentiation of Human Pluripotent Stem Cells to Endothelial Cells

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Abstract

Endothelial cells (ECs) line the interior surface of blood and lymphatic vessels, and play a key role in a variety of physiological or pathological processes such as thrombosis, inflammation, or vascular wall remodeling. Human induced pluripotent stem cell (iPSCs)-derived ECs provide a new opportunity for vascular regeneration and serve as a model to study the mechanism and to screen for novel therapies. We use developmental cues in a monolayer differentiation approach to efficiently generate mesoderm cells from iPSCs via small-molecule activation of WNT signaling in chemically defined media, and subsequent EC specification using vascular endothelial growth factor and fibroblast growth factor for four days. After eight days of further differentiation, mature ECs are further purified using magnetic-activated cell sorting for the EC surface marker CD144. These ECs exhibit molecular and cellular characteristics consistent with native ECs, such as expression of specific surface markers, formation of tube-like structures and acetylated low-density lipoprotein uptake.

Keywords

endothelial cell; induced pluripotent stem cell; monolayer; disease modeling

INTRODUCTION

Endothelial cells (ECs) line the lumen of all blood vessels, and play a critical role in regulation of vascular permeability, angiogenesis, and tissue regeneration. Many cardiovascular diseases are directly associated with endothelial cell (EC) dysfunction, that can lead to limb amputation, and life-threatening strokes, myocardial infarction and heart failure.

Induced pluripotent stem cells (iPSCs) reprogrammed from somatic cells provide a new resource to understand the personal as well as the common genetic underpinnings related to the pathogenesis of disease, and to serve as an unlimited source of cells for high-throughput drug screening (Takahashi et al., 2006). For example, human iPSCs differentiated into cardiomyocytes have proven useful in eliciting features of myocardial disease that can be

used to develop personalized therapies (Burridge et al., 2014; Gu et al., 2017; Lan et al., 2013; Liang et al., 2013; Wu et al., 2015). Moreover, iPSCs can be propagated indefinitely and differentiated into ECs and smooth muscle cells (Gu et al., 2015; Palpant et al., 2017; Sa et al., 2017; Yang et al., 2016) and can be used for cell replacement therapy, disease modeling and drug screening in a personalized manner. However, a highly efficient and cost-effective method to derive ECs from iPSCs must be established before moving into large-scale clinical application.

Here we present a chemically defined method for the differentiation of ECs from human iPSCs with high purity in eight days. This protocol was developed based on fundamental aspects of embryonic developmental signals that control the formation of the vascular system. The protocol consists of three key steps: mesoderm induction, endothelial specification, and EC purification. The first step is to induce mesoderm differentiation by activating WNT signaling, using CHIR99021 which blocks GSK3 β . The second step is to further push the mesoderm cells into the endothelial lineage using the growth factors, vascular endothelial growth factor (VEGF) and Fibroblast Growth Factor (FGF). After eight days of differentiation, more than 60% of cells are CD144⁺ ECs that form tubes on matrigel and exhibit acetylated low-density lipoprotein (Ac-LDL) uptake. These cells can be further enriched with a single step of CD144-based magnetic separation, and these pure and mature iPSC-ECs are capable of 10–15 population doublings without loss of EC characteristics.

Basic Protocol 1 describes the maintenance, passaging, and set up of the human iPSCs for mesoderm induction. **Basic Protocol 2** explains the method for in vitro monolayer endothelial differentiation of human iPSCs. **Basic Protocol 3** is the purification and maintenance of the iPSC-ECs. **Support protocols** describe the characterization of the iPSC-ECs. Taken together these protocols provides a rapid and efficient approach to the generation of iPSC-ECs and identify WNT/ β -catenin signaling as a primary regulator for generating vascular cells from iPSCs.

BASIC PROTOCOL 1. MAINTENANCE, PASSAGING, AND PREPARATION OF iPSCs FOR MESODERM INDUCTION

In this protocol, human iPSCs are maintained on a matrigel-coated plate without any feeder cells. Three days prior to differentiation, iPSCs are passaged at 1:6 ratio and seeded onto a matrigel coated 6-well plate. The mesoderm induction starts once all the wells reach 70–80% confluence. It is critical that the iPSCs maintain an undifferentiated morphology and are actively proliferating before the differentiation.

Materials

Human induced pluripotent stem cells (hiPSC, deposited at WiCell, <http://www.wicell.org/>)

Essential 8^{TM} medium (E8 medium, ThermoFisher, cat. no. A1517001)

BD Matrigel^{TM} hESC-qualified Matrix, 5 mL LDEV-Free (BD Biosciences, cat. no. 354277)

DMEM/F12 medium (Thermo Fisher Scientific, cat. no. 11320-082)

Y-27632, ROCK inhibitor (Stemcell Technologies, cat. no. 72304)

Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (DPBS, ThermoFisher, cat. no. 14190144)

6-well cell culture plates (Thermo Scientific, cat. no. 14-832-11)

15-mL (Corning Falcon, cat. no. 352097) and 50-mL (Corning Falcon, cat. no. 352098) Polystyrene conical tubes

Inverted microscope

37°C water bath

Centrifuge

37°C, 5% CO_2 humidified incubator

Thaw and feeder-free culture of iPSCs

- 1 One day before thawing the iPSCs, thaw 600 μL Matrigel on ice in the refrigerator (4°C) for 4–5 hours.
Matrigel (5mL) is shipped on dry ice. Upon receipt, thaw on ice in the refrigerator (4°C) over-night. Aliquot into sterilized 1.5mL eppendorf tubes (600 μL per vial) and store at –20°C.
- 2 Dilute Matrigel in 40ml ice-cold DMEM/F12 medium in a 50mL conical tube. Keep all reagents and tubes on ice throughout the process.
- 3 Add 2mL of diluted matrigel to each well of a 6-well plate, and incubate at 37°C for 30 minutes.
40mL of the diluted Matrigel will coat three 6-well plates. If using one plate at a time, seal the unused plates with Parafilm and store at 4°C. These plates can be used for a week for further iPSC culture.
- 4 Remove the frozen vial of iPSCs from liquid nitrogen and immediately put it in the 37°C water bath to thaw the cells as fast as possible. Resuspend the cells with 10mL E8 medium and transfer the solution into a 15mL conical tube.
- 5 Wash frozen vial with 1 mL of E8 medium, and transfer the left-over to the same conical tube.
- 6 Centrifuge for 5 minutes at 1000rpm at room temperature.
- 7 During the centrifugation step, add 10 μM Y27632 ROCK inhibitor into 5mL E8 medium. Aspirate the unpolymerized matrigel from one well of a 6-well plate, and add 3mL E8 medium containing the ROCK inhibitor for later usage.
The addition of Y27632 ROCK inhibitor to E8 medium improves cell viability and adhesion after dissociation and replating.

