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Generation of Cardiomyocytes and Endothelial Cells from Human iPSCs by Chemical Modulation of Wnt Signaling

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Abstract

The generation of cardiomyocytes (CMs) and endothelial cells (ECs) from human induced pluripotent stem cells (iPSCs) allows for precise modeling of cardiovascular disease using clinically relevant and patient-specific cells. Differentiation of human iPSCs into cardiomyocytes (iPSC-CMs) and endothelial cells (iPSC-ECs) is governed by small molecules that regulate the WNT signaling pathway. Here we outline the detailed steps to generate iPSC-CMs and iPSC-ECs through small molecule-mediated monolayer differentiation.

Key words Human induced pluripotent stem cells (iPSCs), Cardiomyocytes, Endothelial cells, iPSC-CMs, iPSC-ECs

1 Introduction

The advancement of using patient-specific iPSCs to differentiate into relevant disease cell types is significant to understanding etiologies of cardiovascular disease [1, 2]. Using human iPSC-based models allows for a more accurate disease representation compared to animal models because there are significant differences in the physiology between animals and humans. Patient derived iPSC-CMs and iPSC-ECs can serve as an indefinite resource for modeling cardiovascular diseases and can be further used to study such diseases at the genetic and molecular levels [1, 3, 4].

Monolayer differentiation of human iPSCs to obtain cardiomyocytes and endothelial cells utilizes small molecules targeting the WNT signaling pathway. Wnt signaling is first activated by a GSK3 inhibitor (CHIR99021) and then inhibited to induce cardiomyocyte differentiation [5]. In contrast, continued Wnt signaling activation directs cardiac progenitor cells into endothelial cell lineage when VEGF/FGF is introduced into the differentiation medium [6]. In this protocol, we describe detailed steps to generate beating cardiomyocytes and endothelial cells from human iPSCs using small molecules [6, 7]. We also present the procedures for

immunofluorescence staining and flow cytometry for the characterization of human iPSC-CMs and iPSC-ECs.

2 Materials

All cell culture media should be prepared in a sterile laminar flow biosafety cabinet. The sources of materials are summarized in Table 1.

Table 1
Sources for materials and reagents

Name	Vendor	Catalog number
B27 supplement	Thermo Fisher Scientific	17504044
B27 minus insulin supplement	Thermo Fisher Scientific	A18956
BD Cytofix/cytoperm fixation/permeabilization kit	BD Biosciences	554714
bFGF	Peprotech	100-18B
CD144 microbeads	Miltenyi Biotec	130-097-857
CHIR99021	Selleckchem	S-2924
DMEM/F12	Thermo Fisher Scientific	11330
DPBS (1×)	Thermo Fisher Scientific	14190
E8 medium kit	Thermo Fisher Scientific	A1517001
EGM-2 medium	Lonza	CC-3162
Gelatin solution, 2% in H_2O	Sigma	G1393
Growth factor reduced matrigel	Corning	356231
IWR-1-endo	Selleckchem	S7086
KnockOut Serum Replacement (KSR)	Thermo Fisher Scientific	10828028
LS column	Miltenyi Biotec	130-042-401
QuadroMACS separator	Miltenyi Biotec	130-091-051
RPMI1640 medium	Thermo Fisher Scientific	11875093
SB431542	LC labs	S-7800
RPMI 1640 medium, no glucose	Thermo Fisher Scientific	11879020
SlowFade Gold Antifade Mountant (mounting media)	Thermo Fisher Scientific	S36936
TrypLE express enzyme (1×)	Thermo Fisher Scientific	12605
TrypLE select enzyme 10×, no phenol red	Thermo Fisher Scientific	A1217703
VEGF	Peprotech	100-20
Y-27632	LC Labs	Y-5301

2.1 Preparation of Matrigel-Coated Plates

Thaw a bottle of Matrigel at 4 °C overnight. Add 1 mL of Matrigel solution to 300 mL of DMEM/F12 media (1:300 dilution). Mix them thoroughly. Apply 2 mL of the diluted Matrigel solution into each well of a 6-well plate. Incubate in a 5% CO_2 incubator at 37 °C for at least 30 min before use.

