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Generation of Endothelial Cells from Human Induced Pluripotent Stem Cells

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[Abstract] Endothelial cells (ECs), as important constituent of blood vessel are crucial to maintaining vascular tone, and any dysfunction in ECs could lead to many diseases of the vascular system. Due to limited availability of human samples, most of our research has been limited to animal models. Induced pluripotent stem cells (iPSCs) due to their potential to differentiate into any cell type of the human body, including cells of the cardiovascular system, provide us with a great opportunity to study human vascular disease. This protocol describes an efficient, simple, and step-wise method to differentiate iPSCs to endothelial cells (iPSC-ECs) using small molecules and growth factors. Through the activation of WNT signaling pathway, iPSCs are differentiated towards a mesodermal lineage with the potential to generate endothelial progenitors. These progenitors are further stimulated with growth factors to generate endothelial cells (Figure 1A).

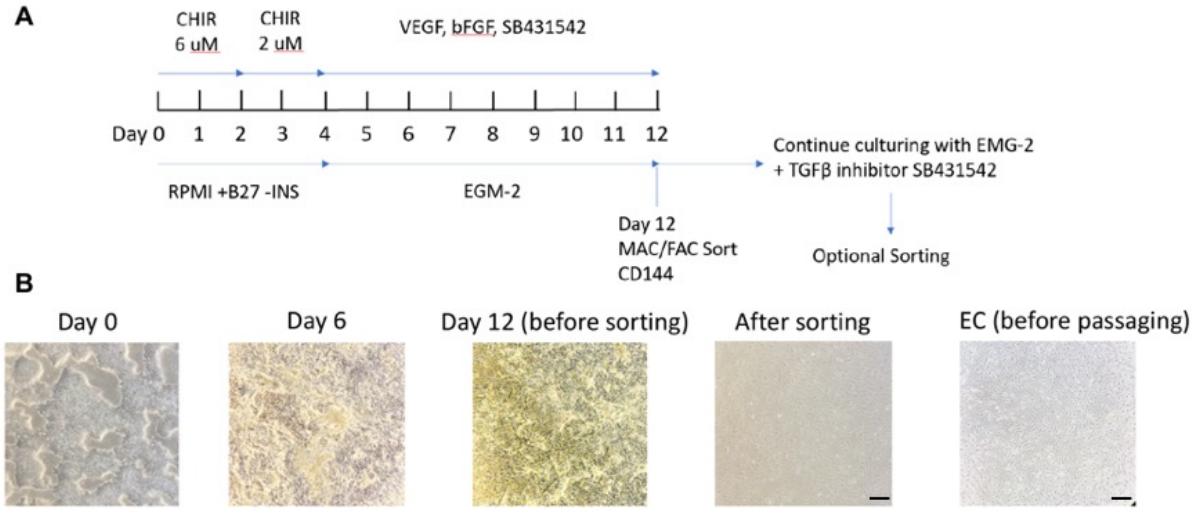

Figure 1. Stepwise differentiation strategy for iPSCs endothelial cell differentiation. A. chemicals/growth factors used at each stage of differentiation. B. Morphology of cells at different stage of differentiation. Scale bars = 500 μm.

