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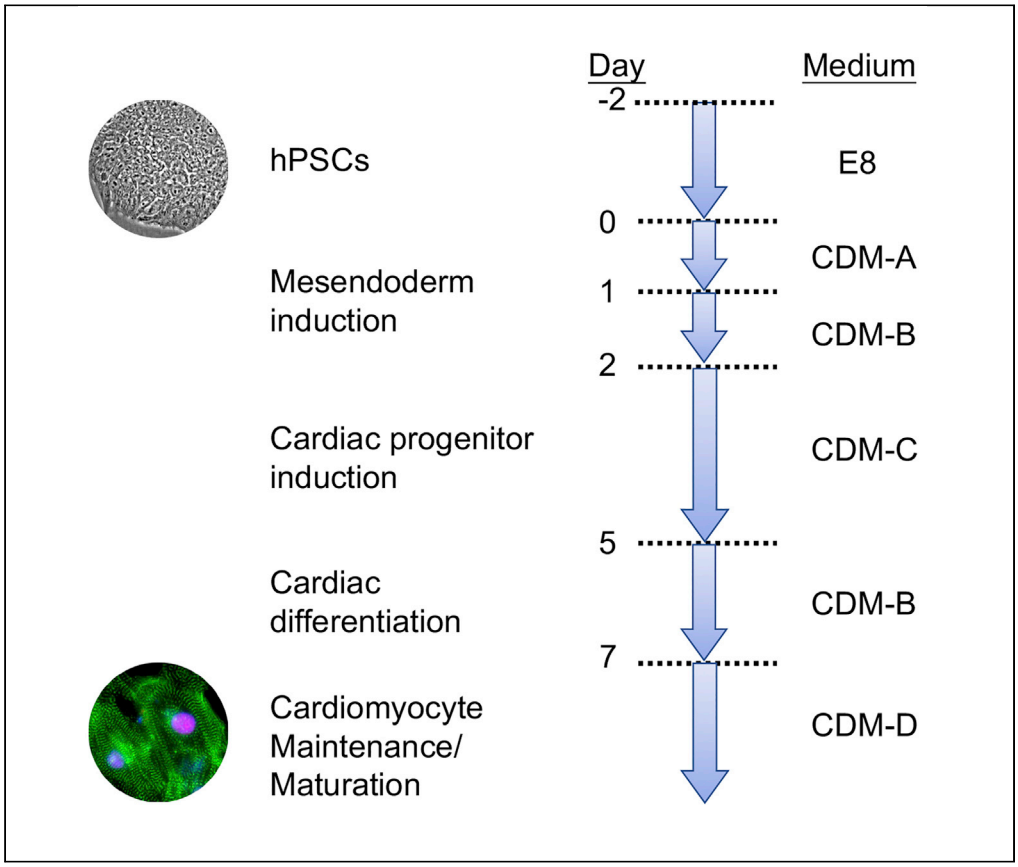
Differentiation of Cardiomyocytes from Human Pluripotent Stem Cells in Fully Chemically Defined Conditions



In the past few years, several different methods for directed cardiomyocyte differentiation from human pluripotent stem cells (hPSCs) in chemically defined conditions have been reported, including our own (Burridge et al., 2014; Lian et al., 2012; Lin et al., 2017). To help researchers adapt to our simple and cost-effective method, here we provide the step-by-step protocols for hPSC-cardiomyocyte differentiation, including hPSC culture, cardiomyocyte differentiation, cardiomyocyte passaging, and cryopreservation.

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HIGHLIGHTS

A cost-effective method to derive cardiomyocytes from human pluripotent stem cells

Albumin-free and chemically defined media for easy protocol optimization

High purity beating cardiomyocytes can be observed within 7 days of differentiation

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Differentiation of Cardiomyocytes from Human Pluripotent Stem Cells in Fully Chemically Defined Conditions

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SUMMARY

In the past few years, several different methods for differentiation of directed cardiomyocytes from human pluripotent stem cells (hPSCs) in chemically defined conditions have been reported, including our own (Burridge et al., 2014; Lian et al., 2012; Lin et al., 2017). To help researchers adapt to our simple and cost-effective method, we provide step-by-step protocols for hPSC-cardiomyocyte differentiation, including hPSC culture, cardiomyocyte differentiation, cardiomyocyte passaging, and cryopreservation.

For complete details on the use and execution of this protocol, please refer to Lin et al. (2017).

BEFORE YOU BEGIN

TIMING: 30 min

1. Plate coating: hPSCs are maintained or differentiated into cardiomyocytes on the following ECM-coated plates/dishes, depending on the researcher's own preference (Chen et al., 2011; Melkoumian et al., 2010; Preissner, 1991; Villa-Diaz et al., 2013).
   a) Matrigel: Thaw a bottle of Growth Factor Reduced Matrigel (~90mg/10ml) on ice to make aliquots in smaller volume in Eppendorf tubes (we usually make 4mg per aliquot) and store at -80°C. Use 40ml ice-cold DMEM/F12 medium to dissolve one aliquot of 4mg frozen Matrigel and dilute it into 100μg/ml in a chilled 50ml conical tube, coat 1ml per well of a 6-well plate or 0.5ml per well of a 12-well plate immediately. Keep the Matrigel tube on ice during the entire coating process, do not allow it to warm to 20°C before coating. After the plates are coated, wait for at least one hour at 20°C or ideally at least 16 hours at 4°C before use. The coated plates could be sealed with foil to prevent dehydration and stored at 4°C for at least one month. Do not use the plate if Matrigel has dried up.
   b) Vitronectin: Thaw one vial (1ml) Thermo Fisher Recombinant Human Vitronectin (VTN-N) Protein (500/ml) stored at -80°C and make four aliquots. Store the aliquots or dilute each aliquot (0.25ml) in 48ml DMEM/F12 and coat 6-well plate with 1ml/well or 12-well plate with 0.5ml/well at 20°C and incubate for 2 hours before use. The coated plates could be stored at 4°C for at least one month before use.
   c) Synthemax: Reconstitute Corning Synthemax II Substrate in cell culture grade water to make 1mg/ml stock solution, store at 4°C for up to 6 months. To coat the plate, dilute the stock solution at 1:20 in cell culture grade water to achieve a 0.05 mg/ml final working solution, coat 6-

