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Methods for the Derivation and Use of Cardiomyocytes from Human Pluripotent Stem Cells

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Summary

The availability of human cardiomyocytes derived from embryonic stem cells (ESCs) has generated considerable excitement, as these cells are an excellent model system for studying myocardial development and may have eventual application in cell-based cardiac repair. Cardiomyocytes derived from the related induced pluripotent stem cells (iPSCs) have similar properties but also offer the prospects of patient-specific disease modeling and cell therapies. Unfortunately, the methods by which cardiomyocytes have been historically generated from pluripotent stem cells are unreliable and typically result in preparations of low cardiac purity (typically <1% cardiomyocytes). We detail here the methods for a recently reported directed cardiac differentiation protocol, which involves the serial application of two growth factors known to be involved in early embryonic heart development, activin A and bone morphogenetic protein-4 (BMP-4). This protocol reliably yields preparations of 30-60% cardiomyocytes, which can then be further enriched to >90% cardiomyocytes using straightforward physical methods.

1. Introduction

Cardiomyocytes from human embryonic stem cells (hESCs) and the related human induced pluripotent stem cells (hiPSCs) have tremendous promise as a model system for heart development and disease, a platform for in vitro drug screening, and a potential cell source for cardiac repair. Both of these pluripotent stem cell types have unquestioned cardiomyogenic potential, which places them in contrast to many adult stem cell types for whom the capacity to differentiate into significant numbers of definitive cardiomyocytes is controversial (for a recent review, please see ref (1)). Moreover, both undifferentiated hESCs and hiPSCs as well as their differentiated cardiac progeny show robust proliferative activity, which makes these cell types particularly attractive for applications requiring large quantities of cells (for example, replacing the $\sim 1 \times 10^9$ host cardiomyocytes lost in a typical human myocardial infarct). hESC- and hiPSC-derived cardiomyocytes have an unambiguous cardiac phenotype, exhibiting spontaneous contractile activity, cardiac-type mechanisms of excitation-contraction coupling, and expression of expected sarcomeric proteins, ion channels, and transcription factors (2-4). Moreover, we and others have shown

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that, following transplantation into rodent infarct models, hESC-derived cardiomyocytes form nascent human myocardium and help preserve cardiac function (5-7).

Despite this progress, the derivation of highly purified populations of cardiomyocytes from pluripotent stem cells remains a significant challenge to the field, particularly for in vivo applications, in which the transplantation of undifferentiated cells can give rise to teratomas or other undesirable non-cardiac derivatives (8, 9). The method by which cardiomyocytes have been historically generated from ESCs involves their spontaneous differentiation in high serum via embryoid bodies, a poorly controlled approach that typically results in preparations of <1% of cardiomyocytes. Our group and others have sought to develop more efficiently cardiogenic guided differentiation protocols, including the procedure described here, which reliably yields preparations of 30-60% cardiomyocytes (6). If a greater degree of cardiac purity is required, additional enrichment steps (e.g. Percoll gradient centrifugation (6, 10)) can be performed, which typically results in preparations of >90% human cardiomyocytes.

2. Materials

2.1 Cells

1. Primary mouse embryonic fibroblasts (pMEFs), not mitotically inactivated (Chemicon/Millipore, Temecula, CA; cat. no. PMEF-CFL).
2. H7 hESC line (Wicell Research Institute, Madison, WI). (See Note 1.)

2.2 Stock Solutions

1. Dulbecco's phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA; cat. no. 14190-250).

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¹The directed cardiac differentiation protocol described here was developed with and optimized for the H7 hESC line (13), so we recommend using this line when first trying this protocol. H7 hESCs are also relatively straightforward to culture in the undifferentiated state and seem to be particularly cardiogenic. That said, this protocol has been tested with and proven successful in the reliable generation of cardiomyocytes from multiple other hESC and hiPSC lines. When adapting our standard protocol to stem cell lines other than H7 hESCs, we have found three relatively minor modifications helpful:

- i. *Number of undifferentiated hESCs seeded per well* (see section 3.2.1.) When setting up cells for cardiac induction, the goal is to obtain an evenly distributed, 80-95% confluent monolayer by 24 hours after dispersion with Versene and re-plating. Stem cell lines vary in terms of plating efficiency, but seeding in the range of $1-10 \times 10^4$ cells per well of a 96-well plate (plated out as 100 μ L/well of suspended cells in MEF-CM plus bFGF) has sufficed for all of the lines we have tried. If one seeds too many cells per well, the cultures tend to pile up rather than form a uniform monolayer.
- ii. *Interval between re-plating and induction with activin A* (see section 3.2.1.). Before the undifferentiated cultures are treated with activin A, they should be allowed to form a very compact, 100% confluent monolayer (see Figure 1). Growth kinetics vary from line to line (and can even vary with increasing passage number within a single line), so it is not surprising that there is some variation in the amount of time the re-plated cells need to form an optimally compact monolayer. We recommend testing a range of intervals from 3-10 days when commencing work with a new line.
- iii. *Supplementation with bFGF or extra medium during BMP-4 treatment.* A significant amount of cell death normally occurs between days 1-5 post-induction with activin A, usually peaking at ~day 3 post-induction (during BMP-4 treatment). With H7 hESCs, the rate of cell death is generally matched by cell proliferation, but we have found some stem cell lines may show an exaggerated death response. In such cases, we have found it sometimes helpful to either add 4ng/mL bFGF on day 1 post-induction and/or an extra 25% volume of fresh RPMI-B27 medium on days 3-4 post-induction. If you do so, do not aspirate the medium already present, and add the supplement very gently to avoid dislodging the cells.

Note that, by performing a pilot experiment in a 96-well plate, one can simultaneously vary these three parameters and quickly adapt the protocol to a new line. We have tested other variables (e.g. the timing or concentration of growth factors), but have never found these to significantly improve the yield of cardiomyocytes.

2. pMEF medium: 89% (v/v) Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA; cat. no. 11965-092), 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA; cat. no. 16140-071), and 2mM L-glutamine (Invitrogen, Carlsbad, CA; cat. no. 25030-081).
3. Pre-conditioned hESC medium: 79% (v/v) Knock-out DMEM (Invitrogen, Carlsbad, CA; cat. no. 10829-018), 20% Knock-out serum replacement (Invitrogen, Carlsbad, CA; cat. no. 10828-028), 1% non-essential amino acids solution (Invitrogen, Carlsbad, CA; cat. no. 11140-050), 1 mM L-glutamine, and 0.1 mM β -mercaptoethanol (Invitrogen, Carlsbad, CA; cat. no. 21985-023). Add 4ng/mL bFGF stock solution (see section 2.3 below) immediately before use.
4. RPMI-B27 medium: 98% (v/v) RPMI 1640 (Invitrogen, Carlsbad, CA; cat. no. 21870-092), 2% B27 serum supplement (Invitrogen, Carlsbad, CA; cat. no. 17504-044), and 2 mM L-glutamine (Invitrogen, Carlsbad, CA; cat. no. 25030-081).
5. Percoll (GE Healthcare/Amersham, Piscataway, NJ; cat. no. 17-0891-02) solutions: shortly before use, prepare 40.5 and 58.5% (v/v) solutions, using the reagents and quantities indicated in Table 1.

2.3 Growth factors

1. Human basic fibroblast growth factor (bFGF) (PeproTech, Rocky Hill, NJ; cat. no. 100-18B): dissolve at 10 μ g/ml in PBS with 0.2% bovine serum albumin (BSA) carrier (Invitrogen, Carlsbad, CA; cat. no. 15260-037), aliquot and store at -20 °C.
2. Activin A (R & D Systems, Minneapolis, MN; cat. no. 338-AC-025): dissolve at 10 μ g/ml in PBS with 0.2% BSA, aliquot and store at -20 °C.
3. Bone morphogenetic protein-4 (BMP-4; R & D Systems, Minneapolis, MN; cat. no. 314-BP-010): dissolve at 1 μ g/mL in PBS with 0.2% BSA carrier (Invitrogen, Carlsbad, CA; cat. no. 15260-037), aliquot and store at -20 °C.

2.4 Enzymes

1. Dispase (Invitrogen, Carlsbad, CA; cat. no. 17105-041): dilute to 0.1 U/mL in PBS, filter-sterilize, aliquot and store at -20°C.
2. Liberase Blendzyme IV (Roche Applied Sciences, Indianapolis, IN; cat. no. 11-988-476-001): dilute to 0.56 U/mL in PBS, aliquot and store at -20°C.
3. Trypsin-EDTA 0.05% (Invitrogen, Carlsbad, CA; cat. no. 25300-112).
4. Defined trypsin inhibitor, 1X (Cascade Biologics, Portland, OR; cat. no. R-007-100).
5. DNase I (Invitrogen, Carlsbad, CA; cat. no. 18047-019).

2.5 Substrates

1. 0.5 % gelatin solution: 2% bovine gelatin (Sigma-Aldrich, St. Louis, MO; cat. no. G1393), warmed at 37 °C for 10 min and then diluted to 0.5% (v/v) with PBS.

2. Matrigel solution: dilute growth factor reduced Matrigel (BD Biosciences, Bedford, MA; cat. no. 356231) 1:60 with cold (4 °C) Knock-out DMEM. Diluted aliquots can be stored at -20 °C, but should be thawed at 4 °C overnight before use.
3. Polyethylenimine (PEI, Sigma-Aldrich, St. Louis, MO; cat. no. P7239): dilute 0.1 % (v/v) in sterile water.