Keywords: iPSCs, Endothelial cells, Differentiation

Materials and Reagents

1. 6-well plates (Fisher Scientific, catalog number: 08-772-1B)
2. 12-well plates (Fisher Scientific, catalog number: 08-772-29)
3. 40 µm strainer (Fisher Scientific, catalog number: 08-771-1)
4. Cryovials
5. Conical tubes
6. Human induced pluripotent cells
7. CD144 (VE-Cadherin) MicroBeads (Miltenyi, catalog number: 130-097-857)
8. TrypLE Express dissociation reagent (Gibco, catalog number: 12605010)
9. DMEM/F-12 basal medium (Gibco, catalog number: 11320033)
10. Essential 8™ Medium (Gibco, catalog number: A1517001)
11. ROCK inhibitor (Selleckchem, catalog number: S1049)
12. B27 supplement minus insulin (Gibco, catalog number: A1895601)
13. RPMI 1640 medium, no glutamine (Gibco, catalog number: 21870076)
14. EGM™ Endothelial Cell Growth Medium BulletKit™ (Lonza, catalog number: CC-3124)
15. VEGF (PeproTech, catalog number: 100-20)
16. bFGF (Thermo Fisher Scientific, catalog number: 13256029)
17. SB431542 (Selleckchem, catalog number: S1067)
18. CHIR99021 (Selleckchem, catalog number: S2924)
19. KnockOut™ Serum Replacement (Gibco, catalog number: 10828028)
20. DMSO (Sigma-Aldrich, catalog number: D2447-50ML)
21. Matrigel (Corning, catalog number: 354248)
22. Gelatin (Sigma-Aldrich, catalog number: G1393-20ML)
23. CD31 (PECAM1) mouse mAb (Cell Signaling, catalog number: 3528S)
24. AutoMACS Rinsing Solution (Fisher Scientific, catalog number: NC9104697)
25. MACS BSA Stock Solution (Miltenyi, catalog number: 130-091-376)
26. DPBS (Gibco, catalog number: 14190)
27. Trypan blue (Life Technologies, catalog number: T10282)
28. iPSC Passage medium (see Recipes)
29. RPMI + B27-INS medium (see Recipes)
30. Sorting Solution MACS buffer (see Recipes)

Equipment

1. 1 ml micropipette
2. Fume hood
3. Micropipettes
4. Autopipetter

5. Water bath
6. Standard table-top centrifuge
7. Cell counting slide (WISBIOMED, catalog number: L12001)
8. MACS sorting apparatus
    a. Magnet plate
    b. Separator
    c. Column
    d. Filter
9. Freezing container
10. Incubator (5% CO_2, 20% O_2)
11. Automated cell counter

Procedure

A. Preparation/Culture of iPSCs
    1. Preparation of Matrigel-coated plates
        a. Thaw vial of frozen Matrigel on ice.
        b. Dissolve 60 µl of Matrigel in 12 ml DMEM/F-12 basil medium (1:200), pipetting up and down to fully dispense. Mix by inversion.
        c. Add 2 ml of the Matrigel and medium mixture to each well of the 6-well plate and incubate at 37 °C in an incubator (overnight, 8-12 h).
    2. Thawing and recovery of iPSC

        Note: Usually, 1 cryovial of frozen cells is made from 1 well of a 6-well plate with ~90% confluency and can be successfully recovered into 1-2 wells of a 6-well plate.
        a. Prepare Essential 8 human ESC/iPSC passage medium (see Recipes).
        b. Warm Essential 8 medium and thaw ROCK inhibitor to room temperature (~25 °C).
        c. Thaw frozen cryovial in a 37 °C water bath for ~1-2 min. Proceed as soon as the vial thaws. Complete the whole recovery proceed as promptly as possible so as to limit cell death.
        d. Prepare 5 ml of iPSC passage medium in a 15 ml conical tube. Use a 1 ml micropipette to transfer the cell solution from the cryovial into the 15 ml conical tube and gently mix.
        e. Pellet the cells by centrifugation at 300 *x g* for 3 min.
        f. After centrifugation, aspirate medium and resuspend the cell pellet in 1 ml of iPSC passage medium with a 1 ml micropipette.
        g. Have an already prepared 6-well Matrigel-coated plate and prepared 2 wells for seeding cells by aspirating the Matrigel DMEM/F-12 basal medium mixture and replacing with 2 ml of iPSC passage medium each.
        h. Dispense 0.5 ml of the cell mixture into each coated well prepared with medium and slide plate horizontally and vertically flat on a surface back and forth a couple times to disperse the cells evenly.

i. Place cell plate into the incubator and incubate without disturbance for 24 h.
j. Change medium to Essential 8 culture medium the next day and continue to change medium every day.