- 8 After centrifugation, aspirate the supernatant carefully without touching the cell pellet. Resuspend the cell pellet in 1mL of E8 medium with ROCK inhibitor and, using a 1000µL pipet and tip, pipet up and down to completely dissociate the cell clusters, Add to the well previously prepared with 3mL E8 medium with ROCK inhibitor.

- 9 Change medium everyday with E8 medium (without ROCK inhibitor).

Do not place E8 medium in a 37°C water bath before use to avoid destabilization of the growth factors in the medium (Chen et al., 2012). Instead, warm up the medium at room temperature for 2–3 hours.

Passaging of iPSCs with EDTA

When the cells reach 85% confluence, split the cells at 1:6 ratio.

- 10 Aspirate culture medium.
- 11 Add 2 mL of 0.5mM EDTA in DPBS to each well, and incubate for 3–5 minutes at room temperature.

Inspect the cells microscopically to assess the dissociation of individual iPSC from neighboring cells, and make sure the cells are not detached from the plate.

- 12 Aspirate EDTA gently to avoid washing the colonies off the plate.
- 13 Add 1 mL of E8 medium with ROCK inhibitor to the well, and pipet up and down using 1000µL pipet and tip at least 5 times to dissociate cells. Top up well to 6 mL with E8 medium with ROCK inhibitor.
- 14 Plate out cells at 1mL per well into a new Matrigel-coated 6-well plate, and top up each well to 4mL with E8 containing ROCK inhibitor.
- 15 Change medium everyday with E8 (without ROCK inhibitor).

The iPSCs will be ready for differentiation after two passages from thawing. Start mesoderm induction (Basic Protocol 2) when the cells have reached 70% to 80% confluence.

BASIC PROTOCOL 2. DIFFERENTIATION OF ECs FROM HUMAN iPSCs

In this protocol, human iPSCs are differentiated into ECs as a monolayer with high efficiency and reproducibility. Differentiation towards mesoderm is induced using small molecules to activate the WNT signaling pathway for two days, and subsequently EC growth factors (VEGF and FGF) are added to the differentiation medium for EC specification (Figure 1A, B). This protocol is designed to be very simple and cost effective. The whole process requires only eight days to obtain CD144⁺ mature ECs from iPSCs.

Materials (also see Basic Protocol 1)

Human iPSCs, 70% to 80% confluent (from Basic Protocol 1)

RPMI medium (Life Technologies, cat. no. 11875-093)
 B-27 supplement minus insulin (Life Technologies, cat. no. A18956-01)
 EGM2 BulletKit (Lonza, cat. no. CC-3124)
 Antibiotic - Antimycotic (100 x, ThermoFisher, cat. no. 15240062)
 5% (vol/vol) Gelatin solution (Sigma-Aldrich, cat. no. G1393-100ml)
 CHIR99021 (Selleckchem, cat. no. S2924)
 SB431542 (Selleckchem, cat. no. S1067)
 Human VEGF-165 (Gemini, cat. no. 300196P100UG)
 Human FGF-basic 154 (Gemini, cat. no. 300113P050UG)
 Phosphate-Buffered Saline (PBS, ThermoFisher, cat. no. 10010023)

Days 1 to 2

- 1 When the iPSCs have reached 75–85% confluency, passage one well of a 6-well plate to a new plate for continuous EC differentiation for the next batch.
Only use iPSCs that are over passage 10. Low passage will significantly reduce differentiation efficiency.
- 2 Prepare 18mL of mesoderm induction medium 1: In a 50mL conical tube, supplement RPMI medium with B-27 supplement minus insulin and 6 μ M CHIR99021.
Insulin-free B27 is expected to improve differentiation efficiency because insulin negatively affects cardiac mesoderm induction (Lian et al., 2013). The concentration for CHIR99021 could be optimized between 5 μ M to 7 μ M for different iPSC lines. If not used immediately, mesoderm induction medium 1 can be stored at 4°C for up to one week.
- 3 Aspirate E8 medium from the 5 remaining wells.
- 4 Wash the plate with PBS, 3mL per well.
- 5 Aspirate PBS and add 3mL of the mesoderm induction medium 1 to each well of the 5 wells containing iPSCs.
Do not change medium for 2 days. Although significant cell death may be noticed, it is fine to proceed provided cell survival is higher than 50%.

Days 3 to 4

- 6 Prepare 18mL of mesoderm induction medium 2: In a 50ml conical tube, supplement RPMI medium with B-27 supplement minus insulin and 3 μ M CHIR99021.

- 7 Aspirate medium from wells, add 3mL of mesoderm induction medium 2 to each well of the 5 wells containing iPSCs.

Do not change medium for 2 days.

Days 5 to 8

- 8 Prepare 40mL of EC differentiation medium: In a 50mL conical tube supplement EGM2 medium with 50ng/mL VEGF and 25ng/mL FGF.
Unused EC differentiation medium can be stored at 4°C for up to one week. To increase the yield of CD144⁺ cells, 10μM of the TGFβ inhibitor SB431542 could be added to the EC differentiation medium because it has been shown that the TGFβ signaling inhibitor SB431542 promoted endothelial cell generation and expansion, and suppressed smooth muscle cell differentiation from endothelial progenitors (James et al., 2010).
- 9 Aspirate medium and add 3–4 mL of EC differentiation medium to each well.
If the confluency of the cells is greater than 85%, add 4mL of the EC differentiation medium. Otherwise, 3mL is sufficient.
- 10 Change EC differentiation medium every other day.

