Chemically Defined Culture and Cardiomyocyte Differentiation of Human Pluripotent Stem Cells

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Since the first discovery that human pluripotent stem cells (hPS cells) can differentiate to cardiomyocytes, efforts have been made to optimize the conditions under which this process occurs. One of the most effective methodologies to optimize this process is reductionist simplification of the medium formula, which eliminates complex animal-derived components to help reveal the precise underlying mechanisms. Here we describe our latest, cost-effective and efficient methodology for the culture of hPS cells in the pluripotent state using a modified variant of chemically defined E8 medium. We provide exact guidelines for cell handling under these conditions, including non-enzymatic EDTA passaging, which have been optimized for subsequent cardiomyocyte differentiation. We describe in depth the latest version of our monolayer chemically defined small molecule differentiation protocol, including metabolic selection–based cardiomyocyte purification and the addition of triiodothyronine to enhance cardiomyocyte maturation. Finally, we describe a method for the dissociation of hPS cell–derived cardiomyocytes, cryopreservation, and thawing. © 2015 by John Wiley & Sons, Inc.

Keywords: human induced pluripotent stem cells • differentiation • cardiac • cardiomyocyte • chemically defined • monolayer

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INTRODUCTION

This unit describes methods for the culture and cardiac differentiation of human pluripotent stem cells (hPS cells), which include human embryonic stem cells (hES cells) and human induced pluripotent stem cells (hiPS cells) (Burridge et al., 2014). In the Basic Protocol of this unit, hiPS cells are grown in a modified version of chemically defined E8 medium on low-density (1:400) Matrigel. E8 medium is changed every day, and the cells are grown for 3 to 4 days, by which time they become 65% to 85% confluent; afterwards, cells are either passaged or differentiated. Cells are passaged non-enzymatically using EDTA, and a Rho-associated protein kinase inhibitor (10 μM Y27632) is used for 24 hr after splitting to improve cell survival and split ratio reliability and to reduce selective pressure. Cells are passaged at a 1:15 split ratio (equal to seeding densities of ~1.25×10^4 cells per cm^2). The timing for splitting and subsequent 3 to 4 days of growth are crucial to the efficiency of the protocol.

Characterization of the resultant cardiomyocytes is described in two support protocols: Support Protocol 1 describes characterization of the cells by flow cytometry, while Support Protocol 2 describes their characterization via immunofluorescent staining.

BASIC PROTOCOL

CULTURE AND CARDIOMYOCYTE DIFFERENTIATION OF HUMAN iPS CELLS

In this protocol, human iPS cells are differentiated as a monolayer, which eliminates embryoid body formation variability (Burridge et al., 2007) but requires careful control of pluripotent cell seeding density. Differentiation towards cardiomyocyte lineage is induced using small molecules to modulate the WNT signaling pathway, first with a GSK3B inhibitor to potentiate WNT signaling and then 2 days later with a WNT inhibitor to attenuate WNT signaling (Gonzalez et al., 2011; Lian et al., 2012; Burridge et al., 2014). The basic culture medium used throughout cardiac differentiation and cardiomyocyte maintenance is CDM3 (Burridge et al., 2014), a chemically defined medium consisting of RPMI 1640, rice-derived recombinant human albumin, and L-ascorbic acid 2-phosphate. This protocol is designed to be very simple and cost effective. It is highly reproducible, and has been shown to generate ~80% to 95% $TNNT2^+$ cells in >200 hiPS cell lines that we have tested. We have empirically demonstrated that higher splitting ratios (lower seeding densities) result in higher $TNNT2^+$ cell yields. At day 0, the medium is changed to CDM3-C containing 6 μM CHIR99021 (GSK3B inhibitor). After 48 hr (day 2), the medium is changed to CDM3-C59 containing 2 μM Wnt-C59 (WNT inhibitor). The medium is then replaced with CDM3 every other day, and contracting cells will be seen from day 8 to day 9. From day 10 to day 16, CDM3 is replaced with CDM3-L (with no D-glucose but with L-lactic acid) to metabolically select and purify cardiomyocytes (Tohyama et al., 2013). From day 20 to day 30, CDM3 is replaced with CDM3-T (with triiodothyronine) to enhance cardiomyocyte maturation (Yang et al., 2014). Beating cardiomyocytes can be maintained indefinitely in CDM3 (>6 months). The time course of pluripotent growth and cardiac differentiation is shown in Figure 21.3.1.

The major optimizable factors are the seeding density (1:12 to 1:20), the number of days of pluripotent growth (3 or 4 days), and the narrow range at which the CHIR99021 is effective in this system (5 to 7 μM). A simple experiment is to seed the cells at densities of 1:12, 1:15, and 1:20, treat them with 5 μM, 6 μM, or 7 μM of CHIR99021 during the day 0 to day 2 window, and assess for cell survival and epithelial to mesenchymal transition. We found only a minimal influence of modification of the later steps on differentiation.