well plate with 1ml/well or 12-well plate with 0.5ml/well and incubate at 20°C for 2 hours before use. The coated plates can be stored at 4°C for at least one month before use.

Note: Since Matrigel starts to form a gel above 10°C and solidify rapidly at 20°C, the conical tube containing Matrigel solution needs to be kept on ice during the whole coating procedure.

Note: Matrigel is a product extracted from mouse sarcoma and therefore not chemically defined as it contains xenogeneic factors, so it may cause concerns for clinical application (Villa-Diaz et al., 2013). Each batch of Matrigel should be tested for its effects on hPSC culture before large-scale use. Vitronectin (VTN-N) and Synthemax II Substrate are fully chemically defined recombinant human proteins that are suitable for clinical application (Chen et al., 2011; Melkoumian et al., 2010; Preissner, 1991).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
α-Actinin primary mouse monoclonal antibody	Sigma-Aldrich	A7811
cTnT (Troponin T) primary mouse monoclonal antibody	Developmental Studies Hybridoma Bank	CT3
cTnI (Troponin I) primary rabbit polyclonal antibody	Santa Cluz	sc-15368
NKX2.5 primary rabbit polyclonal antibody	Abcam	ab35842
AlexaFluor 488–conjugated donkey anti-mouse IgG	Thermo Fisher	A21202
AlexaFluor 488–conjugated donkey anti-rabbit IgG	Thermo Fisher	A21206
AlexaFluor 594–conjugated donkey anti-mouse IgG	Thermo Fisher	A31570
AlexaFluor 594–conjugated donkey anti-rabbit IgG	Thermo Fisher	A31572
Chemicals, Peptides, and Recombinant Proteins		
Ultrapure DNase/RNase-free distilled water	Thermo fisher	10977023
DPBS, no calcium, no magnesium	Thermo Fisher	14190250
DMEM	Thermo fisher	11995065
DMEM/F12	Thermo fisher	11320-032
Essential 8 (E8) medium	Thermo fisher	A1517001
Matrigel Growth Factor Reduced Basement Membrane Matrix. LDEV free	Corning	354230
Synthemax II-SC substrate	Corning	3535
Vitronectin (VTN-N) Recombinant Human Protein, Truncated	Thermo Fisher	A14700
ROCK inhibitor Y-27632	Tocris	1254
Holo-transferrin	Sigma	T0665
Sodium Selenite	Sigma	S5261
L-Ascorbic Acid	Sigma	A8960
Insulin, human, 10mg/ml	Sigma	I9278-5ML

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemically Defined Lipid Concentrate	Thermo Fisher	11905-031
100x Pen Strep	Thermo Fisher	10378-016
Heparin	Sigma	H3149
CHIR99021	Tocris	4423
IWP2	Tocris	3533
Bovine Serum Albumin (BSA)	Sigma	A0281
16% Formaldehyde solution (w/v)	Thermo Fisher	28908
Triton X-100	Sigma	T8787
0.5 M EDTA (pH8.0)	KD Medical	RGF-3130
TrypLE Express	Thermo Fisher	12605-036
CryoStor CS10	StemCell Technology	7930
Experimental Models: Cell Lines		
Human pluripotent stem cells (hPSCs): human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs)	WiCell	hESC lines: H1 (WA01), H9 (WA09), hiPSC line, mND2-0

MATERIALS AND EQUIPMENT

- 0.5 mM EDTA/PBS: In a sterile environment, add 500ul 0.5M EDTA into 500 ml DPBS to make a final concentration of 0.5mM, store in 20°C for up to 6 months.
- 10mM stock Rock Inhibitor (Y27632): In a sterile environment, add 16mg Y27632 into 50ml of DPBS to make 1000X stock solution and store it in 500ul aliquots at -80°C. Dilute the 1000X stock in medium to make 10 μM final concentration before use.
- 5mM stock CHIR99021: In a sterile environment, add 21.2 ml DMSO into 50mg of CHIR 99021 powder to make 1000X stock solution and store it in 500 ul aliquots at -20°C for up to 2 years. After thawing the aliquot, store it at 4°C for up to 2 months and dilute the 1000X stock in medium to make 5 μM final concentration before use.
- 3mM stock IWP2: In a sterile environment, add 35.6 ml DMSO into 50mg of IWP2 powder to make 1000X stock solution and store it in 500 ul aliquots at -20°C for up to 2 years. After thawing the aliquot, store it in 4°C for up to 2 months and dilute the 1000X stock in medium to make 3 μM final concentration before use.
- 600U/ml stock heparin: Add 18KU heparin into 30ml DPBS and filter it to make 1000X stock solution. Divide the solution into 1ml aliquots and store at -20°C for up to 1 year. After thawing the aliquot, store at 4°C for up to one month and dilute the 1000X stock in medium to make 0.6 U/ml final concentration before use.