2.6. Other Reagents

1. Versene (Invitrogen, Carlsbad, CA; cat. no. 15040-066).
2. Cryostor CS10 (BioLife Solutions, Bothell, WA; cat. no. 640221).

1. Methods

3.1 Compatible Methods for Maintaining Undifferentiated hESC Cultures. (See Notes 2 & 3.)

All cell cultures (i.e. pMEFs, undifferentiated hESCs, and differentiated progeny) described below should be maintained at 37 °C in a humidified incubator with 5% CO₂ and ambient O₂.

3.1.1 Preparation of pMEF-Conditioned Medium (MEF-CM), after Xu et al (11)

1. Coat tissue culture flasks with 0.5 % gelatin and air dry.
2. Thaw and plate pMEFs at 2.25×10^4 cells/cm² on gelatin-coated flasks in pMEF medium. pMEFs may be passaged with trypsin-EDTA treatment a maximum of four times prior to irradiation and use in generating MEF-CM. (While we generally use pMEFs purchased from the vendor listed above, pMEFs generated “in-house” typically show more robust growth and less batch-to-batch variability.)
3. Harvest the expanded pMEFs using trypsin-EDTA, and then inactivate them by irradiating the cell suspension. (The amount of irradiation needed to inactivate pMEFs varies somewhat with the cell source and from lot-to-lot. We typically use ~4000 rads.)
4. Re-plate the irradiated pMEFs in gelatin-coated flasks at a density of 5.6×10^4 cells/cm² (which corresponds to $\sim 12.5 \times 10^6$ cells per T-225 flask). Gently agitate the flask to uniformly distribute the cells.

²Many laboratories maintain their undifferentiated hESC cultures in direct contact with pMEFs or other feeder cell types, but the directed cardiac differentiation protocol described here requires undifferentiated hESCs that have been maintained under feeder-free conditions for at least 2-3 passages. To wean undifferentiated hESCs on feeders to feeder-free growth in MEF-CM on Matrigel-coated surface, serially passage the hESCs onto progressively sparser feeder cells in a mixture of pre-conditioned and MEF-CM. Throughout this transition, hESCs are fed daily with media supplemented with usual bFGF (as described in section 3.1.2), and the cells are passaged with dispase (as described in section 3.1.3). Start by passaging the hESCs onto a pMEF layer at 75% normal density and feeding with MEF-CM: preconditioned medium mixed at a 1:3 ratio. With the next passage, re-plate the hESCs onto pMEF feeders at 50% density and culture in 1:1 media, followed by a third passage onto feeders at 25% density and culture in 3:1 media. With the fourth passage, the hESCs are switched to feeder-free culture in undiluted MEF-CM plus bFGF on Matrigel-coated plates.

³Recently, several alternative feeder-free culture systems for maintaining undifferentiated hESCs have been reported, some of which employ defined media formulations with specific growth factors (14-19). We have tried some of the latter for compatibility with this directed cardiac differentiation protocol. Unfortunately, in our hands, the use of hESCs cultured in these media alternatives to MEF-CM either resulted in differentiated preparations of somewhat lower cardiac purity or did not support cardiogenesis at all. We have had success when undifferentiated hESCs were expanded in mTeSR1 medium (Stemcell Technologies, Vancouver B.C., Canada) and then switched back to MEF-CM plus bFGF shortly before induction with activin A. Our lab has ongoing efforts to adapt the protocol to hESCs maintained throughout in an animal-free, defined medium culture system, but, for now, we recommend continued use of MEF-CM.

5. After allowing a minimum of five hours for cell adherence, replace the pMEF medium with pre-conditioned hESC medium plus 4ng/mL of bFGF. We generally use 90 mL of pre-conditioned hESC medium per T-225 flask of confluent pMEFs.
6. Collect the resultant MEF-CM daily, replacing with fresh preconditioned hESC medium for up to a maximum of 7 days. MEF-CM medium can be filter-sterilized for immediate use or combined and sterile-filtered at the end of the 7-day conditioning run (preferred). MEF-CM can be stored at 4 °C for short-term use or at -80 °C for up to a year. Avoid repetitive heat-thaw cycles.

3.1.2 Preparation of Matrigel-Coated Plates

1. Place diluted (1:60) Matrigel in an ice bucket.
2. Add cold, diluted Matrigel to tissue culture plates (at 1 mL per well of a 6-well plate, 0.5 mL per well of a 24-well plate, and 50 μ L per well of a 96-well plate), and then transfer the latter to storage at 4 °C. (During this coating step, we keep plates in a clean, airtight plastic box in the refrigerator.) Plates should be coated by exposure to Matrigel for at least 24 hours, but they can be stored for up to 2 weeks before use.
3. Remove Matrigel by aspiration immediately before use.