Materials

Human induced pluripotent stem cells (hiPS cells; see information in step 1)
E8-Y medium (see recipe)
E8 medium (without Y27632; see recipe)
0.5 mM EDTA (see recipe)
CDM3-C (with CHIR99021; see recipe)
CDM3-C59 (with Wnt-C59; see recipe)
CDM3-L (without D-glucose, with L-lactic acid; see recipe)
Dulbecco's phosphate-buffered saline without Ca or Mg (CMF-DPBS)
TrypLE Express (Life Technologies, cat. no. 12605-036)
Liberase TH, 260 U/50 mg (Roche, cat. no. 05401151001), resuspend in 10 ml WFI water (Corning, cat. no. 25-055-CV) and make 500-μl aliquots; store at −20°C
DNase I, 277 U/μl (Life Technologies, cat. no 18047-019)
CDM3 (see recipe)
Fetal bovine serum (FBS; Life Technologies, cat. no. 10082-147)

Dimethylsulfoxide (DMSO; Fisher Scientific, cat. no. BP231-1)
Liquid N_2
CDM3-T (with T_3; see recipe)

15-ml (Corning Falcon, cat. no. 352097) and 50-ml (Corning Falcon, cat. no. 352098) polystyrene conical tubes
Matrigel-coated (see recipe) 6, 12, 24, 96, 384-well cell culture plates (Greiner, cat. no. 657160, 665180, 662160, 655090, 781091, respectively)
Centrifuge (e.g., Thermo Sorvall ST8)
100-μm cell strainer (Corning Falcon, cat. no. 352360)
Luna Automated Cell Counter (Logos Biosystems, cat. no. L20001)
Cyrovials (Greiner, cat. no. 122261)
Coolcell LX (Biocision, cat. no. BCS-405)

Additional reagents and equipment for generating hiPS cells [*UNIT 4A.1* (Park and Daley, 2009) and *UNIT 4A.2* (Ohnuki et al, 2009)]

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations are carried out in a humidified 37°C, 5% CO_2 incubator (Thermo Scientific Heracell VIOS) unless otherwise specified. We have found that 5% O_2 (hypoxic) incubators are not essential for the success of this protocol.

NOTE: We do not place any media in a 37°C water bath before use due to concerns regarding the temperature stability of the FGF2 in the media (Chen et al., 2011). Bringing the media to room temperature is sufficient, and we have found no noticeable effects on cell growth by using 4°C media.

Thawing and initial plating of hiPS cells

1. Generate hiPS cells in-house (see Park and Daley, 2009; Ohnuki et al., 2009) or obtain from reputable sources such as WiCell (*http://www.wicell.org/*) or Stanford CVI Biobank (*http://med.stanford.edu/scvibiobank.html*).

   Also see McKernan and Watt (2013) and Marx (2015).

2. Remove the vial from liquid nitrogen and place it in a 37°C water bath until only a sliver of ice remains. Fill a 10-ml pipet with E8-Y medium, and use this to remove the contents of the vial: transfer ~5 ml to a 15-ml conical tube, wash out vial with ~1 ml of medium, and transfer the remainder to the conical tube.

   The addition of Y27632 ROCK inhibitor in the E8-Y medium improves cell survival after dissociation, which enhances the consistency of plating.

3. Centrifuge 4 min at 200 × *g*, room temperature. Aspirate supernatant. Resuspend in 4 ml of E8-Y and transfer to 2 wells of a Matrigel-coated 6-well plate.

4. Change medium every 24 hr with E8 (without Y27632).

Passage of hiPS cells with EDTA

Ideally, cells should have reached 65% to 85% confluence in 3 to 4 days (adjust split ratio ~1:12 to 1:20 to achieve this, as higher split ratios result in more efficient differentiations).