Note: heparin sodium salt is usually sold in USP kilounit (KU) with the weight (mg) listed on the bottle as well. When this protocol was developed, we compared four different products of USP grade heparin from Sigma:

H3149 (Lot# SLBL2442V): 100KU / 477mg = 209.6 U/mg
H3149 (Lot# SLBC9940V): 100KU / 519mg = 192.7 U/mg
H3393 (Lot# SLBL1083V): 100KU / 477.4mg = 209.5 U/mg
H5515 (Lot# SLBK0235V): 100KU / 485mg = 206.2 U/mg

We tested all these heparin products at 3 μg/ml and found that their effects on promoting cardiac differentiation were comparable although the U/mg of them were slightly different. To avoid the

possible batch-to-batch variation and standardize the protocol, we chose to use 0.6 U/ml based on the calculation from 3 μg/ml of H3149 (Lot# SLBL2442V).

- 0.1% BSA: Add 100mg of BSA to 100ml of DPBS and filter it, store at 4°C for up to 6 months.
- 3% Formaldehyde solution: Add 9.4ml 16% Formaldehyde solution to 40.6ml DPBS to make 50ml 3% formaldehyde solution. Prepare freshly before each use.
- 0.1% Triton X-100: add 50ul Triton X-100 to 50ml DPBS to make 50ml 0.1% Triton solution, store at 4°C for up to 2 months.
- 3% Formaldehyde solution/0.1% Triton X-100: add 9.4ml 16% Formaldehyde solution and 50ul Triton X-100 to 40.6ml DPBS to make 50ml solution. Prepare freshly before each use.
- E8 basal medium: Add 64mg/L L-ascorbic acid and 13.6μg/L Sodium Selenium to DMEM/F12.

  Note: After preparation, E8 basal medium can be stored at –20°C for up to 1 year.

- CDBM (Cardiac Differentiation Basal Medium): Add 10μg/ml Transferrin, 1x Chemically Defined Lipid Concentrate, 1x Pen Strep to E8 basal medium

  Note: Prepare aliquots of 10mg/ml Transferrin stock and store at –80°C for up to 1 year. After preparation, CDBM can be stored at 4°C for up to 2 months.

- Cardiac differentiation medium:
  CDM-A (day 0): CDBM + CHIR99021 (5 μM)
  CDM-B (day 1,5,6): CDBM + heparin (0.6U/ml)
  CDM-C (day 2,3,4): CDBM + heparin (0.6U/ml) + IWP2 (3 μM)
  CDM-D (day 7+): CDBM + insulin (20μg/ml)

  Note: After preparation, all cardiac differentiation media can be stored at 4°C up to 1 month.

- Freezing container: e.g., Corning CoolCell.
- Benchtop Centrifuge: e.g., Allegra X-14 from Beckman Coulter.
- BSL2 biosafety cabinet: e.g., Purifier Logic+ ClassII, TypeA2 biosafety cabinet from Labconco.
- Cell culture incubator (5% CO_2, 20% O_2): e.g. Heracell 150i from ThermoFisher.
- 37°C water bath: e.g., Precision general purpose bath from ThermoFisher.
- Inverted phase contrast and fluorescence microscope: e.g., EVOS FL microscope from ThermoFisher.
- Flow cytometer: e.g., Accuri C6 Plus from BD Biosciences.

STEP-BY-STEP METHOD DETAILS

Thawing hPSCs

TIMING: 30 min

1. Remove one vial of hPSCs from liquid nitrogen tank and place it in a 37°C water bath until a small piece of ice remains, use 1000ul pipette tip to transfer the contents to 5ml E8 medium in a 15ml conical tube.
2. Centrifuge the conical tube at 200g for 3 minutes, aspirate supernatant, resuspend the pellet with 6ml E8 medium plus Rock inhibitor (Ri) Y-27632 (10 μM) and gently transfer to three wells of a 6-well plate (2ml per well).
3. Gently shake the plate to seed the cells evenly and leave the plate in incubator (37°C, 5% CO_2) for at least 16 hours.

Note: We normally cryopreserve 0.5–1.0 million hPSCs in each vial that is enough to be thawed and seeded in 2–3 wells of a 6-well plate.

Maintain and Passage hPSCs

TIMING: 25 min

4. On the next day after thawing hPSCs, aspirate E8 medium with Ri and change to fresh E8 medium without Ri, culture until cells reach 60-80% confluence.
5. Passage at 1:10 ratio every 3-4 days for routine maintenance with E8 medium changed daily.
6. To passage the hPSCs, aspirate E8 medium and rinse the cells with 1 ml 0.5mM EDTA/PBS per well of a 6-well plate to remove the magnesium and calcium from the medium.
7. Add another 1 ml 0.5mM EDTA/PBS per well of a 6-well plate and leave at 37°C for 2-5 mins within the cell culture incubator.
8. Carefully aspirate EDTA solution from the plate when the colonies are dissociated into small clumps or single cells but still attach to the plate.
9. Use certain amount of E8 medium with Ri to wash the colonies off the plate and dilute the cells before seeding them in 2ml/well in ECM-coated 6-well plates. For examples, use 20ml E8 medium to passage hPSCs from 1 well to 10 wells of 6-well plates.

Note: The duration of EDTA dissociation is determined by the morphology change under microscope that is dependent on cell line and density/confluency of the culture. The best time to remove EDTA is when the colonies are dissociated into small clumps or single cells but still attached to the plate. If the cells are overtreated by EDTA and the colonies are detached from the plate, add 4× volume of E8 medium to dilute and centrifuge the cells at 200 g for 3 min at 20°C, aspirate the supernatant before resuspending and seeding the cells with E8 medium plus Ri.