BASIC PROTOCOL 3. PURIFICATION AND MAINTENANCE OF iPSC-ECs

The aim of this protocol is to purify iPSC-ECs using magnetic-activated cell sorting (MACS) against the mature endothelial surface marker CD144. Briefly, CD144⁺ cells are magnetically labeled with CD144 MicroBeads, which are captured by the column in the magnetic field, and separated from the unlabeled cells. The purified iPSC-ECs can be passaged 10–15 times for different assays, or can be frozen down at –80°C overnight and then transferred to a liquid nitrogen tank for long-term storage.

Materials (also see Basic Protocols 1 and 2)

StemPro® Accutase® Cell Dissociation Reagent (Invitrogen, cat. no. A11105-01)
 TrypLE express enzyme (ThermoFisher, cat. no. 12605-036)
 40-μm cell strainer (Corning Falcon, cat. no. 352340)
 MidiMACS separator (Miltenyi Biotech, cat. no. 130-042-302)
 LS Columns (Miltenyi Biotech, cat. no. 130-042-401)
 CD144 (VE-Cadherin) MicroBeads, human (Miltenyi Biotech, cat. no. 130-097-857)
 FeR Blocking Reagent, human (Miltenyi Biotech, cat. no. 130-059-901)
 Liquid nitrogen
 1.8-mL cryo tubes (Thermo Fisher Scientific, cat. no. 377267)
 Mr. Frosty™ Freezing Container (Thermo Scientific, cat. no. 5100-0001)

Vertex

Cell counter

Bambanker freezing media (Wako Chemicals USA, cat. no. NC9582225)

Cell preparation for MACS sorting

- 1 Remove culture medium and wash the cells three times with PBS.
- 2 Add 2 mL accutase per well of 6-well plate to dissociate the cells. Incubate for 5 minutes at 37°C.
- 3 Add 2 mL EGM2 to neutralize the activity of accutase. Pipet up and down several times to dissociate cell clumps.
- 4 Pass the cell suspension through 40-µm cell strainer to make single cell suspension.
Cell clumps may affect the efficacy of magnetic labeling and block the column.
- 5 Determine total cell number.
- 6 Centrifuge cell suspension in the conical tube at 1,000 rpm for 10 minutes.
- 7 Coat a 6-well plate with 0.2% gelatin for 30 minutes at 37°C for later use.

Magnetic labeling of CD144

- 8 Aspirate supernatant completely from the conical tube.
- 9 Resuspend cell pellet in 80µL MACS buffer per 10⁷ total cells.
- 10 Add 20µL of FcR Blocking Reagent per 10⁷ total cells, pipet up and down to mix the cells with blocking reagent.
- 11 Add 20µL of CD144 MicroBeads per 10⁷ total cells, vortex and mix well.
- 12 Incubate for 15 minutes at 4°C.
- 13 Add 10mL of MACS buffer to wash the labeled cells. Centrifuge at 1,000 rpm for 5 minutes. Aspirate supernatant completely.
- 14 Resuspend the cell pellet in 500µL MACS buffer.

Magnetic separation of CD144⁺ cells

- 15 Place column in the MidiMACS magnetic field, and rinse with 3-mL of MACS buffer (see recipe). Collect flow-through with 15-mL conical tube.
- 16 Apply 500µL cell suspension onto the column.
- 17 Wait until the column reservoir is empty, wash column three times with 3mL MACS buffer.
- 18 Remove column from the MidiMACS separator and place it on 15mL conical tube.

- 19 Add 5mL EGM-2 onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column reservoir.
- 20 Centrifuge at 1,000 rpm for 5 minutes. Aspirate supernatant completely and count cells.
- 21 Aspirate 0.2% gelatin from 6-well plate. Resuspend the cell pellet with EGM2 and replate iPSC-ECs in gelatin-coated plate at the density of 3×10^5 cells per 3mL EGM2 per well.
- 22 Change EGM2 medium (3mL/well) every other day.
- 23 When the cells reach 90% confluency, passage the cells at a ratio of 1:3 with TrypLE express enzyme.

If other cell types start growing out after several passages, resort the cells using CD144 magnetic beads.

Cryopreservation of iPSC-ECs

- 24 Dissociate the iPSC-ECs with TrypLE express enzyme. Centrifuge for 5 minutes at 1,000 rpm, and aspirate the supernatant.
- 25 Resuspend 1×10^6 iPSC-ECs per ml Bambanker freezing medium in 1mL cryovial, and place in Mr. Frosty™ Freezing Container, store overnight at -80°C . Transfer frozen vials to liquid nitrogen the next day for long-term storage.

Thawing iPSC-ECs

- 26 Coat 6-well plate with 0.2% gelatin for 30 minutes at 37°C . Aspirate gelatin right before seeding the cells.
- 27 Thaw frozen iPSC-ECs in a 37°C water bath as fast as possible.
- 28 Transfer all cells to a 15mL conical tube containing 10mL of EGM2 medium. Invert the tube to mix well.
- 29 Centrifuge 5 minutes at 1,000 rpm.
- 30 Aspirate the supernatant, and resuspend cell pellet with EGM2 and replate in gelatin-coated plate at 3×10^5 cells per 3mL EGM2 per well.
- 31 Change EGM2 the next day to get rid of the unattached cells. Then change the medium every other day.

SUPPORT PROTOCOL 1. CHARACTERIZATION OF iPSC-EC BY FLOW CYTOMETRY

We use flow cytometry to analyze the expression of KDR as the marker for cardiac and hematoendothelial progenitors (Kattman et al., 2006; Kennedy et al., 2007; Yang et al., 2008), CD34 as the marker for endothelial progenitor cells (Cheng et al., 2013), and CD144 as the marker for mature endothelial cells (Giannotta et al., 2013) before and after iPSC-EC purification using MACS. Before and after MACS purification, iPSC-ECs from one well of

a six well plate are labeled with PE-KDR, FITC-CD34, and APC-CD144 to evaluate the differentiation efficiency (Figure 2A). Follow the manufacturer's instruction for operating the flow cytometer.