5. Aspirate culture medium.

6. Add 1 ml per well of 0.5 mM EDTA, and incubate for 6 min at room temperature (in sterile hood).

7. Aspirate EDTA from well.

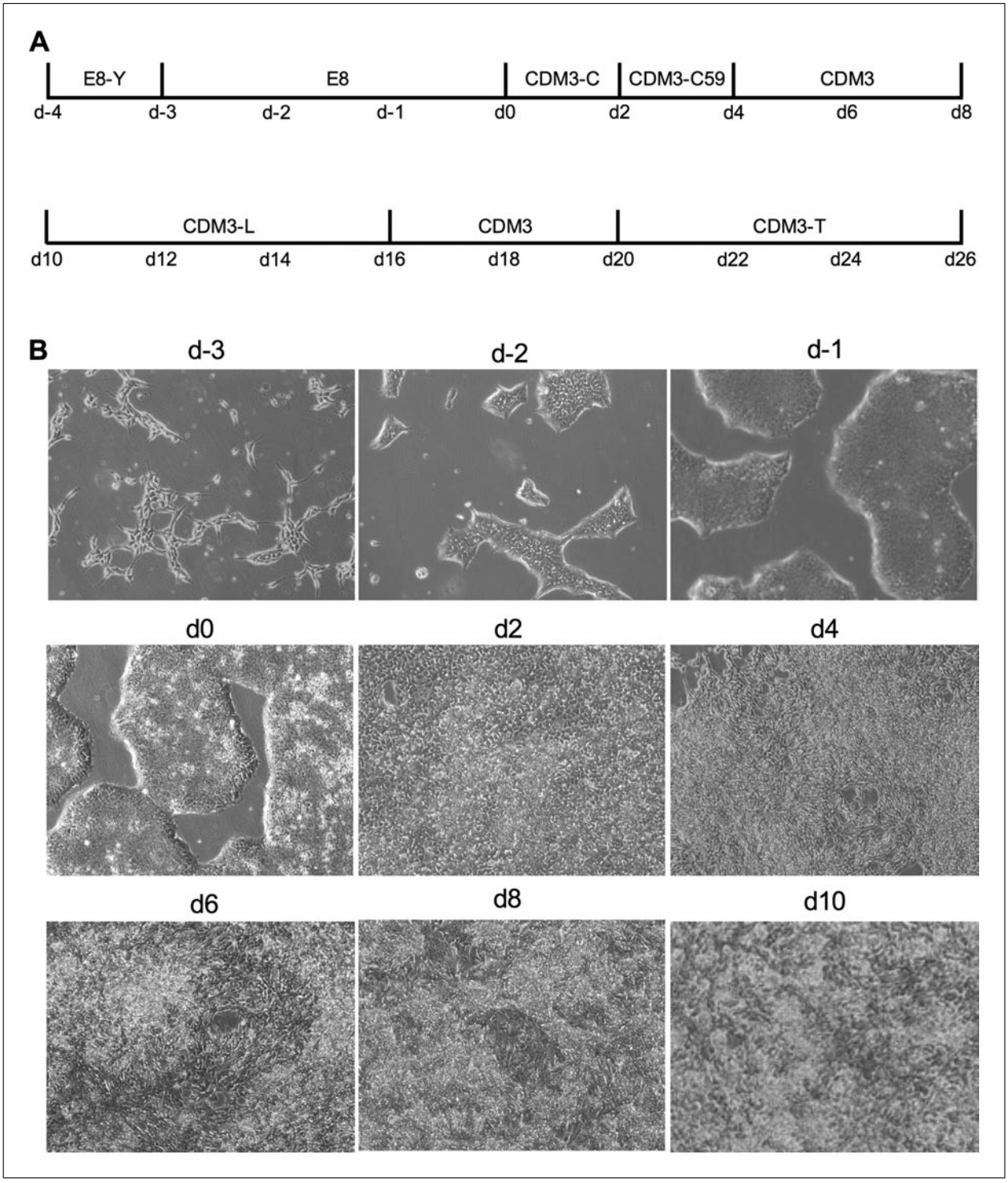

Figure 21.3.1 Human pluripotent growth and cardiac differentiation. (**A**) Time-course of pluripotent growth and subsequent cardiac differentiation showing the medium and small molecules used in each day (d) of differentiation. (**B**) Representative images of hiPS cells seeded at a 1:15 split ratio in E8, followed by differentiation using the CDM3 protocol.

8. With a P-1000 (1-ml) pipet tip, add 1 ml of E8-Y medium to the well, and blast medium against cell surface to dissociate cells (cells should come off easily after pipetting in this way ~5 times). For a 1:15 split, remove 200 µl and discard; for a 1:20 split, remove 400 µl and discard. Top up well to 12 ml with E8-Y.
9. Plate out cells at 1 ml per well into two new Matrigel-coated 6-well plates and top up each well to 2 ml with E8-Y.

In this protocol, we aim to keep the pluripotent cells in the logarithmic growth phase. Cells should not be allowed to become more than 85% confluent (i.e., 85% of the culture surface covered with cells). This prevents cells from becoming contact inhibited, which would result in a slow lag-phase growth after passaging. E8 is less well buffered than other media, and in cells that are overgrown (i.e., >100% confluence), cell death is noted rather than spontaneous differentiation.

Day 0: Beginning differentiation with CDM3-C (with CHIR99021)

10. Aspirate medium from wells.
11. Add 2 ml of CDM3-C.

    Significant cell death may be noted (Fig. 21.3.1). hiPS cells that undergo epithelial to mesenchymal transition at d2 will result in higher cardiomyocyte yields than those that maintain an epithelial morphology (Fig. 21.3.1).

Day 2: Change to CDM3-C59 (with Wnt-C59)

12. Aspirate medium from wells.
13. Add 2 ml of CDM3-C59.

Days 4, 6, and 8: Change to CDM3

14. Aspirate medium from wells.
15. Add 2 ml of CDM3.

Days 10, 12, 14: Change CDM3-L (without D-glucose, with L-lactic acid)

16. Aspirate medium from wells.
17. Add 2 ml of CDM3-L.

    At least 4 days of metabolic selection are required. Some non-cardiomyocyte cell types can survive metabolic selection. Cease metabolic selection if cardiomyocyte death is noted.

Day 15: Dissociation of cardiomyocytes

18. Aspirate medium from wells.
19. Wash cells three times with CMF-DPBS to remove calcium and inhibit contraction.
20. Add 1 ml of TrypLE Express, then incubate for 5 min at 37°C.

    For cells later than day 15, add 0.5 U/ml Liberase TH and 50 U/ml DNase I to the TrypLE to break down deposited collagen.

21. Pipet up and down with a P-1000 (1-ml) pipet tip ~10 times to dislodge cells and to break up aggregates. Avoid forming bubbles.
22. Return cells to incubator for another 5 min at 37°C.
23. Pipet up and down with a P-1000 (1-ml) pipet tip ~10 times to dislodge cells and to break up aggregates. Avoid forming bubbles.
24. Transfer cells to a 15-ml conical tube, top up with CDM3, and centrifuge 5 min at 300 × *g*, room temperature.
25. Resuspend in 1 ml CDM3 and pipet up and down with a P-1000 pipet tip ~10 times to release single cells.

    In our experience, the addition of Y27632 did not improve cardiomyocyte cell survival after dissociation.

26. Pass cells through a 100-μm cell strainer.

27. Count cells with an automated cell counter.
28. Dilute to 1×10^6 per ml with CDM3.
29. Seed into a Matrigel-coated 24-well plate at 750,000 cells per well, a 96-well plate at 100,000 cells per well, or a 384-well plate at 25,000 cells per well.
30. Change medium every other day.

    Cells should begin contraction after ~2 to 5 days.

Freezing cardiomyocytes (day 15)

31. Resuspend at $>2 \times 10^6$ cells per ml in 90% FBS/10% DMSO, transfer 1 ml to a cryovial, and place in a Biocision CoolCell. Place CoolCell at −80°C overnight and then transfer vials to liquid nitrogen.

    BamBanker cell freezing medium is not suitable for the cryopreservation of cardiomyocytes. In our experience, cell survival was superior in cells cryopreserved in FBS when compared to KSR (Knockout Serum Replacement).