PAUSE POINT: If cardiac differentiation is not scheduled yet, the hPSCs can be maintained in E8 medium and passaged every 3–4 days waiting for the start of the differentiation. The hiPSCs can be efficiently differentiated into cardiomyocytes even after 50 passages, as long as they are well maintained in fully pluripotent state.

Prepare hPSCs for Cardiac Differentiation

TIMING: 25 min

10. When hPSCs reach around 70% confluence, cardiac differentiation can be performed.
11. Split the cells at 1:3 ratio to 6-well or 12-well plates (4x104-6x104 cells/cm2) in E8 medium plus Rock inhibitor (10 μM)
12. Change to E8 medium without Rock inhibitor on the next day.
13. The hPSCs should reach 80-90% confluence in two days.

Note: There is variation of the proliferation rates among different hPSC lines. Control the cell number for each line when they are passaged to ensure they reach the required density in two days.

Cardiomyocyte Differentiation from hPSCs

TIMING: 5 min/day

14. Day 0: When the hPSCs reach ~ 80-90% confluent, aspirate E8 medium and replace them with differentiation medium CDM-A (2ml per well of a 6-well plate or 1ml per well of a 12-well plate), count the day as day 0 of differentiation.
15. Day 1: After the cells have been cultured in CDM-A for 24 hours, count it as day 1 and change the medium to differentiation medium CDM-B.
16. Day 2-4: Change the medium to differentiation medium CDM-C daily on day 2, 3, and 4.
17. Day 5-6: Change the medium to differentiation medium CDM-B daily on day 5 and 6.
18. Day 7: Change the medium to CDM-D. Then change CDM-D every other day in the first week, but twice per week after day 14 if long-term culture is needed. CDM-D is the medium used for long-term culture.

(Please see Figure 1 for representative morphologies from Day 0 to Day 7 of differentiation)

⚠ CRITICAL: There is large amount of cell death during days 2–5, it is a normal process. Shake the plate before aspirating the old medium to remove the floating dead cells and cell debris as much as possible.

Dissociate and Passage the hPSC-Derived Cardiomyocytes for Characterization or Other Experiments

TIMING: 1 h

19. Aspirate CDM-D, gently wash cells once with DPBS.
20. Add 1ml TrypLE per well of a 6-well plate, incubate at 37°C for 5-15 minutes. The incubation time depends on the density of the derived cardiomyocytes on the plate.
21. Aspirate TrypLE when the cardiomyocytes are dissociated into single cells but still attached to the plate under the microscope.
22. wash off the cells and gently pipette up and down with 2ml CDM-D per well of a 6-well plate.
23. transfer the cell suspension to 15ml or 50ml conical tube and count the cells before centrifuge them at 200g for 3 minutes.
24. For cryopreservation, see the below steps.
25. For passaging, resuspend the cells with CDM-D plus 10 μM Rock inhibitor and seed them on ECM (Matrigel, Vitronectin or Sythemax, depending on the researchers' preference) -coated tissue culture plates at $2x10^5$ cells/cm2.
26. Shake the plate on the next day to remove floating dead cells before aspirating the medium and changing to CDM-D without Rock inhibitor. Maintain the cells in CDM-D for characterization or other experiments.

Note: The hPSC-derived cardiomyocytes have the highest recovery rate when they are dissociated and split between days 10–15.

Note: TrypLE treating time is critical for the survival rate after passaging. If it is too short, the cells will not be dissociated into single cells and still attach tightly to the plate and could not be easily washed off via medium. Under this circumstance, increasing the force of pipetting may damage the cells. On the other hand, the cells will go into apoptosis if the treatment of TrypLE is too long. The optimal time point to stop TryLE treatment is when the cells are dissociated into single cells but still lightly attached to the plate (right before detachment). Since there is variation of derived cell density among different hPSC lines (Figure 2), the TrypLE treatment time is variable and could be determined by the observation under microscope. The recovery rate of live cell passaging using this protocol is expected to be over 70%.