B. Differentiation of endothelial cells (ECs)

Human iPSC cells cultured in Essential 8 medium generally will be ready for passaging or differentiation in about 4 days when the cells reach 85-90% confluent.

1. Day 0
   a. Prepare RPMI + B27-INS medium (see Recipes) with 6 μM CHIR99021 (*i.e.,* mix 7.2 μl of a 10 mM CHIR99021 stock to 12 ml of RPMI + B27-INS medium).
   b. Aspirate previous medium from iPSC wells and add 2 ml of RPMI + B27-INS medium with 6 μM CHIR99021 to each well.
   c. Incubate in a 37 °C incubator for 48 h.
2. Day 2
   a. Prepare RPMI + B27-INS medium with 2 μM CHIR (*i.e.*, mix 2.4 μl of a 10 mM CHIR stock to 12 ml of RPMI + B27-INS medium).
   b. Aspirate previous medium from EC wells and add 2 ml of RPMI + B27-INS medium with 2 μM CHIR to each well. Cells will be induced toward mesodermal stage as previously reported (Lian *et al*., 2014).
   c. Incubate at 37 °C in the incubator for 48 h.
3. Day 4
   a. Prepare EGM-2 medium supplied with VEGF, bFGF, SB431542 at the concentration of 50 ng/ml, 20 ng/ml, and 10 μM respectively.
      Note: Extra medium can be prepared for future use, but freshly prepared medium is preferred.
   b. Aspirate the previous medium from differentiation wells and add 2 ml of EGM-2 medium supplied with VEGF, bFGF, SB431542.
   c. Incubate in a 37 °C incubator for 48 h.
4. On Days 6, 8, and 10, aspirate previous medium and change to the same EGM-2 medium supplied with VEGF, bFGF and SB431542.

C. Sorting of ECs

ECs generally are ready for sorting via MACS (magnetic-activated cell sorting) on Day 12 of differentiation.

1. Preparation of materials and reagents
   a. Sterilize an ice bucket well with 70% ethanol spray and keep MACS buffer and magnetic antibody (CD144) on ice in a fume hood.
   b. Preparation of gelatin plates
      i. Produce a 0.2% gelatin coating mixture by adding appropriate ratio of gelatin and DPBS,

and mix.

ii. Add 1 ml of 0.2% gelatin coating to each well of a 12-well plate and incubate at 37 °C in hypoxia-capable incubator.

Note: Preparation of EC cells for sorting will provide enough time (≥ 10 min) for gelatin to set and be ready for use.

2. Suspension of ECs
   a. Aspirate old culture media and wash each well with 1 ml PBS per well.
   b. Aspirate PBS and add 1 ml of TrypLE per well then incubate at 37 °C in an incubator for 10 min.
   c. Use a 1 ml micropipette to pipette suspension up and down to break free cell clusters.
   d. Neutralize lysis by adding 1 ml EGM-2 medium per well.
3. Filtering EC cells
   a. Pre-wet a 40 μm strainer with 2-3 ml of MACS buffer.
   b. Filter cell suspension through a 40 μm strainer into a 50 ml conical tube.

      Note: Change to a new strainer when clogging is observed; typically, 1 strainer is used for every 2-4 wells.
   c. Centrifuge the collected cells at 300 *x g* for 5 min
   d. Aspirate the medium without disrupting the cell pellet and resuspend the cell pellet in 5 ml of MACS buffer.
4. Counting cells and antibody attachment
   a. In a 96-well plate, add 10 μl of resuspended cell solution and 10 μl trypan blue each into a well and pipette up and down to mix well.
   b. Load 10 μl of the cell and dye mixture onto each side of the cell counting slide for duplicate measure.
   c. Adjust the automated cell counter to the correct settings and insert the slide into slot to count cells.