Materials

Day 8 iPSC-ECs before MACS sorting and Day 10 iPSC-ECs two days after purification (see Basic Protocol 3)

1% (w/v) PFA in DPBS (prepare from 20% PFA; Electron Microscopy Sciences, cat. no. 15713-S)

0.5% (w/v) BSA (Sigma-Aldrich, cat. no. A3311) in DPBS

PE Mouse Anti-Human CD309 (VEGFR-2) Clone 89106 (BD Pharmingen™, cat. no. 560872)

FITC Mouse Anti-Human CD34 Clone 581 (BD Pharmingen™, cat. no. 555821)

APC anti-human CD144 (VE-Cadherin) Antibody Clone BV9 (BioLegend, cat. no. 348508)

Flow cytometry tubes with cell strainer cap (Corning Falcon, cat. no. 352235)

Centrifuge accommodating TX-750 rotor

5/7 mL tube buckets with decanting aid for TX-750 rotor (Thermo, cat. no. 75003732)

Flow cytometer capable for analyzing FITC, PE and APC such as Aria (BD Biosciences)

1. Dissociate iPSC-ECs using accutase for 2–3 minutes at 37°C. Once all the cells detach from the bottom of the plate, neutralize the enzyme with EGM2.
2. Apply cell suspension to the cell strainer of the FACS tube to remove cell clumps.
3. Centrifuge for 5 minutes at 1,000 rpm at room temperature. Aspirate supernatant carefully and do not disturb cell pellet.
4. Add 1mL of 1% PFA (prepared from 20% stock) in DPBS, vortex, incubate for 20 minutes at room temperature, centrifuge as described in step 3, and aspirate supernatant.

Do not permeabilize the cells with Triton X100. This will destroy the cell surface protein and affect antibody labeling.

5. Wash with 2mL 0.5% BSA in DPBS, centrifuge as described in step 3, and aspirate supernatant. Repeat twice.
6. Resuspend the fixed cells in 100µL of 0.5% BSA in DPBS containing a 1:200 dilution of PE-KDR, FITC-CD34, and APC-CD144 antibodies, vortex, incubate for 30 minutes at 4°C, centrifuge as described in step 3, and aspirate supernatant.

7. Wash with 2mL 0.5% BSA in DPBS, centrifuge as described in step 3, and aspirate supernatant. Repeat twice.
8. Resuspend cells in 300µL of 0.5% BSA in DPBS.
9. Analyze with flow cytometer Aria (BD Biosciences), following instrument manufacturer's instructions.

SUPPORT PROTOCOL 2. CHARACTERIZATION OF iPSC-EC BY IMMUNOFLUORESCENT STAINING

After the differentiation and purification of iPSC-ECs, we further evaluate the expression of EC marker CD31 and CD144 by immunofluorescent staining, as shown in Figure 2B.

Material

Day 10 iPSC-ECs two days after purification (see Basic Protocol 3)

4% (w/v) PFA in DPBS (prepare from 20% PFA; Electron Microscope Sciences, cat. no. 15713-S)

5% (w/v) BSA (Sigma-Aldrich, cat. no. A3311) in CMF-DPBS

Anti-VE Cadherin antibody (CD144), rabbit polyclonal IgG (Abcam, cat. no. ab33168)

Anti-CD31 primary antibody, mouse monoclonal IgG1 (Abcam, cat. no. ab24590)

AlexaFluor 488–conjugated goat anti-rabbit IgG (Life Technologies, cat. no. A11008)

AlexaFluor 594–conjugated goat anti-mouse IgG1 (Life Technologies, cat. no. A21125)

Prolong Diamond with DAPI (Life Technologies, cat. no. P36962)

Nunc® Lab-Tek™ II Chamber Slide™ System, Sterile, 4-WELL (Thermo Nunc, cat. no. 62407-294)

Fluorescence microscopy (Leica, Wetzlar, Germany)

1. Seed cells on a 0.2% gelatin-coated 4-well chamber slide and allow cells to recover from passaging for one day. When ready for fixation, remove medium from cells.
2. Fix cells with 4% PFA in DPBS for 15 minutes at room temperature.
3. Block with 5% BSA in DPBS, and incubate for 60 minutes at room temperature.
4. Stain cells with CD31 and CD144 antibodies at 1:100 dilution in 5% BSA in DPBS. Incubate overnight at 4°C.
5. Wash three times with DPBS, each time for 5 minutes.

6. Stain with secondary antibodies at 1:1000 dilution in 5% BSA in DPBS for 60 minutes at room temperature, in the dark.
7. Wash three times with DPBS, each time for 5 minutes.
8. Adhere coverslip with 1 to 2 drops of Prolong Diamond (with DAPI) and evaluate by fluorescence microscopy.

SUPPORT PROTOCOL 3. DIL-CONJUGATED ACETYLATED LDL UPTAKE

Endothelial cells and macrophages take up acetylated-low density lipoprotein (Ac-LDL) through the LDL scavenging mechanism (Voyta et al., 1984). The ability to identify endothelial cells based on their increased metabolism of Ac-LDL was examined using Ac-LDL labeled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL), as shown in Figure 2C. This is a very simple yet robust assay to confirm endothelial function.

Material

Day 10 iPSC-ECs two days after purification (see Basic Protocol 3)

Low Density Lipoprotein from Human Plasma, Acetylated, DiI complex (DiI-Ac-LDL) (Life Technologies, L-3484)

Nunc® Lab-Tek™ II Chamber Slide™ System, Sterile, 4-WELL (Thermo Nunc, cat. no. 62407-294)

Fluorescence microscopy (Leica, Wetzlar, Germany)

1. Seed 10,000 iPSC-ECs per well in 4-chamber slides (LabTek II) coated with 0.2% gelatin. Allow 6–24 hours for the cells to attach and proliferate.
2. Add 250µL/well 5% FBS ECM containing 10µg/ml DiI-Ac-LDL and leave in incubator for 6 hours.
3. Wash with PBS twice and add 500µL PBS. Image with inverted fluorescence microscope.

SUPPORT PROTOCOL 4. TUBE FORMATION OF iPSC-EC

One of the most widely used *in vitro* assays to model the reorganization of angiogenesis is the tube formation assay (DeCicco-Skinner et al., 2014). This assay measures the ability of endothelial cells, plated on extracellular matrix, to form capillary-like structures, as shown in Figure 2D. Upon plating, endothelial cells attach and generate mechanical forces on the surrounding extracellular support matrix to create guidance pathways that facilitate cellular migration.

