Stem Cell Differentiation into Endothelial Cells

This protocol describes the differentiation of human induced pluripotent stem cells (hiPSCs) into endothelial cells (ECs) using a chemically defined, feeder-free differentiation protocol. The procedure begins with undifferentiated hiPSCs cultured on Matrigel-coated plates and concludes with endothelial cell differentiation over an eight-day period. This protocol has been modified from Schnellmann, et. al., Adv.Sci. 2022. https://doi.org/10.1002/advs.202201483

Principle

Undifferentiated hiPSCs are maintained under feeder-free conditions using mTeSR1 on Matrigel-coated culture vessels. Differentiation is initiated by activation of the Wnt signaling pathway using CHIR99021 in Essential 6 medium. Following mesoderm induction, cells are replated onto collagen type I-coated culture vessels and cultured in endothelial growth medium supplemented with VEGF and SB-431542 to promote endothelial specification. A ROCK inhibitor (Y-27632) is included during the first 24 hours after replating to enhance cell survival.

Materials

- Human induced pluripotent stem cells (hiPSCs)
- mTeSR1
- Essential 6 (E6) Medium
- Endothelial Cell Growth Medium (EGM-2)
- Matrigel
- Collagen Type I
- CHIR99021
- SB-431542
- Vascular Endothelial Growth Factor (VEGF)
- Y-27632 (Rocki)
- TrypLE Express
- Tissue culture-treated plates
- Cell culture incubator (37°C, 5% CO_2)
- Biosafety cabinet
- Inverted microscope

Preparation

Prior to differentiation:

- Coat culture vessels with Matrigel according to the manufacturer's recommendations (see ***Culture of Human-induced Pluripotent Stem Cells (hiPSCs)*** protocol).
- Maintain hiPSCs in mTeSR1 medium until cultures reach approximately **80% confluency**.
- Prepare 20ug/mL collagen type I-coated culture plates for endothelial specification.

Procedure

Maintenance of Undifferentiated hiPSCs

1. Culture hiPSCs on Matrigel-coated tissue culture plates.
2. Maintain cells in mTeSR1 medium.
3. Monitor cultures daily to ensure colonies remain undifferentiated and free of spontaneous differentiation.
4. Initiate differentiation once cultures reach approximately **80% confluency**.

Mesoderm Induction (Days 0–2)

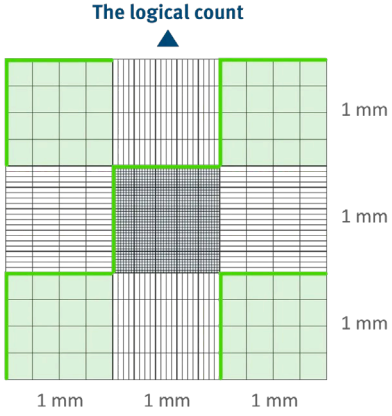
1. Replace mTeSR1 medium with Essential 6 medium supplemented with 10uM CHIR99021.
2. Continue induction for **48 hours**.
3. Replace differentiation medium daily during this period.
4. Monitor cultures for morphological changes associated with early mesoderm commitment.

Cell Harvest and Replating (Day 2)

1. Dissociate cells using TrypLE Express for 5 min.
2. Transfer the cell suspension to a 15 mL conical tube.
3. Take a small aliquot of the cell suspension medium 15uL to count cells using a hemocytometer.

Cells/mL = Average count x Dilution Factor x 10,000

Total number of cells = cell/mL x Volume



4. Calculate the required volume to seed the cells at your desired density (e.g., $2x10^4$ cells per cm^2, or 1,100,000 cells for a 100 mm dish).
5. Centrifuge at 200 x g for 5 minutes at room temperature.
6. Carefully aspirate the supernatant without disturbing the pellet.
7. Gently resuspend cell pellet in an appropriate volume of complete EGM-2 with SB-431542 and VEGF (e.g., 1 mL).
8. Seed cells onto 20ug/mL collagen type I-coated culture plates at a density of: **2×10^4 cells/cm^2**

Endothelial Specification (Days 2–8)

Following replating:

1. Culture cells in Endothelial Cell Growth Medium (EGM-2) supplemented with:
    - 10uM SB-431542
    - 50ng/mL VEGF
2. Include Y-27632 during the first **24 hours** after replating to enhance cell survival.
3. After the initial 24-hour period, discontinue Y-27632 supplementation.
4. Replace the differentiation medium every other day for an additional **6 days**.

Expected Morphological Changes

Throughout differentiation, cells should progress through the following stages:

Differentiation Stage	Expected Morphology
Undifferentiated hiPSCs	Compact colonies with well-defined borders
Mesoderm induction	Loss of colony morphology; increased cell spreading
Early endothelial specification	Cobblestone-like cell clusters begin to appear
Mature endothelial cells	Uniform cobblestone morphology characteristic of endothelial monolayers

Quality Control

Following differentiation, endothelial identity may be evaluated using:

Morphology

- Cobblestone endothelial morphology

Endothelial Marker Expression

Examples include:

- CD31 (PECAM-1)
- VE-Cadherin (CD144)
- CD34
- KDR (VEGFR2)
- eNOS
- vWF

Functional Assays (Optional)

- Acetylated LDL uptake
- Tube formation assay

- Nitric oxide production
- **Flow cytometry**
- **Immunofluorescence staining**

Notes

- Begin differentiation only with healthy, undifferentiated hiPSC cultures.
- Uniform cell confluency at the start of differentiation improves reproducibility.
- Careful monitoring of morphology throughout the differentiation process is recommended.
- Endothelial identity should be confirmed using appropriate phenotypic and functional assays before downstream applications.