      Alternatively, a hemocytometer can be used to count the cells if automated cell counter is not available.
   d. Centrifuge the 50 ml conical tube of ECs again at 300 *x g* for 5 min. While waiting for centrifugation, calculate the total number of cells in suspension with the concentration provided by the cell counter, and then evaluate the amount of MACS buffer and CD144 antibody needed for the sorting solution. Per 10 million cells, add 80 μl of MACS buffer and 20 μl of antibody.
   e. Resuspend the cell pellet in sorting solution. Suspension can be transferred into a 15 ml conical tube for convenience.
   f. Incubate suspended cells in a dark fridge at 4 °C for 15 min.
5. Sorting
   a. Rinse the cells by diluting the suspension with 1 ml of MACS buffer per 10 million cells.
   b. Centrifuge the diluted cell suspension at 300 *x g* for 5 min.

c. During centrifugation, set up MACS sorting apparatus (Figure 2)
   i. Sterilize MACS magnet stand, separator and conical tubes with 70% ethanol spray and place in a hood.
   ii. Clip columns into separator correctly and attach separator to magnet stand at the appropriate height.
   iii. Take a 50 ml conical tube and put it under the column to collect filtered fluid.
d. Aspirate diluted sorting solution and resuspend cell pellet in 1 ml of MACS buffer.
e. Wet MACS column by passing through 3 ml of MACS buffer.
f. Run 500 µl of resuspended cells per column.
g. Rinse column with 3 ml MACS buffer; repeat 3 times to assure unlabeled cells are flushed out.
   Note: Wait for almost all buffer to flow through before adding more.
h. Once rinsed, remove the column from separator and place onto a new 15 ml conical tube.
i. Add 5 ml of MACS buffer and immediately elute labeled cells by forcing buffer through the column with plunger.

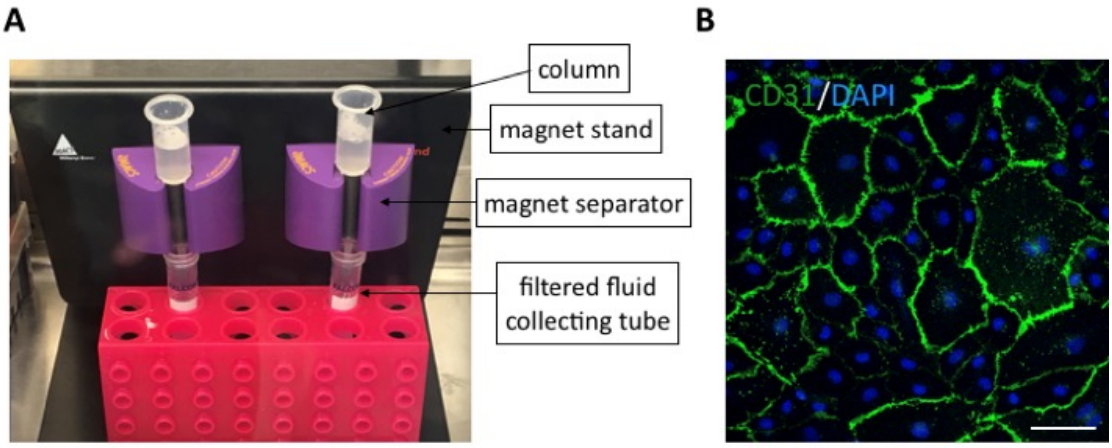

Figure 2. MACS sorting apparatus and iPSCs-ECs characterization. A. MACS sorting apparatus setup. Arrows indicate each item used for MACS sorting. B. Immunostaining on iPSC-ECs with EC surface marker CD31. Green: CD31, blue: DAPI. Scale bar = 50 µm.

6. Replating sorted EC
   a. Centrifuge MACS buffer with positively labeled cells at 300 *x g* for 5 min.
   b. Prepare EGM-2 medium with 10 µM SB431542, which will be the medium used to culture the current and following passages of ECs.
   c. Aspirate gelatin solution from previously prepared 12-well gelatin plates and replace with 500 µl EGM-2 medium supplied with 10 µM SB431542.
      Note: Label plate as Passage 0. The sorting efficiency of iPSC-ECs usually ranges from 50% to 80% among different iPSC lines.
   d. Resuspend centrifuged cells, and seed the cell as 50, 000 cells per well in 1 ml EGM-2

medium supplied with SB431542 and distribute evenly amongst the gelatin-coated wells

D. (Optional) Additional sorting of ECs

If cells are still impure after the first sorting, additional sorting can be proceeded. Procedure is the same as Sorting of ECs (Procedure C).