Material

Day 10 iPSC-ECs two days after purification (see Basic Protocol 3)

24-well cell culture plates (ThermoFisher, cat. no. 144530)

Corning Matrigel™ Membrane Matrix, Growth Factor Reduced (ThermoFisher, cat. no. CB-40230)

1. Thaw 10mL of growth factor reduced Matrigel in ice bath in 4°C cold room overnight.
2. Pre-cooling tips and 24 well plate at 4°C to help prevent solidify of Matrigel. Pipette 300µL of Matrigel in each well of a 24-well plate. Pipet slowly to avoid air bubbles. Centrifuge the plate at 1000rpm for 5 minutes at 4°C.
3. Incubate 24 well plate in 37°C incubator for 30–60 minutes to solidify the Matrigel.
4. Suspend cells in EGM2. Seed 6×10^4 cells per well of 24-well plate, incubate and observe tube formation after 6, 12, and 24 hours respectively.

The iPSC-ECs will start forming tubes 3 hours after seeding onto Matrigel. The tubes will regress after 24 hours. These timelines may vary depending on specific iPSC-EC lines.

5. Obtain 6 images per well. Use ImageJ to quantify number and length of the tubes.

Cultrex in vitro angiogenesis assay tube formation kit (Trevigen, cat. no. 3470-096-K) is an alternative assay that can be used for improved image quality and simplified procedure.

REAGENTS AND SOLUTIONS

EDTA, 0.5 mM

To 500mL of DPBS (Corning, cat. no. 21-031-CV) add 500µL of 0.5 M EDTA (Corning 46-034-CI) for a final concentration of 0.5 mM. Store up to 6 months at room temperature.

FGF, 25 µg/mL

In a sterile hood, reconstitute a 50-µg vial of human FGF-basic 154 using 2-ml of 10 mM Tris (pH 7.6) in 0.1% (wt/vol) BSA–H₂O. Divide the solution into 200-µl aliquots in 1.5-ml tubes and store them at –80°C for up to 1 year.

VEGF, 50 µg/mL

In a sterile hood, reconstitute one vial (100µg) of VEGF powder in 2mL of 0.1% (wt/vol) BSA–H₂O. Divide the solution to 100µL aliquots and store at –80 °C for up to 1 year. Use the aliquots immediately after thawing.

Y-27632, 10 mM

In a sterile hood, add the appropriate volume of PBS to a 5mg vial and dissolve it to a concentration of 10mM. Divide the solution to 100µL aliquots and store at –20°C for up to 1 year.

CHIR-99021, 12 mM

In a sterile hood, add 895 μ L DMSO to 5mg of CHIR-99021. Divide the solution to 50 μ L aliquots and store at -20°C for up to 1 year.

SB431542, 10 mM

Reconstitute 10mg SB431542 (Selleckchem, cat. no. S1067) with 2.6 mL DMSO. Filter the reconstituted SB solution through a 0.22 μ m Millex filter unit. Divide to 50 μ L aliquots and store at -20°C for up to 6 months.

0.2% (vol/vol) Gelatin

Warm up the 5% (vol/vol) stock gelatin solution in a 37°C water bath until it has fully dissolved. In a sterile hood, mix 20mL of 5% (vol/vol) Gelatin solution with 490mL of ultrapure water. The solution can be stored at 4°C for up to 6 months.

EGM2

In a sterile hood, thoroughly mix the EGM supplements from the BulletKit with EBM (500mL). Add 5mL Antibiotic-Antimycotic (100x, ThermoFisher, cat. no. 15240062) to prevent bacterial and fungal contamination. The medium can be stored at 4°C for up to 2 weeks.

MACS Buffer

Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2mM EDTA by diluting MACS BSA Stock Solution (#130-091-376) 1:20 with autoMACS® Rinsing Solution (#130-091-222). Keep buffer at 4°C . Degas buffer before use, as air bubbles could block the column.

COMMENTARY**Background Information**

ECs have been successfully generated from human embryonic stem cells as well as iPSCs (Levenberg et al., 2010; Nourse et al., 2010; Orlova et al., 2014; Rafii et al., 2013). Despite substantial effort, attempts to generate high-purity endothelial cells from human iPSCs have met with modest success, with efficiencies ranging from 5 to 30% cells positive for lineage markers, and the long and complicated procedure usually takes 2–3 weeks. Compared with previous protocols, the current protocol provides a simple and cost-effective method to generate iPSC-ECs with high purity.

The next step is to generate tissue-specific, functionally distinct endothelial cell subtypes from iPSCs. This would allow us to better understand the contribution of a specific cell type to the development of the disease, and to develop a more precise way to determine the appropriate therapy. Developmental studies have shown distinct origins and pathways for specifying endothelial subtypes such as endocardial, vascular, valve ECs, and lymphatic ECs (Barnes et al., 2010; de la Pompa et al., 1998; Misfeldt et al., 2009; Peterkin et al., 2009; Ranger et al., 1998). Recently, Palpant et al. developed a monolayer-directed differentiation

protocol using different concentrations of activin A and bone morphogenetic protein 4 (BMP4) to polarize cells into mesodermal subtypes that reflect mid-primitive-streak cardiogenic mesoderm and posterior-primitive-streak hemogenic mesoderm (Palpant et al., 2017). This differentiation platform provides a basis for generating distinct cardiovascular progenitor populations that enable the derivation of cardiomyocytes and functionally distinct EC subtypes from cardiogenic versus hemogenic mesoderm with high efficiency, and without cell sorting. This will allow us to study fundamental questions about lineage specification during development, and provide a scalable cell-specific source for tissue engineering, cell therapy, disease modeling, and drug discovery.

Additionally, other protocols provide important guidance for using these iPSC derived vascular cells in preclinical animal studies including myocardial infarction (Gu et al., 2012), hindlimb ischemia (Lai et al., 2013; Rufaihah et al., 2011; Yoo et al., 2013), retinopathy (Park et al., 2014), carotid artery injury (Giordano et al., 2016), and dermal wound healing (Kim et al., 2013; Lee et al., 2015). These studies demonstrated that transplantation of iPSC-ECs improved the disease condition through either integration into the host vasculature or paracrine activation.

Critical Parameters

Quality of the iPSCs—We found that the proliferation rate and pluripotency of the iPSCs are the most important factors for differentiation efficiency. In our protocol, we use EDTA to dissociate the cells and grow them as monolayers. This method simplified the passaging process and significantly improved cell survival and homogeneity (Beers et al., 2012). We also found that by shortening the incubation time with EDTA, we can separate good iPSC clones from the differentiated cells because the good clones come off the plate faster.