Thawing cardiomyocytes

32. Remove vial from liquid nitrogen and place in a 37°C water bath for approximately 1 min until there is just a sliver of ice left.
33. Transfer vial contents to a 15-ml conical tube and add ~10 ml of CDM3 medium supplemented with 20% FBS dropwise.
34. Invert to mix, then centrifuge 4 min at $200 \times g$, room temperature.
35. Resuspend pellet in CDM3 at a ratio of ~1 ml per million cells to be plated in a Matrigel-coated 12-well plate or equivalent.
36. After 48 hr, replace medium with CDM3 and then change medium every other day.

    Expect ~60% survival after thawing.

Days 16 and 18: Change CDM3 medium

37. Aspirate medium in wells.
38. Add 2 ml of CDM3.

Days 20, 22, 24, 26, 28: Change medium to CDM3-T (with T_3)

39. Aspirate medium in wells.
40. Add 2 ml of CDM3-T.

    We have not validated the concentrations and timing of T_3 treatment. It is possible further optimization may improve maturation.

SUPPORT PROTOCOL 1

CHARACTERIZATION OF CARDIOMYOCYTES BY FLOW CYTOMETRY

Cardiomyocytes can be analyzed using flow cytometry (see Fig. 21.3.2). Day 20 cardiomyocytes are first stained with troponin T (TNNT2) antibodies and labeled with AlexaFluor 488–conjugated goat anti-mouse IgG_1. Follow the manufacturer's instructions for operation of the flow cytometer.

For this procedure, we use a Thermo 5/7 ml bucket with decanting aid, allowing the simultaneous processing of a large number of tubes.

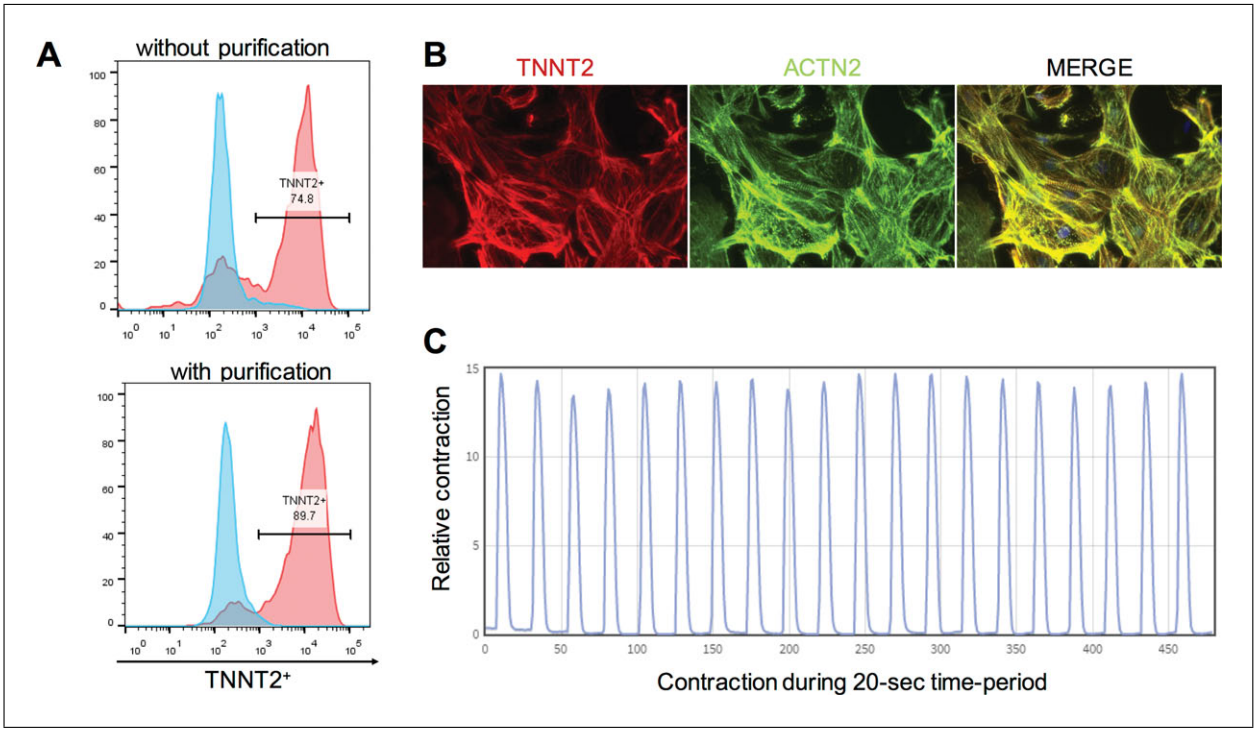

Figure 21.3.2 Characterization of cardiomyocytes produced using CDM3. (**A**) Flow cytometry of day-20 cardiomyocytes stained with troponin T (TNNT2) with or without metabolic purification. (**B**) Immunofluorescent staining of day-15 cardiomyocytes for troponin T (TNNT2) and α-actinin (ACTN2). (**C**) Measurement of contraction by video capture using Cellogy Pulse System.