2.2 Preparation of Cardiomyocyte Differentiation Media

1. CM Media I: Mix 500 mL of RPMI1640 medium with 10 mL of B27 minus insulin supplement (50×).
2. CM Media II: Add 60 μL of CHIR99021 stock (10 mM in DMSO) to 100 mL of CM Media I. The final concentration of CHIR99021 is 6 μM. Mix thoroughly.
3. CM Media III: Add 50 μL of IWR-1 stock (10 mM in DMSO) to 100 mL of CM Media I. The final concentration of IWR-1 is 5 μM. Mix thoroughly.
4. CM Media IV: Mix 500 mL of RPMI1640 medium with 10 mL of B27 supplement (50×).
5. CM Media V: Mix 500 mL of RPMI1640 medium (no glucose) with 10 mL of B27 supplement (50×).
6. CM Passaging Media: Add 10 mL of knockout serum replacement (KSR) to 90 mL of CM Media IV. The final concentration of KSR is 10%. Mix thoroughly.

2.3 Preparation of Endothelial Differentiation Media

1. EC Media I: Add 60 μL of CHIR99021 stock (10 mM in DMSO) to 100 mL of CM Media I. The final concentration of CHIR99021 is 6 μM. Mix thoroughly.
2. EC Media II: Add 30 μL of CHIR99021 stock (10 mM in DMSO) to 100 mL of CM Media I. The final concentration of CHIR99021 is 3 μM. Mix thoroughly.
3. EC Media III: Add 50 μL of VEGF stock (concentration: 100 μg/mL; final concentration: 50 ng/mL), 25 μL of bFGF stock (concentration: 100 μg/mL; final concentration: 25 ng/mL), and 100 μL of SB431542 stock (concentration: 10 mM in DMSO; final concentration: 10 μM) to 100 mL of EGM-2 medium. Mix thoroughly.
4. EC Media IV: Add 25 μL of VEGF stock (concentration: 100 μg/mL; final concentration: 25 ng/mL) and 100 μL of SB431542 stock (concentration: 10 mM in DMSO; final concentration: 10 μM) to 100 mL of EGM-2 medium. Mix thoroughly.

2.4 Preparation of Gelatin-Coated Plates

Dilute 2% gelatin in DPBS (1:10) and filter by a 0.2 μm filter. Apply 2 mL of 0.2% gelatin solution to each well of a 6-well plate. Incubate in a 5% CO_2 incubator for 30 min before use.

2.5 Preparation of Solutions for Immunofluorescence Staining

1. Fixation solution (4% paraformaldehyde, PFA): Dilute 16% PFA to 4% PFA using PBS (1:4 dilution). Mix well.
2. Permeabilization solution (0.1% Triton X-100): Add 1 mL of 10% Triton X-100 into 99 mL of PBS. Mix well.
3. Blocking solution (0.2% BSA): Dissolve 0.2 g of BSA in 100 mL of PBS. Wait until BSA is completely dissolved. Filter through a 0.2 μm filter in a sterile hood. Store at 4 °C.

2.6 Preparation of Solutions for Flow Cytometry

1. FACS buffer: Add 4 mL of FBS to 96 mL of PBS. Filter through a 0.2 μm filter in a sterile hood. Store at 4 °C.

3 Methods

3.1 Chemically Defined Cardiomyocyte Differentiation

3.1.1 Cardiomyocyte Differentiation from Human iPSCs

1. Culture human iPSCs in complete E8 media in a Matrigel-coated 6-well plate. Adjust the seeding density so that they can reach 90–95% confluent in 3–4 days (Fig. 1a).
2. Remove old media and add 2 mL of CM Media II to each well (*see* **Note 1**). This serves as day 0 (D0). Do not touch on D1.
3. On D2, replace old media with 2 mL of CM Media I.
4. On D3, replace old media with 2 mL of CM Media III.
5. On D5, replace old media with 2 mL of CM Media I.
6. On D7, replace old media with 2 mL of CM Media IV. Repeat on D9 and D11 as well.