Cell density before mesoderm induction—Three-to-four days before differentiation, we split the 90% confluent iPSCs at 1:6 ratio in a new Matrigel coated plate. We noticed that if the density is too low after passaging, the iPS clones lose cross talk and it takes longer for the cells to reach 70–80% confluency. Also, when a single clone grows too big, they will start differentiating spontaneously, and this will negatively impact the directed differentiation of iPSCs to a specific lineage. After 3–4 days, the iPSCs should reach 70–80% confluency and be ready for mesoderm induction. If the density is higher than 80%, significant cell death may happen due to the over-growth. Once the cells reach the endothelial progenitor stage, they will proliferate very fast, and that is another reason to start at low cell density.

Optimize CHIR99021 concentration for each cell line—Some iPSC lines may be more sensitive to CHIR99021 than others in terms of the WNT activation, so we optimize the concentration from 5 μ M to 7 μ M based on the specific cellular response. If there is extensive cell death upon CHIR99021 treatment, we reduce the concentration down to 5 μ M; if we don't see significant morphology change by day 4, we increase the concentration to 7 μ M. Although the window for adjusting CHIR concentration is relatively small, we do find it has great impact on differentiation efficiency.

Single cell suspension for MACS sorting—To enrich mature iPSC-ECs population after differentiation, we utilize MACS sorting against the EC surface marker CD144. To increase the purity of cells after sorting, it is critical to dissociate the cells thoroughly to achieve single cell suspension for antibody labeling. Having clumps in the suspension will not only block the column during cell sorting, but also pull down cells that are not magnetically-labeled together with the CD144⁺ cells. However, do not over digest the cells with enzyme, as this may affect surface protein localization.

Seed the cells at high density after sorting—After MACS sorting, the cells are fragile and require careful handling. Fetal Bovine Serum in EGM2 could be increased to 10–20% to improve cell adhesion and survival after sorting. Seed the iPSC-ECs at 3–4×10⁵ cells per well in a 6-well plate. If fewer cells are obtained after differentiation and purification, scale down to 12-well or 24 well plates. Change medium the next day to get rid of unattached cells, and then change the medium every other day.

Troubleshooting

Extensive cell death with CHIR99021 treatment—There could be several reasons for the cell death upon CHIR99021 treatment.

1. The iPSCs are contaminated with mycoplasma. To avoid the contamination, test mycoplasma in the culture medium once a week or biweekly using MycoAlert™ Mycoplasma Detection Kit (Lonza, cat. no. LT07-318). Discard the mycoplasma positive cells immediately after detection. For precious cell lines, Plasmocin™ (InvivoGen, cat. code. ant-mpp) can be used to eliminate mycoplasma contamination. Stop using Plasmocin one week before EC differentiation.
2. The starting density of the iPSC is too high. Make sure the iPSC culture does not exceed 80% confluency.
3. iPSCs are at early passage (less than 10). Further expand the cells to at least passage 15 before starting differentiation.
4. The concentration of CHIR is too high for this specific cell line. Optimize it starting with a lower dose.

Low yield of CD144⁺ cells—The individual's genetic background may affect EC differentiation efficiency, thus it is necessary to optimize the protocol for each cell line. Test higher or lower splitting ratio to get 50%–80% starting cell density, or vary the level of CHIR99021 (5μM to 7μM), VEGF (50–100ng/mL), and FGF (10–50ng/mL). During EC specification from Day 4 to Day 8, FBS could also be withdrawn from EGM2 to eliminate spontaneous differentiation. SB431542 could be added to EGM2 from Day 4 to Day 8 to reduce smooth muscle cell differentiation and proliferation.

Low proliferation rate of the purified iPSC-ECs after MACS sorting—It is normal that the cells do not grow as well as before MACS sorting, due to the manipulations involved in the procedure. However, they should recover overnight, or at most in 2–3 days. Additional FBS could be added to the EGM2 to support the cell adhesion and survival for a couple

of days, and keep VEGF at 50ng/mL and FGF at 25ng/mL in EGM2 until the cells start growing at the normal rate again. Withdraw the additional FBS and growth factors one week before performing any functional assays.

Batch effect of small molecules—The current protocol requires the use of Matrigel, B-27 supplement, and recombinant proteins, which are prone to batch effects, making differentiation efficiency variable. It is important to test the efficacy of the small molecules for differentiation, and adjust the concentration for different batches and cell lines.

Understanding Results

Ideally, this differentiation protocol should produce 2×10^6 iPSC-ECs from 5 wells of a 6-well plate. iPSC-EC purity after differentiation should be around 50–70%, and over 85% after MACS sorting against CD144. These iPSC-ECs will stain positive for the endothelial cell surface markers CD31 and CD144 and exhibit LDL uptake. The cells are also capable of forming tubes 12 hours after seeding on growth factor reduced Matrigel. After cryopreservation, more than 80% of the cells should be recovered, and cells will regain endothelial characteristics after one passage.

Time Considerations

Human iPSC thawing and passaging will take 10–15 days, depending on the quality of the cell lines. The differentiation and purification process from iPSCs to iPSC-ECs will take 8 days.

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LITERATURE CITED

- Barnes RM, Firulli BA, Conway SJ, Vincentz JW, Firulli AB. 2010; Analysis of the Hand1 cell lineage reveals novel contributions to cardiovascular, neural crest, extra-embryonic, and lateral mesoderm derivatives. *Dev Dyn*. 239 (11) 3086–3097. DOI: 10.1002/dvdy.22428 [PubMed: 20882677]
- Beers J, Gulbranson DR, George N, Siniscalchi LI, Jones J, Thomson JA, Chen G. 2012; Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. *Nat Protoc*. 7 (11) 2029–2040. DOI: 10.1038/nprot.2012.130 [PubMed: 23099485]
- Burridge PW, Matsa E, Shukla P, Lin ZC, Churko JM, Ebert AD, ... Wu JC. 2014; Chemically defined generation of human cardiomyocytes. *Nat Methods*. 11 (8) 855–860. DOI: 10.1038/nmeth.2999 [PubMed: 24930130]
- Chen G, Gulbranson DR, Yu P, Hou Z, Thomson JA. 2012; Thermal stability of fibroblast growth factor protein is a determinant factor in regulating self-renewal, differentiation, and reprogramming in human pluripotent stem cells. *Stem Cells*. 30 (4) 623–630. DOI: 10.1002/stem.1021 [PubMed: 22213113]
- Cheng CC, Chang SJ, Chueh YN, Huang TS, Huang PH, Cheng SM, ... Wang HW. 2013; Distinct angiogenesis roles and surface markers of early and late endothelial progenitor cells revealed by