Materials

Cardiomyocytes (Basic Protocol)
1% (w/v) PFA in CMF-DPBS (prepare from 20% PFA; Electron Microscopy Sciences, cat. no. 15713-S)
Dulbecco's phosphate-buffered saline without Ca or Mg (CMF-DPBS)
90% (v/v) methanol (Fisher, cat. no. A412-1)
0.5% (w/v) BSA (Sigma-Aldrich, cat. no. A3311) in CMF-DPBS
0.5% (w/v) BSA (Sigma-Aldrich, cat. no. A3311) in CMF-DPBS containing 0.1% (v/v) Triton X-100 (Sigma Aldrich, cat. no. X100)
TNNT2 mouse monoclonal (13-11) primary antibody (Thermo Scientific, cat. no. MS-295-P)
AlexaFluor 488–conjugated goat anti–mouse IgG_1 (Life Technologies, cat. no. A21121)

Flow cytometry tubes (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 and Texas Red such as Beckman Coulter CytoFLEX

1. Dissociate cells as above, transfer 1×10^6 cells to a flow cytometry tube and centrifuge 4 min at $300 \times g$, room temperature. Decant supernatant.
2. Add 1 ml of 1% PFA (prepared from 20% stock) in CMF-DPBS, vortex, incubate for 20 min at room temperature, centrifuge as described in step 1, and decant supernatant.

3. Add 1 ml of cold 90% methanol, incubate for 15 min at 4°C, centrifuge as described in step 1, and decant supernatant.
4. Wash with 2 ml 0.5% BSA in CMF-DPBS, centrifuge as described in step 1, and decant supernatant.
5. Repeat step 4.
6. Resuspend the fixed cells in 100 µl of 0.5% BSA/0.1% Triton X-100 in CMF-DPBS containing a 1:200 dilution of TNNT2 mouse monoclonal (13-11) primary antibody, vortex, incubate for 1 hr at room temperature, centrifuge as described in step 1, and decant supernatant.
7. Wash with 2 ml 0.5% BSA/0.1% Triton X-100 in CMF-DPBS, centrifuge as described in step 1, and decant supernatant.
8. Resuspend in 100 µl of 0.5% BSA/0.1% Triton X-100 in CMF-DPBS containing a 1:1000 dilution of AlexaFluor 488 goat anti-mouse IgG_1, vortex, incubate for 30 min at room temperature, centrifuge as described in step 1, and decant supernatant.
9. Wash with 2 ml 0.5% BSA/0.1% Triton X-100 in CMF-DPBS, centrifuge as described in step 1, and decant supernatant.
10. Repeat step 9.
11. Resuspend cells in 300 µl of 0.5% BSA in CMF-DPBS.
12. Analyze with flow cytometer such as Beckman Coulter CytoFLEX, following instrument manufacturer's instructions.

Expected results are approximately 85% TNNT2 positive cells.

SUPPORT PROTOCOL 2

CHARACTERIZATION BY IMMUNOFLUORESCENT STAINING

Day 15 cardiomyocytes can be evaluated by immunofluorescent staining using antibodies against troponin T (TNNT2) and α-actinin (ACTN2), as shown in Figure 21.3.2.

Material

Cardiomyocytes (Basic Protocol)
Dulbecco's phosphate-buffered saline without Ca or Mg (CMF-DPBS)
4% (w/v) PFA in CMF-DPBS (prepare from 20% PFA; Electron Microscope Sciences, cat. no. 15713-S)
0.5% (v/v) Triton X-100 (Sigma-Aldrich, cat. no. X100) in CMF-DPBS
3% (w/v) BSA (Sigma-Aldrich, cat. no. A3311) in CMF-DPBS
TNNT2 (Troponin T) primary antibody, rabbit polyclonal IgG (Abcam, cat. no. ab45932)
ACTN2 (α-actinin) primary antibody, mouse monoclonal IgG_1, clone EA-53 (Sigma-Aldrich, cat. no. A7811)
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)

8-well Lab-Tek II chamber slides (Thermo Nunc, cat .no. 154534)
12-well Matek glass No. 1.5 plates (Matek, cat no. P12G-1.5-14-F)

1. Plate cells on a Matrigel-coated 8-well chamber slide or 24-well glass-bottom Matek plate and allow cells to grow for >2 days. When ready for fixation, remove medium from cells.
2. Fix cells by adding 4% PFA in CMF-DPBS, and incubating 15 min at room temperature.
3. Permeabilize with 0.5% Triton X-100 in CMF-DPBS. Incubate for 15 min at room temperature.
4. Block with 3% BSA in CMF-DPBS, and incubate for 30 to 60 min at room temperature.
5. Stain cells with TNNT2 and ACTN2 antibodies at 1:200 and 1:500 dilution, respectively, in 3% BSA in CMF-DPBS. Incubate for 1 to 3 hr at room temperature or overnight at 4°C.
6. Wash three times with CMF-DPBS, each time for 2 to 3 min.
7. Stain with secondary antibodies at 1:1000 dilution in 3% BSA in CMF-DPBS for 30 to 60 min at room temperature in dark.
8. Wash three times with CMF-DPBS, each time for 2 to 3 min.
9. Adhere coverslip with 1 to 2 drops of Prolong Diamond (with DAPI) and evaluate by fluorescence microscopy.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps.

CDM3-C

To 1 liter RPMI 1640 (Corning 10-040-CM), add:
10 ml CDM3 supplement (see recipe)
600 μl of 10 mM CHIR99021-HCl (Biorbyt, cat. no. orb154612; 6 μM final) in DMSO
There is no need to filter sterilize
CDM3-C is stable at 4°C for >4 weeks

CDM3-C59

To 1 liter RPMI 1640 (Corning, cat. no. 10-040-CM), add:
10 ml CDM3 supplement (see recipe)
200 μl of 10 mM Wnt-C59 (Biorbyt, cat. no. orb181132; 2 μM final) in DMSO
There is no need to filter sterilize
CDM3-C59 medium is stable at 4°C for >4 weeks

CDM3-L

To 1 liter RPMI 1640 (no glucose; Invitrogen, cat. no. 11879-020), add:
10 ml CDM3 supplement (see recipe)
4 mM L-lactic acid (Wako Chemicals, cat. no. 129-02666)
There is no need to filter sterilize
CDM3-L is stable at 4°C for >4 weeks

To prepare this medium, make a 1 M stock solution from 10 M L-lactic acid (Wako Chemials, cat. no. 129-02666) by adding 1 M HEPES (Life Technologies, cat. no. 15630-080). Filter sterilize. Add 4 ml of 1 M L-lactic acid to 1 liter CDM3 (see recipe). 1 M L-lactic acid can be stored for >4 weeks in 4°C.