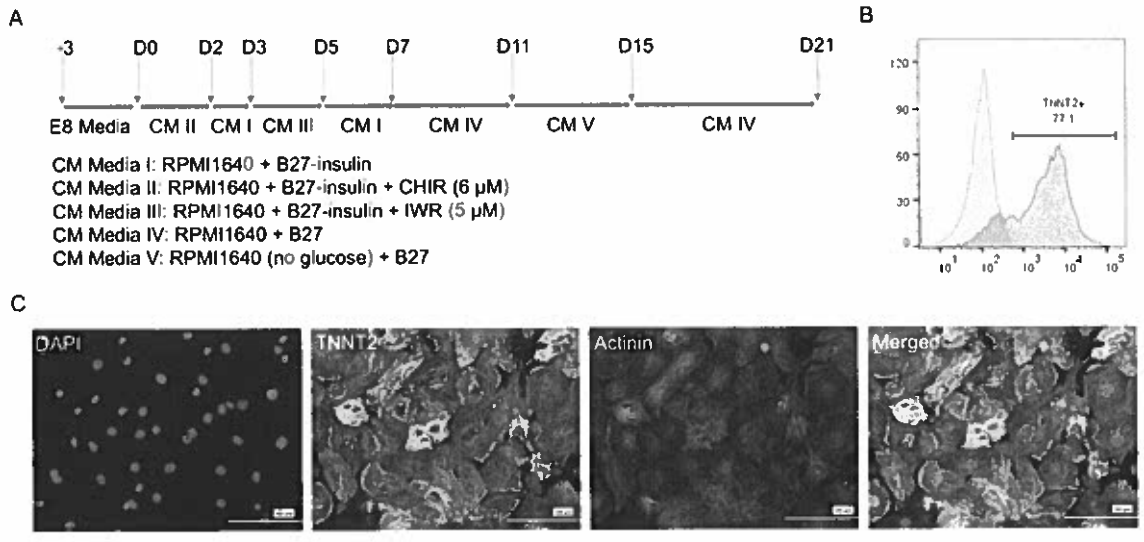

Fig. 1 Cardiac differentiation of human iPSCs by sequential modulation of Wnt signaling. (**a**) Schematic overview of cardiomyocyte differentiation. (**b**) FACS analysis of human iPSC-CMs using an anti-cardiac troponin T antibody. (**c**) Sarcomere structure of human iPSC-CMs revealed by TNNT2 and a-actinin staining. Scale bars: 100 μm

7. On D13, when contracting cells are observed, replace old media with 2 mL of CM Media V. Replace with 2 mL of fresh CM Media V on D15 (*see* **Note 2**). Media should turn pink during this process because cells are going through a glucose starvation phase (*see* **Note 3**).
8. On D17, replace old media with 2 mL of CM Media IV.
9. From D19 onward, replace old media with 2 mL of CM Media IV every other day.

3.1.2 Passage Human iPSC-CMs

1. Remove old media and rinse each well with 3 mL of DPBS (*see* **Note 4**).
2. Add 1 mL of TrypLE Select Enzyme (10×) to each well and incubate in 5% CO_2 at 37 °C for 5–8 min to dislodge cells.
3. Mechanically dissociate iPSC-CMs into single cells by pipetting vigorously with a 1000-μL pipette.
4. Transfer cells to a 15-mL conical tube and add 2× volumes of CM Passaging Media to neutralize TrypLE enzyme.
5. Spin at 300 × *g* for 5 min at room temperature (RT).
6. Discard the supernatant and resuspend in 4 mL of CM Passaging Media to allow for thorough resuspension. Seed cells into 2 wells of a Matrigel-coated 6-well plate. Refresh the media every other day with CM Media IV. Cardiomyocytes should resume beating in 1–3 days post passage (*see* **Note 5**).

3.2 Chemically Defined Endothelial Cell Differentiation

1. Culture human iPSCs in complete E8 media in a Matrigel-coated 6-well plate until 95% confluent (3–4 days post passage) (Fig. 2a).
2. Remove the old media. Add 2 mL of EC Media I to each well of a 6-well plate. This is D0. Do not touch on D1.
3. On D2, replace with 2 mL of EC Media II. Do not touch on D3.
4. On D4, replace with 2 mL of EC Media III. Refresh every other day thereafter.
5. On D12, remove the old media and rinse cells with 3 mL of DPBS each well once.
6. Add 1 mL TrypLE Express Enzyme to each well of the 6-well plate and incubate in 5% CO_2 at 37 °C for 5 min.
7. Make single-cell suspension using a 1000-μL pipette to dislodge cells (*see* **Note 6**).
8. Apply the above cell suspension to a 40-μm cell strainer on top of a 50-mL conical tube pre-loaded with 5 mL of EGM-2 media.
9. Count cells using a Bio-Rad TC20 automated cell counter.