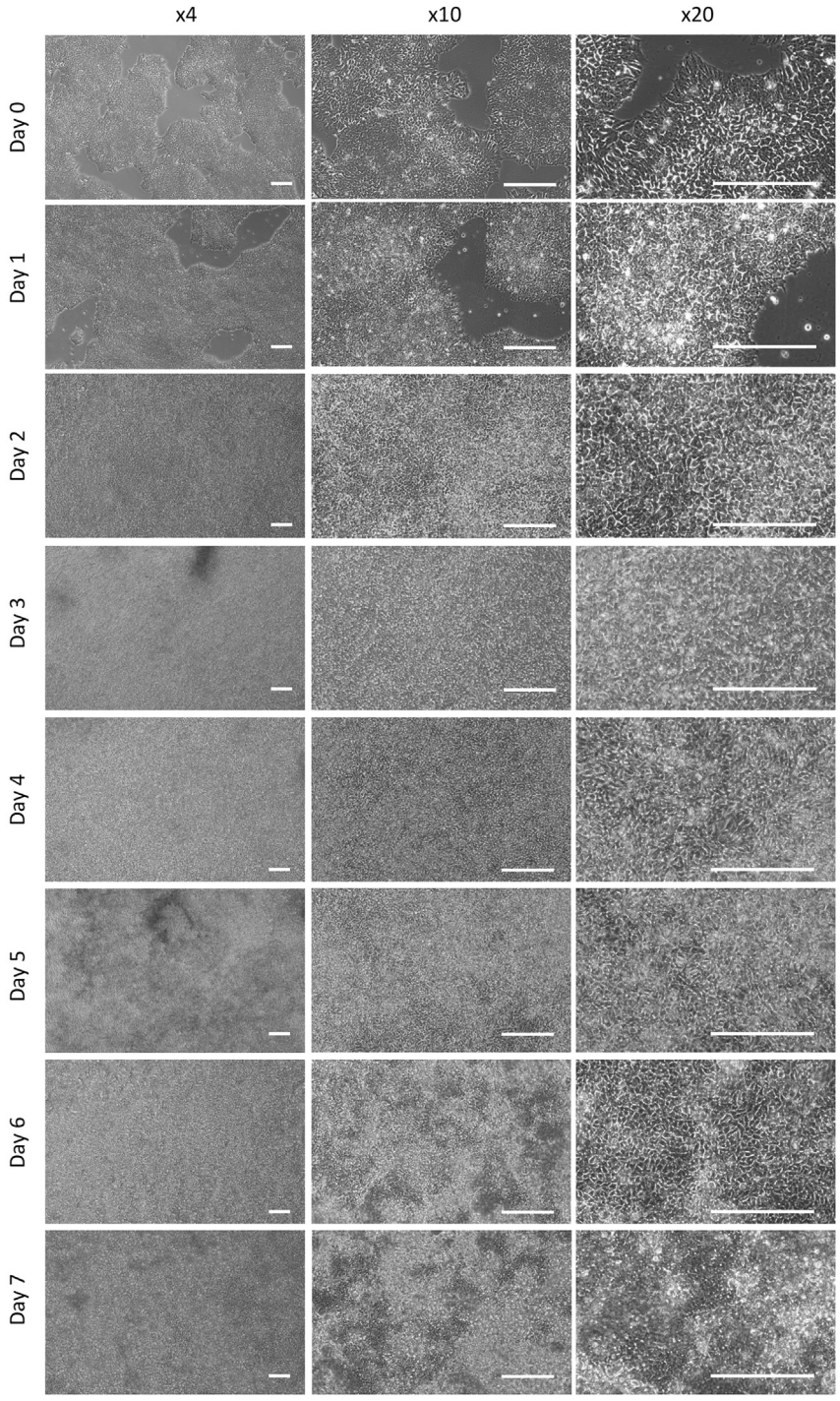

Figure 1. hPSC Cardiac Differentiation

Representative phase contrast images show the chronological morphology change from day 0 (iPSCs) to day 7 (beating cardiomyocytes) under the current differentiation protocol. Scale bar, 100μm.

Characterization of hPSC-Derived Cardiomyocytes

At day 10 after differentiation, the cells should show characteristics of cardiomyocytes, such as spontaneous contraction, cardiac marker expression, cardiac action potential, which could be detected by video recording, immunofluorescence (IF) staining, and patch clamping, respectively.

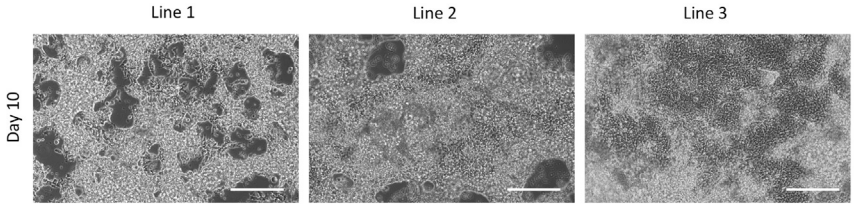

Figure 2. Cell Density of Cadiomyocytes Derived from hiPSCs

Phase contrast images show the density variation of derived cardiomyocytes among three different lines at day 10 after differentiation. Scale bar, 100μm.

Immunofluorescence (IF) staining of cardiac markers (Troponin T, alpha- actinin, NKX2.5, etc) for fluorescence microscopy (Figure 3A) or flow cytometry (Figure 3B) are the easiest ways to characterize and quantify the hPSC-derived cardiomyocytes.

If facility and expertise are available, patch clamping can be used to detect action potentials for different subtypes of derived cardiomyocytes, and transmission electron microscope (TEM) can be used to detect the ultrastructure of cardiac sarcomeres.

Fluorescence Microscopy

TIMING: 5 h

27. Passage the hPSCs-derived cardiomyocytes from 6-well plate at day 10 to a desired size of plate (24- or 48-well plate).
28. After 2 days of recovery and growth, remove medium and gently wash cells with DPBS once.
29. Aspirate DPBS, fix cells with in 3% Formaldehyde solution for 5 min at 20°C.
30. Aspirate fixative, wash cells with DPBS once and permeabilize cells with 0.2% Triton X-100 for 5 min at 20°C (skip this step for cell surface protein staining).
31. Aspirate Triton X-100, washes with DPBS once, block nonspecific antibody binding with 10 mg/ml Bovine Serum Albumin (BSA) for at least 30 min at 20°C.
32. Aspirate BSA, add cardiac specific primary antibody (diluted in BSA at required concentration) for 1 hour at 20°C or at least 16 hours at 4°C.
33. Wash off primary antibody by DPBS twice and incubate with secondary antibody (diluted in DPBS at 1:1000) for 1 hour at 20°C.
34. Aspirate secondary antibody and directly incubate 2 minutes at 20°C with DAPI (1:20 diluted in DPBS by adding 1 drop of DAPI to 1ml DPBS) for nuclei staining.
35. Wash off DAPI with DPBS twice, the stained cells can be imaged under fluorescence microscope or stored in DPBS at 4°C (avoid light). The immunofluorescence staining can be maintained for more than one month if the cells are not dried out.

