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Directed cardiomyocte generation from human pluripotent stem cells using a chemically defined protocol (GiWi2)

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We use this protocol and it's working

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Abstract

The GSK3 inhibitor and Wnt inhibitor, named GiWi2, are utilized for robust differentiation of human pluripotent stem cells (hPSCs) into cardiomyocytes under serum-free and growth factor-free conditions. This differentiation protocol, developed previously by Dr. Xiaojun Lian (Nat Methods. 2015 Jul; 12(7): 595–596), applies two small molecules at precise developmental stages to sequentially promote mesoderm formation and ventricular cardiomyocyte (vCM) specification. The protocol produces 88–98% cTnT+ cells with yields exceeding 1×10^6 cardiomyocytes/cm² across multiple human embryonic stem cell (hESC) and human induced pluripotent stem cell (hiPSC) lines. The resulting cardiomyocytes exhibit spontaneous contraction for over eight months, demonstrating their functionality and long-term viability.

Materials

1. **Matrigel** (*life science, cat# 354230*)
2. **Y27632** (*Tocris, cat# 1254*), 5 mM Y27632 stock (1:1000)
3. **TeSR-E8** (*Stem Cell Technologies, cat# 05990*): Thaw TeSR-E8 25X Supplement at room temperature (15-25°C) or overnight at 2 - 8°C. Add 20 mL of TeSR-E8 25X Supplement to 480 mL of TeSR-E8 Basal Medium. Mix thoroughly. Store complete medium at 2 - 8°C for up to 2 weeks.
4. **Accutase** (*Innovative Cell Technology, cat# AT104)*
5. **CHIR99021** (*Selleckchem, cat# S1263-25mg*): 10 mM CHIR99021 stock
6. **IWP2** (*Tocris, cat# 3533-10mg*): 5 mM IWP2 stock
7. **Insulin** (*Sigma, cat# 91077C-250MG*): 5 mg/mL in stock (1:500)
8. **RPMI** (*Life Technologies, cat# 11875-085*)
9. **B-27 Minus Insulin** (Gibco, *cat# A18956-01*), 50X for RPMI-B27 [RPMI1640 (Life Technologies); 2% B-27 Supplement Minus Insulin (B27-Insulin)]
10. **L-Ascorbic acid 2-phosphate sesquimagnesium** (*Sigma, cat# A8960-5G*): 50 mg/mL in stock (1:250)
11. **Cardiomyocyte Maintenance Medium (CMM)**: RPMI1640 supplemented with 10 µg/mL of insulin and 200 µg/mL of L-Ascorbic acid 2-phosphate sesquimagnesium. Add 0.5 mL of insulin stock (5 mg/mL) and 1 mL of ascorbic acid (50 mg/mL) to 250 mL of RPMI1640 basal medium. Sterilize by filtration and store at 4°C.
12. **Freezing medium (2X)**: 20% DMSO, 60% defined FBS, 20% hES cell culture medium, and need to add fresh 10 µM Y27632 (ROCKi).

Materials

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Feeder-free culture of hPSCs

2 Prepare a Matrigel-coated 6-well plate and store it at 4°C, put it at room temperature at least 1 hour before use.

3 Remove a frozen cell vial of hPSCs, from a liquid N_2 tank and immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently for 2 - 3 min until the contents are almost completely thawed.

4 Spray the vial with 70% (v/v) ethanol and place it in a tissue culture hood. Use a sterile 1-mL pipette to gently transfer the cells into a sterile 15-mL conical tube containing 5 mL of room temperature mTeSR1 medium. Rinse the cell tube a few times without disturbing "little" cell clusters.

5 Centrifuge the cells at 200 g for 5 min at room temperature. Aspirate and discard the supernatant with a sterilized electronic pipette. Resuspend the cell pellet in 2 mL of TeSR-E8

medium(with 5 µM ROCKi), and gently resuspend the cells without disturbing "little" cell clusters.

6 Aspirate the liquid from the wells of the Matrigel-coated plate.

7 Slowly add the cell suspension to the Matrigel-coated 6-well plate. Put the plate back into the 37°C, 5% CO_2 incubator.

8 The next day, aspirate the medium in each well and replace it with 2 mL of fresh room temperature mTeSR-E8 medium (without ROCKi). Repeat this medium replacement daily until cells are ready for passage.