E. Passaging ECs

Note: After sorting and culture, confluent ECs can be either passaged or frozen for future use once confluent.

1. Preparation of 12-well gelatin plates (same as Step C1b).
2. Aspirate medium and wash with 1 ml of DPBS for each well.
3. Add 1 ml of TrypLE per well and incubate in a 37 °C incubator for 5 min.
4. While incubating the cells, prepare the medium that will be used for culturing the passaged ECs. Add SB431542 (10 μM) to EGM-2 medium.
5. Use a 1 ml micropipette to resuspend the cells.
6. Add 1 ml per well of EGM-2 medium with SB431542 to neutralize.
7. Collect suspension from each well into a 50 ml conical tube and centrifuge at 300 *x g* for 5 min.
8. While waiting for centrifugation, aspirate the gelatin and DPBS mix from the previously prepared 12-well gelatin plates and add 1 ml of EGM-2 medium with SB431542 per well.
9. Aspirate the supernatant gently and resuspend in 1 ml of EGM-2 medium with SB431542 using a 1 ml micropipette.
10. Distribute the resuspended cells evenly into new 12-well gelatin plates; usually the passaging ratio is 1:3.

    Note: SB431542 usually will be added till passage 2 to inhibit non-ECs proliferation. And differentiated ECs can be characterized by immunostaining of typical EC surface markers (e.g., CD31). Further characterizations can refer to a manuscript of EC characterization (Li et al., 2011). The morphology of related ECs is similar to the cells seeded after sorting.

F. Freezing ECs

Note: If ECs are not needed immediately, they can be frozen for future use.

1. Aspirate culture medium from wells and wash cells with 500 μl DPBS per well.
2. Aspirate DPBS and add 500 μl of TrypLE per well, then incubate cells in a 37 °C hypoxia-capable incubator for 5-8 min.

   Note: Check cells after 5 min to see if cells require further digestion.
3. Pipette cells up and down to break up clusters.
4. Add an equal volume (500 μl) of EGM-2 media to neutralize the TrypLE and transfer the mixture into a 15 ml conical tube.
5. Centrifuge cells at 300 rcf for 4 min.
6. While waiting for centrifugation, create freezing buffer of KnockOut™ serum supplied with 10%

DMSO.

7. After centrifugation, aspirate supernatant and resuspend cells as 1 million cells per 1 ml of freezing buffer.
8. Transfer 1 ml of cell suspension into a cryogenic storage vial and place vials into a cell freezing container.
9. Store cell freezing container in -80 °C overnight, and then transfer frozen cells to liquid nitrogen for storage afterward.

G. Recovering frozen ECs

1. Remove the cryovial containing frozen ECs from liquid nitrogen and immediately thaw it in a 37 °C water bath.
2. Thaw the ECs while swirling the cryovial till almost no ice left in the vial.
3. Transfer the ECs into a 15 ml tube with pre-warmed EGM-2 medium (1 ml per vial) and centrifuge the cell suspension at 300 *x g* for 3 min.
4. Resuspend the cell pellets in pre-warmed EGM-2 medium, and transfer ECs into appropriate plates as per experiment plan.

Recipes

1. iPSC Passage medium
   E8 medium plus 10 μM ROCK inhibitor
2. RPMI + B27-INS medium
   RPMI 1640 medium plus B27 supplement minus insulin
3. Sorting Solution MACs buffer
   AutoMACS Rinsing Solution supplemented with MACS BSA Stock Solution (1:20)

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Competing interests

Authors have no conflict of interest or competing interest.

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