CDM3

To 1 liter RPMI 1640 (Corning, cat. no. 10-040-CM), add 10 ml CDM3 supplement (see recipe). There is no need to filter sterilize. CDM3 medium is stable at 4°C for >4 weeks.

CDM3 supplement

Slowly add 10.56 g of L-ascorbic acid 2-phosphate (Wako Chemicals, cat. no. 321-44823) to a 500-ml bottle of WFI water (Corning, cat. no. 25-055-CV), inverting intermittently. Mix until clear. Add 25 g of rice-derived recombinant human albumin (ScienCell, cat. no. OsrHSA-100) and, mix until dissolved by inverting intermittently. Filter sterilize. Make 10-ml aliquots in 15-ml conical tubes and store up to 6 months at −20°C.

CDM3-T

To 1 liter RPMI 1640 medium (Corning, cat. no. 10-040-CM), add:
10 ml CDM3 supplement (see recipe)
1 ml of 2 μg/ml triiodo-L-thyronine (Sigma, cat. no. T6397-100MG)
There is no need to filter sterilize
CDM3-L is stable at 4°C for >4 weeks

Make a 2 μg/ml stock solution by adding 1 ml 1 N NaOH (Fisher Scientific, cat. no. SA48-500) to 100 μg 3,3′,5-triiodo-L-thyronine (Sigma-Aldrich, cat. no. T6397); mix and top up to 50 ml with WFI water (Corning, cat. no. 25-055-CV). Make 100-μl aliquots and store up to 6 months at −20°C.

E8 medium

To 1 liter DMEM/F12 with L-glutamine and HEPES (Corning, cat. no. 10-092-CM), add one thawed 1.5-ml aliquot of E8 supplement prepared as described below for the following final concentrations of additives:
64 μg/ml L-ascorbic acid 2-phosphate (Wako Chemicals, cat. no. 321-44823)
20 μg/ml insulin (Life Technologies, cat. no. A11382ij)
5 μg/ml transferrin (Sigma-Aldrich, cat. no. T3705-1G)
14 ng/ml sodium selenite (Sigma-Aldrich, cat. no. S5261-10G)
100 ng/ml recombinant human FGF2 (Peprotech, cat. no. 100-18B)
2 ng/ml recombinant human TGFβ1 (Peprotech, cat. no. 100-21)
100 ng/ml heparin sodium salt (Sigma H3149-250KU; add from 10 mg/ml stock solution in WFI water)
There is no need to filter sterilize
The medium is stable at 4°C for >4 weeks

Shown below are directions for making 100 1.5-ml E8 supplement aliquots. This will generate 100 liters of E8 medium.

1. Add 50 ml of room temperature WFI water (Corning, cat. no. 25-055-CV) to 150 ml cell culture bottle, then slowly add 6.4 g of L-ascorbic acid 2-phosphate (Wako Chemicals, cat. no. 321-44823), inverting intermittently. Mix until clear.

2. Place 46 ml of room temperature WFI water (Corning, cat. no. 25-055-CV) in a sterile 100-ml glass beaker with a stir bar on a stir plate, add 2 g of insulin (Life Technologies, cat. no. A11382ij), adjust pH to 3 with ∼1.4 ml of 1 N HCl (Fisher Scientific, cat. no. SA48-500) to dissolve, adjust pH to 7.4 with 10 N NaOH (Fisher Scientific, cat. no. SS255-1) (∼200 μl), then add 500 mg of transferrin (Sigma-Aldrich, cat. no. T3705-1G), 1 ml of 10 mg/ml heparin sodium salt (Sigma, cat. no. H3149-250KU), and 1 ml of 1.4 mg/ml sodium selenite (Sigma-Aldrich, cat. no. S5261-10G). Make up to 50 ml and add to the ascorbic acid solution.

Note that insulin will go back through its isoelectric point and come back out of solution as it progresses from pH 3 through to pH 7.4. As it passes pH 7, it will go back in to solution.

If the mixture does not stay in solution, then increase the pH with additional 10 N NaOH (Fisher Scientific, cat. no. SS255-1) (~200 μl).

3. Add 48 ml of WFI water (Corning, cat. no. 25-055-CV) to a 50-ml conical tube, and use this to resuspend the contents of ten 1-mg vials of FGF2 (Peprotech, cat. no. 100-18B).

4. Add 2 ml of WFI water to two 100 μg vials of TGFβ1 (Peprotech, cat. no. 100-21).

5. Add the growth factors to the 150-ml cell culture bottle, mix well, and filter sterilize. Prepare 1.5-ml aliquots in 2 ml microcentrifuge tubes and store at –20°C.

6. Add one thawed aliquot to a 1-liter bottle of DMEM/F12 to prepare the E8 medium; there is no need to filter sterilize. The medium is stable at 4°C for >4 weeks.

E8-Y

To 1000 ml E8 medium (see recipe), add 1 ml of 10 mM Y27632 (Biorbyt, cat. no. orb154626) for a final concentration of 10 μM. There is no need to filter sterilize. E8-Y is stable at 4°C for >4 weeks.