- functional group analyses. *BMC Genomics*. 14: 182. doi: 10.1186/1471-2164-14-182 [PubMed: 23496821]
- de la Pompa JL, Timmerman LA, Takimoto H, Yoshida H, Elia AJ, Samper E, ... Mak TW. 1998; Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature*. 392 (6672) 182–186. DOI: 10.1038/32419 [PubMed: 9515963]
- DeCicco-Skinner KL, Henry GH, Cataisson C, Tabib T, Gwilliam JC, Watson NJ, ... Wiest JS. 2014; Endothelial cell tube formation assay for the in vitro study of angiogenesis. *J Vis Exp*. (91) e51312. doi: 10.3791/51312 [PubMed: 25225985]
- Giannotta M, Trani M, Dejana E. 2013; VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity. *Dev Cell*. 26 (5) 441–454. DOI: 10.1016/j.devcel.2013.08.020 [PubMed: 24044891]
- Giordano S, Zhao X, Xing D, Hage F, Oparil S, Cooke JP, ... Chen YF. 2016; Targeted delivery of human iPSC-ECs overexpressing IL-8 receptors inhibits neointimal and inflammatory responses to vascular injury in the rat. *Am J Physiol Heart Circ Physiol*. 310 (6) H705–715. DOI: 10.1152/ajpheart.00587.2015 [PubMed: 26801304]
- Gu M, Mordwinkin NM, Kooreman NG, Lee J, Wu H, Hu S, ... Wu JC. 2015; Pravastatin reverses obesity-induced dysfunction of induced pluripotent stem cell-derived endothelial cells via a nitric oxide-dependent mechanism. *Eur Heart J*. 36 (13) 806–816. DOI: 10.1093/eurheartj/ehu411 [PubMed: 25368203]
- Gu M, Nguyen PK, Lee AS, Xu D, Hu S, Plews JR, ... Wu JC. 2012; Microfluidic single-cell analysis shows that porcine induced pluripotent stem cell-derived endothelial cells improve myocardial function by paracrine activation. *Circ Res*. 111 (7) 882–893. DOI: 10.1161/CIRCRESAHA.112.269001 [PubMed: 22821929]
- Gu M, Shao NY, Sa S, Li D, Termglinchan V, Ameen M, ... Rabinovitch M. 2017; Patient-Specific iPSC-Derived Endothelial Cells Uncover Pathways that Protect against Pulmonary Hypertension in BMPR2 Mutation Carriers. *Cell Stem Cell*. 20 (4) 490–504. e495. doi: 10.1016/j.stem.2016.08.019 [PubMed: 28017794]
- James D, Nam HS, Seandel M, Nolan D, Janovitz T, Tomishima M, ... Rafii S. 2010; Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGFbeta inhibition is Id1 dependent. *Nat Biotechnol*. 28 (2) 161–166. DOI: 10.1038/nbt.1605 [PubMed: 20081865]
- Kattman SJ, Huber TL, Keller GM. 2006; Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell*. 11 (5) 723–732. DOI: 10.1016/j.devcel.2006.10.002 [PubMed: 17084363]
- Kennedy M, D'Souza SL, Lynch-Kattman M, Schwantz S, Keller G. 2007; Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. *Blood*. 109 (7) 2679–2687. DOI: 10.1182/blood-2006-09-047704 [PubMed: 17148580]
- Kim KL, Song SH, Choi KS, Suh W. 2013; Cooperation of endothelial and smooth muscle cells derived from human induced pluripotent stem cells enhances neovascularization in dermal wounds. *Tissue Eng Part A*. 19 (21–22) 2478–2485. DOI: 10.1089/ten.TEA.2012.0768 [PubMed: 23790124]
- Lai WH, Ho JC, Chan YC, Ng JH, Au KW, Wong LY, ... Tse HF. 2013; Attenuation of hind-limb ischemia in mice with endothelial-like cells derived from different sources of human stem cells. *PLoS One*. 8 (3) e57876. doi: 10.1371/journal.pone.0057876 [PubMed: 23472116]
- Lan F, Lee AS, Liang P, Sanchez-Freire V, Nguyen PK, Wang L, ... Wu JC. 2013; Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells. *Cell Stem Cell*. 12 (1) 101–113. DOI: 10.1016/j.stem.2012.10.010 [PubMed: 23290139]
- Lee SJ, Park C, Lee JY, Kim S, Kwon PJ, Kim W, ... Yoon YS. 2015; Generation of pure lymphatic endothelial cells from human pluripotent stem cells and their therapeutic effects on wound repair. *Sci Rep*. 5: 11019. doi: 10.1038/srep11019 [PubMed: 26066093]
- Levenberg S, Ferreira LS, Chen-Konak L, Kraehenbuehl TP, Langer R. 2010; Isolation, differentiation and characterization of vascular cells derived from human embryonic stem cells. *Nat Protoc*. 5 (6) 1115–1126. DOI: 10.1038/nprot.2010.31 [PubMed: 20539287]