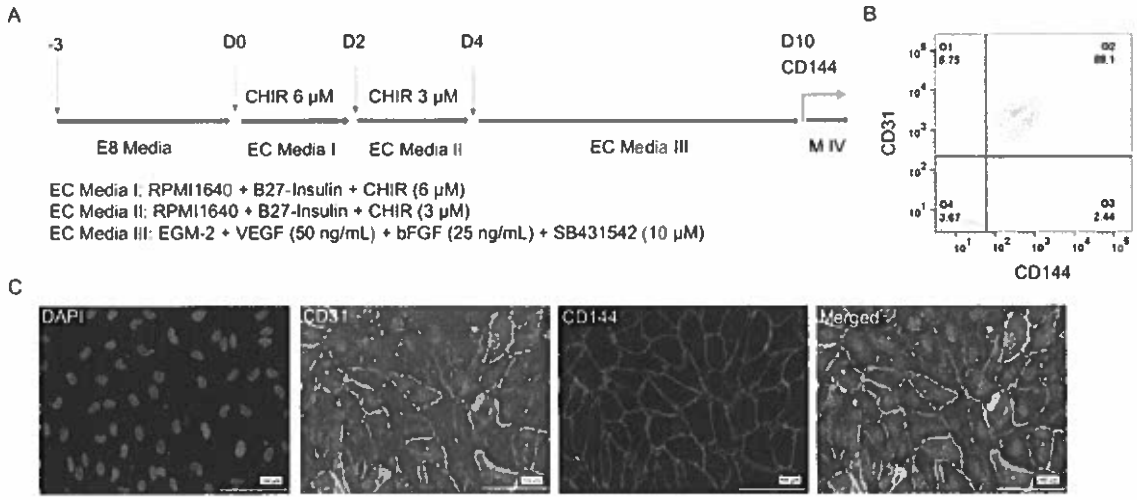

Fig. 2 Small chemical induction of vascular endothelial differentiation of human iPSCs. (**a**) Schematic overview of vascular endothelial differentiation by small chemicals. (**b**) FACS analysis of human iPSC-ECs using anti-CD31 and anti-CD144 antibodies. (**c**) Endothelial markers CD31 and CD144 are expressed in human iPSC-ECs. Scale bars: 100 μm

10. Centrifuge the cell suspension at 300 × *g* for 5 min. Discard the supernatant.
11. Resuspend cell pellets in 80 μL of MACS wash buffer per 10^7 live cells.
12. Add 20 μL of CD144 (VE-Cadherin) microbeads per 10^7 cells (*see* **Note 7**). Mix and incubate for 15 min at a 4 °C refrigerator.
13. Wash cells by adding 1 mL of MACS wash buffer per 10^7 cells and centrifuge at 300 × *g* for 5 min. Discard the supernatant.
14. Resuspend cell pellets (up to 10^8 cells) in 500 μL of MACS wash buffer.
15. Place an LS column in the magnetic field of a suitable MACS Separator and follow the instructions.
16. Rinse the column with 3 mL of MACS wash buffer.
17. Apply cell suspension to the column and let the cells pass through gravity (*see* **Note 8**).
18. Add 3 mL of MACS wash buffer to the column when the column reservoir is empty. Repeat twice.
19. Remove the column from the separator and place it in a collection tube.
20. Add 5 mL of MACS wash buffer into the column. Immediately flush out cells by firmly pushing the plunger into the column. This step must be performed without the magnetic field (*see* **Note 9**).

21. Centrifuge cell suspension at 300 × *g* for 5 min. Discard the supernatant.
22. Resuspend cells with the EC Media IV and seed them onto a new plate pre-coated with 0.2% gelatin. Refresh the media every other day (*see* **Note 10**).

3.3 Immunofluorescence Staining

1. Before immunofluorescence staining, seed human iPSC-CMs onto Matrigel-coated coverslips that are placed in a 24-well plate (seeding density: 0.5–1 × 10^6 cells/mL). Refresh with CM Media IV every other day.
2. After a week of maintaining iPSC-CMs, wash cells using 1 mL of PBS. Repeat once.
3. Add 0.5 mL of fixation solution (4% paraformaldehyde, PFA) and incubate for 15 min at RT.
4. Remove the fixation solution. Wash cells using 1 mL of PBS. Repeat once.
5. Add 0.5 mL of permeabilization solution (0.1% Triton X-100) and incubate for 20 min at RT (*see* **Note 11**).
6. Remove the permeabilization solution. Wash cells using 1 mL of PBS. Repeat once.
7. Add 0.5 mL of blocking solution (0.2% BSA in PBS). Incubate at RT for 1 h with shaking.
8. Add 200 μL of a primary antibody diluted with the blocking solution (1:500). Incubate at 4 °C overnight on a shaker.
9. Remove the primary antibody. Wash cells using 0.5 mL of blocking solution for 3 min with shaking. Repeat twice.
10. Add 200 μL of a secondary antibody diluted in the blocking solution (1:1000). Incubate at RT for 1 h with shaking.
11. Remove the secondary antibody. Rinse cells with 0.5 mL of PBS for 3 min with shaking. Repeat twice.
12. Counterstain the nuclei with DAPI (1:1000 dilution in PBS) and incubate for 5 min at RT.
13. Remove the DAPI solution. Rinse cells with 0.5 mL of PBS. Repeat once.
14. Mount the coverslips onto a microscope slide using 5 μL of the mounting media. Store at 4 °C and protect from light (*see* **Note 12**).