EDTA, 0.5 mM

To 500 ml of CMF-DPBS (Corning, cat. no. 21-031-CV) add 500 μl 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.

Matrigel-coated plates

1. Thaw a bottle of growth factor-reduced Matrigel (Corning, cat. no. 356230) overnight at 4°C, then store it at 4°C. There is no need to aliquot or leave on ice. The bottle of Matrigel will be stable at 4°C for > 3 months.
2. Add 1.25 ml of Matrigel to 500 ml of 4°C DMEM/F12 (enough for 42 plates; Corning, cat. no. 10-092-CM). Return the Matrigel bottle to 4°C quickly to prevent gelling.
3. Mix the bottle by inversion and plate at 2 ml per well of a 6-well plate (or equivalent amounts for other size plates).
4. Place plates at 37°C for at least 30 min. Plates may be kept here at 37°C for >2 weeks without risk of wells drying out and being unusable.

We use 2 ml per well for 6-well plates so that the plates do not dry out during extended storage at 37°C.

We use Matrigel at a 1:400 dilution. Matrigel is supplied at a 10 to 12 mg/ml stock concentration (see product insert). At a 1:400 dilution and using 2 ml per well, this equates to ~5 μg/cm^2, which is well within the suitable range (>3 μg/cm^2) as previously described (Burridge et al., 2011; Miyazaki et al., 2012).

COMMENTARY

Background Information

Numerous techniques now exist for the cardiac differentiation of hiPS cells, yet only minimal differences in the cardiomyocytes produced have been demonstrated (Burridge et al., 2014). The chemically defined methodology described here was specifically designed to provide improved control and understanding of the constituents required for cardiomyocyte differentiation while simultaneously providing an 'inert' platform for subsequent drug testing assays and manipulation of factors controlling maturation and subtype specification. It has yet to be demonstrated what effect differentiation in CDM3 has on the cardiomyocytes it produces in comparison to RPMI+B27 and various other media such as StemPro-34 (Life Technologies) or APEL (StemCell Technologies), each of which has a different basal medium. It is likely that differences in metabolism exist due to the single energy source (glucose) in CDM3 and the lack of pyruvate, galactose, and fatty acids (such as linoleic, linolenic, or oleic acid), resulting in cardiomyocytes relying on glycolysis rather than pyruvate decarboxylation, fatty acid oxidation, the TCA cycle, and/or oxidative phosphorylation. The lack of lipids in the

medium may impact cell membrane structures, which may affect successful patch clamp experiments. In addition, the lack of retinoic acid, which is a component of B27, may influence subtype specification, although we have not noted this to date (Burridge et al., 2014). It has been demonstrated that the addition of creatine, carnitine, taurine, and insulin may be useful for long-term cardiomyocyte culture (Xu et al., 2006), and this combination is commonly used for rat neonatal ventricular myocytes (RNVMs). We have previously shown that hiPS-cell-derived cardiomyocytes can adhere to a variety of surfaces such as Matrigel, fibronectin, laminin, and collagen, and what role these surfaces have on maturation and subtype specification is still to be established. CDM3 provides a suitable platform for the analysis of each of these variables and high level of control over the cell environment during differentiation. Finally, the cost-effectiveness of this protocol makes it highly suitable for large-scale differentiation techniques, both adherent- and suspension-based.

Critical Parameters

The chemically defined pluripotent culture medium E8 (Chen et al., 2011) is used to provide a more reproducible environment for pluripotent growth. In our experience, suitable pluripotent growth is the most important variable in achieving efficient subsequent cardiac differentiation. We have made small modifications to the E8 formula. These include (1) replacing human transferrin with a recombinant version and reducing the concentration; (2) selecting a DMEM/F12 with higher levels of sodium bicarbonate so that it does not have to be supplemented (this DMEM/F12 is also supplied in 1-liter bottles, thus eliminating the need for transferring to a larger bottle and filtration); (3) and adding heparin sulfate, which has been demonstrated to stabilize FGF2 at 37°C, preventing the transient decrease in FGF2 levels over 24 hr as previously noted (Chen et al., 2012). Our experience indicates that cells grow exceptionally well in this medium, and by negotiating with vendors, we have reduced the cost of E8 by 75% compared to commercial media such as Essential 8 (Life Technologies) or TeSR-E8 (StemCell Technologies).

Passaging hPS cells as single cells and growing them as monolayers is well established (Denning et al., 2006). The use of EDTA simplifies this process by eliminating the need for centrifugation and dissociating cells to small clumps to improve cell survival (Yu et al., 2011; Beers et al., 2012). We add 10 μM Y27632 for the first 24 hr after passaging to improve consistency of plating and minimize selective pressure. A number of Rho kinase inhibitors have been shown to improve survival of dissociated hiPS cells, although Y27632 has been demonstrated to be more effective than others such as thiazovivin (Chen et al., 2014). We use either atmospheric (~20%) O_2 or physiological (5%) O_2 for pluripotent culture and have not detected a significant impact on subsequent differentiation efficiency. Physiologic 5% O_2 has been demonstrated to improve reprogramming efficiency (Yoshida et al., 2009), enhance expression of pluripotency genes (Forristal et al., 2010; Narva et al., 2013), and reduce spontaneous differentiation (Ezashi et al., 2005), and is optimal when used with mTeSR1 (Stem Cell Technologies) (Ludwig et al., 2006) and E8 (Chen et al., 2011). Low O_2 also pushes hPS cells to anaerobic glycolysis, resulting in the production of less reactive oxygen species (ROS) and DNA damage, as well as improved genetic stability, and is therefore recommended where available. In particular, even when used for just the first 2 days of the differentiation time course, 5% O_2 can result in inhibition of cardiac differentiation. In our original publication, we demonstrated that a variety of matrices were suitable for cardiac differentiation, although adhesion of cardiomyocytes after day 12 became problematic with all except Matrigel and laminin 511 or 521 (Burridge et al., 2014). We commonly use Matrigel at a 1:400 dilution, and have not found a more cost-effective alternative. We have found no added benefit in using hES cell–qualified Matrigel.