- Lian X, Zhang J, Zhu K, Kamp TJ, Palecek SP. 2013; Insulin inhibits cardiac mesoderm, not mesendoderm, formation during cardiac differentiation of human pluripotent stem cells and modulation of canonical Wnt signaling can rescue this inhibition. *Stem Cells*. 31 (3) 447–457. DOI: 10.1002/stem.1289 [PubMed: 23193013]
- Liang P, Lan F, Lee AS, Gong T, Sanchez-Freire V, Wang Y, ... Wu JC. 2013; Drug screening using a library of human induced pluripotent stem cell-derived cardiomyocytes reveals disease-specific patterns of cardiotoxicity. *Circulation*. 127 (16) 1677–1691. DOI: 10.1161/CIRCULATIONAHA.113.001883 [PubMed: 23519760]
- Misfeldt AM, Boyle SC, Tompkins KL, Bautch VL, Labosky PA, Baldwin HS. 2009; Endocardial cells are a distinct endothelial lineage derived from Flk1+ multipotent cardiovascular progenitors. *Dev Biol*. 333 (1) 78–89. DOI: 10.1016/j.ydbio.2009.06.033 [PubMed: 19576203]
- Nourse MB, Halpin DE, Scatena M, Mortisen DJ, Tulloch NL, Hauch KD, ... Murry CE. 2010; VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering. *Arterioscler Thromb Vasc Biol*. 30 (1) 80–89. DOI: 10.1161/ATVBAHA.109.194233 [PubMed: 19875721]
- Orlova VV, van den Hil FE, Petrus-Reurer S, Drabsch Y, Ten Dijke P, Mummery CL. 2014; Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. *Nat Protoc*. 9 (6) 1514–1531. DOI: 10.1038/nprot.2014.102 [PubMed: 24874816]
- Palpant NJ, Pabon L, Friedman CE, Roberts M, Hadland B, Zaunbrecher RJ, ... Murry CE. 2017; Generating high-purity cardiac and endothelial derivatives from patterned mesoderm using human pluripotent stem cells. *Nat Protoc*. 12 (1) 15–31. DOI: 10.1038/nprot.2016.153 [PubMed: 27906170]
- Park TS, Bhutto I, Zimmerlin L, Huo JS, Nagaria P, Miller D, ... Zambidis ET. 2014; Vascular progenitors from cord blood-derived induced pluripotent stem cells possess augmented capacity for regenerating ischemic retinal vasculature. *Circulation*. 129 (3) 359–372. DOI: 10.1161/CIRCULATIONAHA.113.003000 [PubMed: 24163065]
- Peterkin T, Gibson A, Patient R. 2009; Common genetic control of haemangioblast and cardiac development in zebrafish. *Development*. 136 (9) 1465–1474. DOI: 10.1242/dev.032748 [PubMed: 19297410]
- Rafii S, Kloss CC, Butler JM, Ginsberg M, Gars E, Lis R, ... James D. 2013; Human ESC-derived hemogenic endothelial cells undergo distinct waves of endothelial to hematopoietic transition. *Blood*. 121 (5) 770–780. DOI: 10.1182/blood-2012-07-444208 [PubMed: 23169780]
- Ranger AM, Grusby MJ, Hodge MR, Gravallese EM, de la Brousse FC, Hoey T, ... Glimcher LH. 1998; The transcription factor NF-ATc is essential for cardiac valve formation. *Nature*. 392 (6672) 186–190. DOI: 10.1038/32426 [PubMed: 9515964]
- Rufaihah AJ, Huang NF, Jame S, Lee JC, Nguyen HN, Byers B, ... Cooke JP. 2011; Endothelial cells derived from human iPSCs increase capillary density and improve perfusion in a mouse model of peripheral arterial disease. *Arterioscler Thromb Vasc Biol*. 31 (11) e72–79. DOI: 10.1161/ATVBAHA.111.230938 [PubMed: 21836062]
- Sa S, Gu M, Chappell J, Shao NY, Ameen M, Elliott KA, ... Rabinovitch M. 2017; Induced Pluripotent Stem Cell Model of Pulmonary Arterial Hypertension Reveals Novel Gene Expression and Patient Specificity. *Am J Respir Crit Care Med*. 195 (7) 930–941. DOI: 10.1164/rccm.201606-1200OC [PubMed: 27779452]
- Takahashi K, Yamanaka S. 2006; Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 126 (4) 663–676. DOI: 10.1016/j.cell.2006.07.024 [PubMed: 16904174]
- Voyta JC, Via DP, Butterfield CE, Zetter BR. 1984; Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J Cell Biol*. 99 (6) 2034–2040. [PubMed: 6501412]
- Wu H, Lee J, Vincent LG, Wang Q, Gu M, Lan F, ... Wu JC. 2015; Epigenetic Regulation of Phosphodiesterases 2A and 3A Underlies Compromised beta-Adrenergic Signaling in an iPSC Model of Dilated Cardiomyopathy. *Cell Stem Cell*. 17 (1) 89–100. DOI: 10.1016/j.stem.2015.04.020 [PubMed: 26095046]

- Yang L, Geng Z, Nickel T, Johnson C, Gao L, Dutton J, ... Zhang J. 2016; Differentiation of Human Induced-Pluripotent Stem Cells into Smooth-Muscle Cells: Two Novel Protocols. *PLoS One*. 11 (1) e0147155. doi: 10.1371/journal.pone.0147155 [PubMed: 26771193]
- Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, ... Keller GM. 2008; Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature*. 453 (7194) 524–528. DOI: 10.1038/nature06894 [PubMed: 18432194]
- Yoo CH, Na HJ, Lee DS, Heo SC, An Y, Cha J, ... Cho YS. 2013; Endothelial progenitor cells from human dental pulp-derived iPS cells as a therapeutic target for ischemic vascular diseases. *Biomaterials*. 34 (33) 8149–8160. DOI: 10.1016/j.biomaterials.2013.07.001 [PubMed: 23896001]

Figure 1.

Generation of endothelial cells from iPSCs. (A) Schematic of the differentiation procedure with the medium, extracellular matrices, and small molecules used in each step. (B) Representative images of hiPSC cells at 80% confluency on day 0, followed by mesoderm induction and endothelial cell specification. After MACS sorting against CD144, the iPSC-ECs show homogeneous cobble-stone morphology characteristic of ECs.

Figure 2.

Characterization of iPSC-ECs. (A) Flow cytometry of iPSC-ECs before and after purification. The population of mature iPSC-ECs (CD144⁺) is significantly enriched after MACS sorting. (B) iPSC-EC differentiated from skin fibroblast-derived iPSC express the endothelial surface marker CD144 (green) and CD31 (red). Nuclear staining using DAPI is in blue. (C) iPSC-ECs incorporate acetylated low-density lipoprotein (Ac-LDL, red; DAPI, Blue). (D) Angiogenesis assays: representative images of tube formation 6 hours, 12 hours, and 24 hours after seeding cells in Matrigel. iPSC-ECs start forming tubes at 6 hour time point, and will regress after 24 hours.