9 Passage the cells with EDTA-method: Asprite the medium and wash once with DPBS. Add 1 mL 0.5 mM EDTA-DPBS buffer to each well to dissociate the cells. Incubate for 3 - 5 min at 37°C and observe with microscopy. Asprite the EDTA buffer. Add 1 mL TESR-E8 with Rock-inhibitor (1:1000) to the cells and gently detach them by pipetting them up and down. Transfer the cells to a 15 mL tube with 5 mL TESR-E8 (with ROCKi), and rinse the well with 1 mL medium. Add the cells to a Matrigel-coated plate (6 wells).

Differentiatie hPSCs into Cardiomyocytes

10 **(Day -2) Passaging hPSCs using Accutane:**

10.1 Take hPSCs cultured on Matrigel-coated 6-wells in mTeSR1 medium at 80-90% confluence. Aspirate medium and wash once with DPBS. Add 1 mL of room-temp Accutase to each 6-well. Place plate in 37°C, 5% CO_2 incubator for ~5 min.

10.2 Add 0.5 mL mTeSR1 to each well and gently detach the cells by pipetting the cells up and down and pool the cells in a 15-mL conical tube. Count total cells and centrifuge at 200 g for 5 minutes at room temp.

10.3 Aspirate supernatant, resuspend cells in TeSR-E8 + 5 µM Y27632, and plate 200 k cells/cm^2 (i.e. ~700 k cells/3.5 cm^2 for 12-well) onto Matrigel-coated plate. Add TeSR-E8 + 5 µM Y27632 medium to each well to obtain a final volume of 1 mL in each well of the 12-well plate. Incubate the cells. Move the plate in quick, short, back-and-forth, and side-to-side motions to disperse the cells across the surface of the wells.

Note

1) This time point corresponds to Day -2 of differentiation.
2) Seeding density is crucial: recommended cell density by day 0 should be 250-400 k cells/cm^2.

11 **Day -1 Maintenance:** On day -1 of differentiation, aspirate the medium and replace it with 2 mL of room temperature TeSR-E8 per well of the 12-well plate.

12 **Day 0 Add GSK3 inhibitor CHIR99021:** On day 0 of differentiation, prepare 2 mL of 6 µM CHIR99021 in RPMI-B27 medium for each well of 12-well. Aspirate the old medium and add 2 mL of this mixture to each
well. **Record the time, as the medium needs to be changed exactly 24 hours later.**

13 **Day 1 Remove GSK3 inhibitor CHIR99021.** After 24 h, aspirate medium from each well and replace with 2 mL of room-temp RPMI-B27. Put the plate back into 37°C, 5% CO_2 incubator.

Note

If a lot of cell death/detachment is observed, which might be less than the optimal initial seeding density or too much CHIR (concentration vs time).

14 **Day 3 add Wnt inhibitor IWP2:** On day 3 of differentiation, prepare the combined medium by using a 5 mL pipette to collect 1 mL of medium from the 12-well plate; mix it with 1mL of fresh RPMI-B27 medium in a 15 mL conical. Add 1 µl of 5 mM IWP2 (final conc. is 2.5 µM) into the 2 mL of combined medium. Before aspirating 1 mL of the remaining medium in the well of the 12-well plate, gently rock the plate back and forth to get cell debris into suspension, ensuring that the cell debris will be discarded via aspiration. Aspirate the remaining 1 mL of medium from each well of the 12-well plate, and then add 2 mL per well of the combined medium containing IWP2 to each well and incubate the cells.

15 **Day 5 Remove Wnt inhibitor IWP2:** On day 5 of differentiation, aspirate the medium from each well of the 12-well plate and add room-temperature RPMI-B27 medium at a volume of 2 mL per well. Put the plate back into the 37°C, 5% CO_2 incubator.

16 **Day 7 Change medium with CMM:** On day 7 of differentiation and every 2 days thereafter, aspirate the medium from each well of the 12-well plate and add room-temperature RPMI medium supplemented/filtered together with B27(with insulin) and 10 µg/mL insulin and 200 µg/mL L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate at a volume of 2 mL per well. Put the plate back into the 37 C, 5% CO_2 incubator.

17 Robust spontaneous contraction should occur by day 12. The cells can be maintained with this spontaneously beating phenotype for more than 6 months.

References

18 1. Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. Nat Protoc. 2013 Jan;8(1):162-75. doi: 10.1038/nprot.2012.150. Epub 2012 Dec 20. PMID: 23257984; PMCID: PMC3612968.

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