For cardiac differentiation, we have now used the protocol described here for >200 hiPS cell lines. The protocol is not 100% reproducible, and we have found that some lines go through periods of very successful differentiation followed by periods when they become refractory as they progress from passage 25 to beyond passage 90. Nevertheless, we have not found an hiPS cell line that is completely recalcitrant to differentiation. In 6-well plates, we have also commonly observed that some wells differentiate acceptably, and that others do not differentiate at all or are subject to total cell death. Taken together, these observations suggest that a controlling factor in differentiation efficiency is a combination of passage number, cell density, proliferation rate, and the microenvironment established in response to CHIR99021 and Wnt-C59 treatment.

During the continued development of our protocols, we have used numerous suppliers of recombinant human albumin, and currently use one from ScienCell based on cost. We did not notice any significant differences in differentiation efficiency among the various alternative manufacturers. Similarly, we selected an L-ascorbic acid 2-phosphate that can be purchased in large volumes. Despite continued assessment of CHIR99021 and Wnt-C59 dosing alternatives and timing in a large number of lines, we have not discovered a clearly superior regimen. The differentiation protocol described above was simply the one that most commonly worked with the largest number of the lines.

Metabolic purification is the process of replacing glucose in the medium with lactate, based on the premise that only cardiomyocytes in the culture are able to use the TCA cycle to produce ATP (Tohyama et al., 2013). As first demonstrated, this method used α-MEM and FBS and showed that cardiomyocytes could survive long term without glucose. The method has not proven to be as straightforward in CDM3 and we have found high levels of variability in survival among lines and differentiations during the metabolic selection. To counteract this, we have reduced the length of time the cells are treated to 4 to 6 days so that we could observe cells to identify early signs of cardiomyocyte death. Additionally, we noted that some differentiation runs contain contaminating non-cardiomyocyte cell types that cannot be removed by metabolic selection. In our original publication (Burridge et al., 2014), we used sodium DL-lactate to overcome the lack of membrane permeability of L-lactic acid as previously described (Tohyama et al., 2013), but we have found that cardiomyocytes cannot be maintained long term using either approach.

Passaging of cardiomyocytes is relatively simple at ≤ day 15, but we have found that the cells lay down a layer of collagen over time in culture that must be broken down before cells can be isolated, and therefore we recommend the use of Liberase TH and DNase I. By analyzing cryopreservation techniques, we found that cells that are more effectively broken up to single cells have better survival upon thawing, that 10% DMSO+90% FBS is an effective cryopreservation solution, and that the addition of higher percentages of FBS (up to 40%) to the CDM3 for 48 hrs post-thaw improved plating efficiency. Other cryopreservation media such as CryoStor10 have proven effective for the cryopreservation of hiPS cell-derived cardiomyocytes, but they are not cost-efficient. We found that the addition of Y27632 for 24 hr after thawing had a negative effect on cell survival, and this compound is therefore not included.

Troubleshooting

Interline variability and passage number variability in differentiation efficiency: Pluripotent cells must be undifferentiated and growing at a fast rate, ideally achieving 75% to 85% confluence in 3 to 4 days. We have found that lines over passage 25 have a higher differentiation success rate.

Cell death after CHIR99021 treatment: This is commonly due to too high a starting density. For some early-passage lines (<p25), a lower level of CHIR99021 (e.g., 5 μM) may be more suitable.

No signs of epithelial to mesenchymal transition at day 2 were found: Clear signs of EMT are indicative of efficient mesodermal differentiation, and the lack of EMT is likely due to overconfluence of starting cells.

Cell death during late differentiation (days 6 to 8): This can be observed in low-passage (<p25) lines, and therefore we recommend repeating differentiation.

No beating cells: If cells are not contracting by day 10, discard the plate and repeat differentiation. If this occurs repeatedly, try higher (1:20) or lower split ratio (1:12) or varying the level of CHIR99021 (5 to 7 μM).

Total cell death during metabolic selection: There is variability in how long cells can survive the metabolic (L-lactic acid) selection, and we recommend reducing the number of days in CDM3-L medium.

Anticipated Results

Differentiation should produce 1 to 2 million cardiomyocytes per well for a 6-well plate. Cardiomyocyte purity after metabolic selection will be >90% based on TNNT2 flow cytometry. Cardiomyocytes will stain positive for cardiac markers such as TNNT2 and ACTN2. Cells will beat at ~50 beats per minute—slower at lower temperatures, and faster at higher temperatures. Cardiomyocytes will demonstrate chronotropic responses to drugs such as norepinephrine. Toxicity to known cardiotoxic drugs will increase as the cells age (e.g., from day 30 to day 90). After cryopreservation, cell survival will be ~60%, and cells will regain contraction in 2 to 5 days.

Time Considerations

This full protocol takes 19 days from passage of hiPS cells through to dissociation/cryopreservation of cardiomyocytes.

After thawing cells, it can take 2 weeks for proliferation to reach a suitable rate for successful differentiation. Medium should be changed daily for pluripotent cells; this protocol is not compatible with skipping days or 'weekend-free' schedules. Contracting cells will be noted at approximately day 9 of differentiation.

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