Note: Step 29 and 30 can be combined to one step by preparing a 3% Formaldehyde solution +0.2%Triton X-100 solution for non-cell surface protein staining.

Note: To save antibody, we recommend culturing cells on small wells (24- or 48-well plate) for IF staining. However, when using vacuum to remove the solution for so many steps during this procedure in such small wells, the cells are prone to detach from the plate even after fixation. To avoid the detachment of cells, we recommend using manual pipetting to gently remove or add solution (antibody or DPBS).

Flow Cytometry

TIMING: 6 h

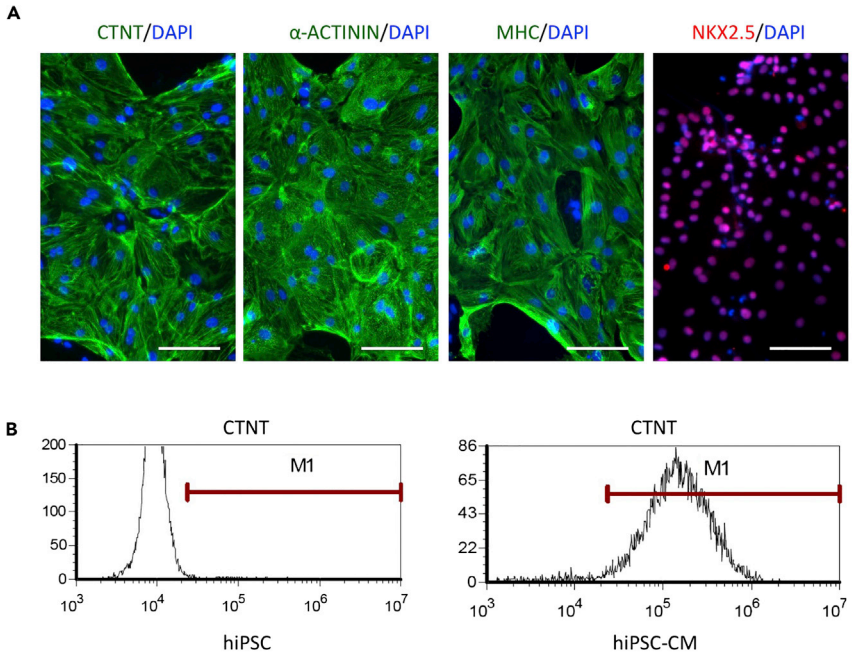

Figure 3. Characterization of hiPSC-Derived Cardiomyocytes

(A) Immunofluorescence staining of cardiac markers (cTnT, α-Actinin, MHC, and NKX2.5) shows the cardiomyocytes differentiated from iPSCs at day 30.

(B) Flow cytometry using cTnT antibody indicates high purity (95%) of cardiomyocytes in the differentiated cells at day 10 after induction of differentiation. Scale bar, 50 μm.

36. Aspirate the culture medium and wash live hPSC-derived cardiomyocytes with DPBS once, incubate with TrypLE (0.5ml / one 12-well) at 37°C for 4-8 minutes, TrypLE incubation time depends on the cell density.
37. After the cells are dissociated into single cells and partially detached from the plate, add culture medium (1ml / one 12-well) to dilute TrypLE, gently pipette up and down to wash off and suspend the cells, make a total volume of 1.5 ml / one 12-well, move the suspension to 1.5ml Eppendorf tube.
38. Centrifuge the cells at 200g for 3 minutes in Eppendorf centrifuge at 20°C.
39. Add 3% Formaldehyde solution / 0.1% Triton X-100 to resuspend the pellets, fix and permeabilize the cells for 5 minutes at 20°C (0.3ml / pellet from one 12-well).
40. Add 1ml DPBS to dilute and wash the suspension, centrifuge at 200g for 3 minutes.
41. Add 1% BSA (50μl / pellet from one 12-well) to resuspend the pellet and block nonspecific binding of antibody for 30 minutes at 20°C. If using 96-well plate for FACS, transfer the 50μl suspension to 96-well plate.
42. Add 50μl primary cardiac specific antibody (diluted in 1% BSA) to the original 50μl blocking solution to make a total volume of 100μl, incubate at 20°C for 60 minutes or 4°C for at least 16 hours.
43. Add 100μl DPBS to dilute and wash off the primary antibody, centrifuge the cells at 200g for 3 minutes.
44. Suspend the pellet with 100μl AlexaFluor 488 or 594–conjugated second antibody (diluted 1:1000 in DPBS), incubate at 4°C for 60 minutes.
45. Add 100μl DPBS to wash off the second antibody and centrifuge the cells at 200g for 3 minutes.
46. Resuspend the pellet with 120μl DPBS for flow cytometry analysis.

Note: Negative control cells (non-cardiomyocytes) need to be prepared by following the same procedure for flow cytometry, we recommend using undifferentiated hiPSCs as negative control.

Note: Lots of cells will be lost after several steps of washing and transferring of the cell suspension and pellets, as such, at least 1 million cells (dissociated from one 12-well) are required for each flow cytometry experiment.