3.4 Flow Cytometry

1. Wash human iPSC-CMs with 3 mL of DPBS once.
2. Add 1 mL of TrypLE Select Enzyme (10×) per well of a 6-well plate. Incubate in 5% CO_2 at 37 °C for 7 min.
3. Do not discard the dissociation enzyme at this moment. Dislodge cells using a 1000-μL pipette. Transfer cell suspension to

a round FACS tube through a strainer cap. The FACS tube is pre-filled with 1 mL of CM passaging media (10% KSR) to neutralize the enzyme activity.

4. Spin at 300 × *g* for 5 min.
5. Remove the supernatant without disturbing cell pellets. Add 250 µL of the Fixation/Perm solution. Incubate for 20 min at 4 °C (*see* **Note 13**).
6. Add 1 mL of Perm/Wash buffer. Vortex briefly and spin at 300 × *g* for 4 min.
7. Discard the supernatant. Add 100 µL of diluted primary antibodies (1:200 to 1:500) in 1× Perm/Wash buffer. Vortex briefly and incubate overnight at 4 °C.
8. Wash cells by adding 1 mL of 1× Perm/Wash buffer. Vortex briefly and spin at 300 × *g* for 4 min.
9. Discard the supernatant. Add 100 µL of diluted secondary antibodies (1:500 to 1:1000) in Perm/Wash buffer. Vortex briefly and incubate at RT for 1 h. Protect from light if secondary antibodies are conjugated with light-sensitive fluorescence.
10. Wash cells by adding 1 mL of 1× Perm/Wash buffer. Vortex briefly and spin at 300 × *g* for 4 min.
11. Discard the supernatant. Resuspend cells with 400 µL of FACS buffer (4% FBS in PBS). Store at 4 °C until loading to a FACS instrument (*see* **Note 14**).

4 Notes

1. To increase chances of producing contracting cardiomyocytes, continual differentiation is encouraged. When a 6-well plate of iPSCs are confluent, use 1 well for passaging into a new Matrigel-coated 6-well plate. The remaining 5 wells are used for cardiac differentiation. Repeat the same procedure for the next 6-well plate. This should be repeated for 5 times for each iPSC line to obtain the maximal cardiomyocyte production.
2. Cells usually begin beating around D8–12 after CM Media IV is introduced. However, the dates of onset beating vary among different iPSC lines. If beating is not observed after D15, the plate should be discarded as beating is unlikely thereafter.
3. It is possible that beating cardiomyocytes are not produced after going through this differentiation protocol. Continual differentiation will guarantee the production of contracting cardiomyocytes for most iPSC lines.

4. It is recommended to replate iPSC-CMs around D22–25 when they recover from glucose starvation. The replating process will facilitate the purification of iPSC-CMs and eliminate dead cells.
5. After replating iPSC-CMs into new wells, they usually resume beating in 2–3 days. If they don't beat or non-cardiomyocytes take over the majority, these wells of cardiomyocytes should be discarded.
6. Do not remove or neutralize the dissociation enzyme at this step. Dislodge cells in TrypLE solution until they are completely dissociated into single-cell suspension. Otherwise, large cell clumps could clog the column during MACS sorting.
7. The volume of microbeads should be calculated based on the number of live cells. Dead cells should not be included. Live and dead cells can be easily distinguished using Trypan Blue staining.
8. Do not apply any external pressure to the column. Cell suspension should go through the column smoothly by gravity. External pressure can damage the column and significantly reduce the efficiency of MACS sorting.
9. The MACS separator should be removed out of the MACS sorting environment. Any magnetic field can interfere the final elution of CD144+ iPSC-ECs.
10. Human iPSC-ECs are usually pure in passage 1 after MACS sorting (Fig. 2b). However, the percentage of CD31+ CD144+ iPSC-ECs can substantially decrease with extensive passaging due to spontaneous endothelial-to-mesenchymal transition. TGF-β inhibitor SB431542 can prevent and delay this transition, but can not completely block this process [8].
11. For immunofluorescence staining targeting cell membrane proteins such as CD31 and CD144, permeabilization is not required.
12. For human iPSC-CMs, intercalated distribution of cardiac troponin T (TNNT2) and α-actinin can be visualized in the sarcomere unit (Fig. 1c). For human iPSC-ECs, cell surface markers CD31 and CD144 are expressed (Fig. 2c).
13. For FACS targeting cell membrane proteins such as CD31 and CD144, steps for fixation and permeabilization are optional.
14. Examples of FACS analysis in human iPSC-CMs and iPSC-ECs are shown in Figs. 1b and 2b, respectively.

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