Optional Steps

Cryopreservation of hPSC-Derived Cardiomyocytes

TIMING: 1 h

47. Dissociate the differentiated cardiomyocytes from plate, see the above steps in Dissociate and passage the hPSC-derived cardiomyocytes.
48. After counting the cells and centrifuging the suspension at 200g for 3 minutes, resuspend the pellet in CryoStor CS10 with 10 μM Rock inhibitor at $10x10^6$ cells/ml, transfer 1ml/cryovial in Corning CoolCell alcohol-free freezing container and place at -80°C for at least 16 hours, then transfer vials to liquid nitrogen tank for long-term storage.

Note: The hPSC-derived cardiomyocytes have the highest recovery and survival rate when they are dissociated and cryopreserved between days 10–15 of differentiation.

Thawing and Recovery of hPSC-Derived Cardiomyocytes

TIMING: 1 h

49. Remove one vial of hPSC-derived cardiomyocytes from liquid nitrogen tank and place it in a 37°C water bath until a small piece of ice remains, use 1000μl pipette to transfer the content to 5ml 37°C warmed CDM-D in a 15ml conical tube.
50. Count the cell viability using cell counter, resuspend the cells in CDM-D plus Rock inhibitor (10 μM) and seed them in desired Matrigel-coated tissue culture plates at a density of $3x10^5$ Trypan Blue negative cells /cm^2.
51. Remove Rock inhibitor on the next day by changing medium to fresh CDM-D without Rock inhibitor.
52. The hPSC-derived cardiomyocytes can be long-term cultured for different purposes.

Note: The recovery and survival rate of hPSC-derived cardiomyocytes after freeze-and-thaw in our hands is highly variable and ranges between 40%–80%, based on the cell numbers counted before cryopreservation and day 2 after thawing and plating.

Note: Adding Rock inhibitor (10 μM) for at least 16 h in culture medium significantly improves the survival rate after freeze-and-thaw.

Note: When the hPSC-derived cardiomyocytes were dissociated and cryopreserved at days 10–12, the cells still have the capacity to proliferate. As such, the thawed and reseeded cells will be getting more confluent in the next couple of days due to cell proliferation and increase of cell size (Figure 4).

EXPECTED OUTCOMES

The spontaneous contraction of derived cardiomyocytes usually can be observed under microscope for the first time between day 6-8, sometimes only in certain areas, sometimes in the whole well.

Normally we start seeing the contraction of some cells around day 7 of differentiation, when the cells already express cardiac specific markers like cTnT, alpha actinin, and NKX2.5. The percentage of cardiac marker positive cells continually increases from day 7 to day 10 based on flow cytometry analysis, in coincidence with the increase of the expression level of these markers that can be detected by

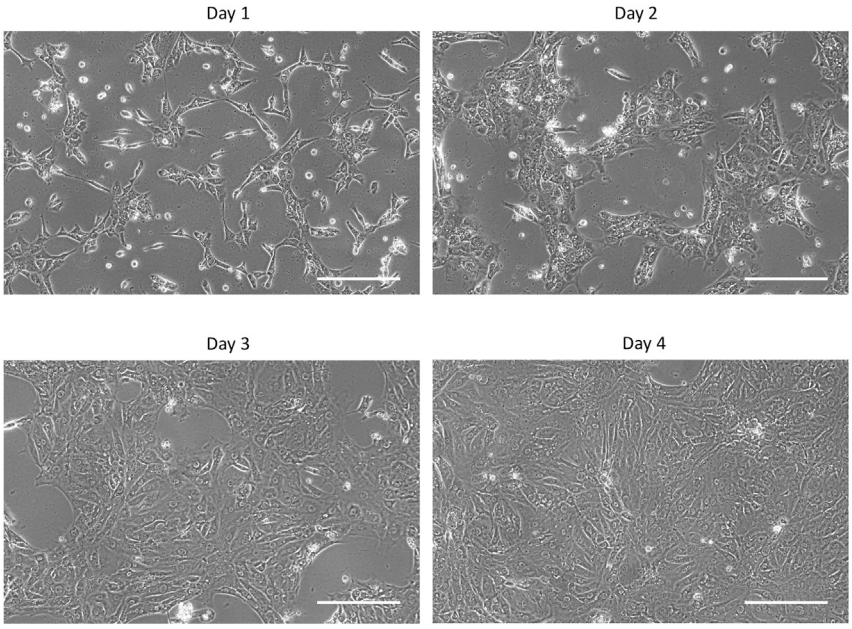

Figure 4. The Growth of hiPSC-derived Cardiomyocytes after Freezing and Thawing

Phase-contrast images show continuous expansion of hiPSC-derived cardiomyocytes during the first four days after thawing when they were cryopreserved at day 10 of differentiation. Scale bar, 50μm.

RT-PCR or immunofluorescence staining. The hPSC-derived cardiomyocytes are considered fully differentiated at day 10.

Sometimes, even if the differentiation efficiency is high the cardiomyocytes don't beat spontaneously, or the frequency of the contraction is not stable and consistent in our albumin-free culture medium. The hPSC-derived cardiomyocytes may beat, stop beating, and then beat again.

Like other cardiac differentiation protocols in the literature, this protocol generates cardiomyocytes that include all three subtypes (Nodal, atrial, and ventricular-like cardiomyocytes) according to patch clamping analysis at day 20 of differentiation. However, ventricular-like cells dominate with around 70% of the whole population. It is worthwhile to note that ventricular-like cardiomyocytes express MLC2a, an atrial cardiomyocyte marker, at early stage (day 10) but gradually loose MLC2a expression and gain expression of MLC2v, a ventricular cardiomyocyte marker, during maturation.

The purity of cardiomyocytes after day 10 varies among different iPSC lines and different batch of differentiation. However, majority of differentiation experiments under this protocol can reach >85% purity without further metabolic selection, which can be confirmed by cell morphology under phase contrast microscope and quantified by cardiac marker immunofluorescent staining, imaging, or flow cytometry.

The density of derived cardiomyocytes also varies, especially among different iPSC lines (Figure 2). The cell number of a 6-well plate at day 10 after differentiation may range between 30 – 60 million.

LIMITATIONS

Our lab has performed hundreds of batches of cardiac differentiation from more than fifty different hPSC lines (most of them were hiPSCs) using this protocol in the past four years. Overall the rate of successful differentiation (cardiomyocyte purity > 85%) is over 80%. However, for a very few certain

hiPSC lines the differentiation efficiency was low, and modification of the protocol was needed. This happened when the proliferation rate of certain hPSC line was significantly different (faster or slower) from majority of the hPSC lines. The specific modification for certain cell line could include the adjustment of the dosage (3-5 μM) and treatment timing (18 hours to 36 hours) of CHIR99021, or the timing of IWP2/heparin treatment (3-4 days).

The advantage of our differentiation media is they are serum and albumin free, which not only makes it cost effective, but also produces a chemically defined background for basic research and clinical application as well as avoids batch to batch variation of serum or albumin. However, the cardiomyocytes derived under this protocol could not consistently beat and we believe it is due to the components of media, as we have found that adding FBS or BSA to our maintenance medium could promote spontaneous contraction.

The heparin used in this protocol (Sigma, H3149) was heparin sodium salt purified from porcine intestinal mucosa, which is not a xeno-free product. However, we demonstrated that heparin was the key factor in the purified product to promote cardiac differentiation by the observation of the effect of heparinase I treatment (Lin et al., 2017). If fully xeno-free condition is required for the researcher, a synthetic heparin may be used as the substitute in the future.

Although most of time the differentiation efficiency (purity of cardiomyocyte) is consistent (>85%), the final density of differentiated cardiomyocytes is variable between different hPSC lines. This may be due to the variation of proliferating rates among lines. When the cardiomyocytes need to be dissociated, the variation of cell density requires different treating time of dissociating solution (TrypLE), which also causes the variation of recovery rate, especially after cryopreservation.

Like in other hPSC differentiation protocols from the literature, the cardiomyocytes derived in this protocol at day 10 are fully differentiated but immature, and they are closer to fetal rather than adult cardiomyocytes. For cetain research purpose, mature cardiomyocytes could be achieved by long-term (at least 2 months) *in vitro* culture or other methods (Robertson et al., 2013).

TROUBLESHOOTING

Problem

The hPSCs or differentiating cells could not grow in some area of the plate.

Potential Solution

Since the volume of Matrigel to coat the plate is small, make sure the entire surface of each well was covered by Matrigel during coating. Also, avoid Matrigel drying out in some area of the well when the plates are stored in long-term. Cells cannot grow on those areas without Matrigel coating or where Matrigel dried out.

Problem

The hPSCs cannot reach 80-90% confluence after two days culture. Should I start differentiation at lower cell density than 80% or wait for more time to start the differentiation?

Potential Solution

The hPSC density and time in culture before starting the differentiation are critical for differentiation efficiency. In our experience, the starting density of >80% confluence is essential for efficient differentiation and we prefer the cells were only seeded for two days before induced differentiation. If the density could not reach the level after two days culture, the hPSCs can be cultured for one more day to wait for the required confluency. However, if the seeding density was too low and it needs at least 4-5 days to reach 80-90% confluency, the hPSCs are

not in a good condition to start differentiation. In that scenario we recommend making one more passage and adjust the seeding density of hPSCs.

Problem

There are lots of cell death during the differentiation process. Is that normal?

Potential Solution

It is normal and expected. The large number of cell death, especially during day 2-5, is caused by two factors: 1) the differentiation starts at a cell density of around 90% confluence; since the cells are continually proliferating during differentiation, they are over-confluent on the plate which causes cell death; 2) The differentiation protocol applies IWP2 to inhibit Wnt signaling at day 2-5 that favors cardiac lineage differentiation. Under this circumstance, the cells that spontaneously turn into other lineages but need the support of Wnt signaling will be cultured in a nonpermissive environment and dying.

Problem

There is no spontaneous contraction of cardiomyocytes observed under the microscope even after 10 days of differentiation.

Potential Solution

There are two possibilities regarding to this observation: 1) the direct differentiation failed, and few cells were differentiated into cardiomyocytes; 2) the cells do differentiate into cardiomyocytes that can be confirmed by cardiomyocyte marker staining, but they don't spontaneously beat in a consistent way. Since our culture medium is extremely simple (no serum, no albumin), we found that the contraction of the derived cardiomyocytes cultured in this medium is not stable or consistent. To enhance the consistency of spontaneous contraction, we recommend adding 2% FBS or BSA into maintenance medium.

Problem

The recovery rate of hPSC-derived cardiomyocytes after freeze-and-thaw is highly variable. How to solve this problem?

Potential Solution

At this point we could not produce a consistent recovery rate after freezing and thawing for different set of differentiation. To acquire consistent cell number for the downstream application, we thaw one vial out to calculate the recovery rate and save a record after every set of cryopreservation. By this way we can track the cell number and know how many cells we need to thaw for the downstream use.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.L. and J.Z.; Investigation, Y.L.; Writing – Original Draft, Y.L.; Writing – Review & Editing, Y.L. and J.Z.; Funding Acquisition, J.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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