3.1.3. Routine Culture and Passage of Undifferentiated hESCs under Feeder-Free Conditions

—General information on the routine culture of undifferentiated hESCs can be found elsewhere in this volume, but specific instructions on how to transition undifferentiated hESC cultures on feeders to feeder-free conditions in MEF-CM can be found below in Note 2. Once feeder-free hESC cultures are established, we strongly recommend maintaining these in a 6-well plate format, as this provides maximal flexibility for both passaging and setting up cultures for cardiac induction.

1. Once feeder-free undifferentiated hESC cultures are established, maintain these by feeding daily with MEF-CM supplemented with 4 ng/mL of fresh bFGF (at 4 mL/well of a 6-well plate). Once the undifferentiated hESC colonies occupy ~75% of the well surface area, the cultures should be passaged as detailed in steps 2-8.
2. Aspirate MEF-CM media, and rinse with 2 mL/well PBS.
3. Aspirate PBS, replace with 1mL /well dispase (0.1 U/mL) and incubate the cells in dispase at 37 °C, until the edges of the hESC colonies begin to curl. This typically requires ~1.5-2 minutes incubation in the enzyme.
4. Gently aspirate the dispase without dislodging the cells, and replace the enzyme solution with 2mL/well MEF-CM supplemented with 4 ng/mL bFGF.
5. Using a cell scraper, collect the hESCs, which should readily detach in small clumps. Gently triturate and break up the larger clumps until they no longer gravity-settle. (Avoid over-trituration!)

6. Dilute the cell suspension in an appropriate volume of MEF-CM with 4ng/mL bFGF, and then re-plate at 2mL/well in Matrigel-coated 6-well plates. We generally split our H7 hESCs at a 1:3 to 1:6 ratio, passaging every 5-7 days.
7. To ensure an even distribution of the dispersed clumps of hESCs on the new plate surface, alternate sliding the plate left-to-right and front-to-back on the incubator shelf. (Do not swirl, which will place cells either at the periphery or center of the well.)
8. Allow at least 4-6 hours for cell adhesion before moving the plate or re-feeding. Maintain the newly passaged cultures in MEF-CM plus bFGF as described in step 1.

3.2 Directed Cardiac Differentiation of hESC Cultures

3.2.1 Setting up hESC Cultures for Cardiac Induction

1. Maintain undifferentiated hESC cultures in MEF-CM, as detailed in section 3.1. When the undifferentiated colonies occupy ~75% of the well surface area, they are ready for either routine passaging to maintain undifferentiated cultures or set up for cardiac induction. (Our standard practice is to maintain our undifferentiated hESCs in a 6-well plate. When the plate is ready for passaging, we split 1-2 wells at either 1:3 or 1:6, thereby generating a new plate. The remaining 4-5 wells are induced into cardiomyocytes following steps 2-7.)
2. To set up cultures for cardiac induction, aspirate the MEF-CM, and gently rinse the cells with PBS at 2 mL/well of a 6-well plate.
3. Aspirate the PBS, replace with 2 mL/well of Versene, and incubate the cells in Versene at 37 °C until they become rounded up and loosely adherent, but not yet detached. This typically requires 3-7 minutes. (See Note 4.)
4. Gently aspirate the Versene and replace with 1 mL/well of pre-warmed MEF-CM supplemented with 4 ng/mL fresh bFGF. Dislodge the cells by gently flowing MEF-CM over them with a 1000 uL micropipette.
5. Collect the dispersed hESCs, gently triturate them into a single-cell suspension, and quantitate by hemacytometer. Add MEF-CM supplemented with 4 ng/mL bFGF to reach a final concentration of 4×10^5 cells /mL.
6. Re-plate the dispersed hESCs on Matrigel-coated plates at a density of 4×10^5 cell/cm², which corresponds to roughly 100 μ L of cell suspension per well of a 96-well plate well or 0.5 mL per well of a 24-well plate well. (See Note 1.)

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⁴When setting up hESCs for subsequent cardiac induction, their dispersion with Versene is a critical step that requires close monitoring under the microscope and a bit of practice. The goal is to incubate the cells in Versene until they become rounded up and loosely adherent, but not yet fully detached. If this is done properly, the overall shape of the initial colony should still be apparent, and the hESCs are easily dislodged by gently flowing MEF-CM over them. A cell scraper should not be needed. On the other hand, excessive incubation in Versene adversely affects the viability and plating efficiency of the hESCs and may prevent successful cardiac induction. In general, lower-passage hESC cultures are more strongly adherent and require longer incubation times in Versene (~5-7 minutes), while higher-passage hESCs are easier to dislodge (~3-5 minutes). As noted above, re-plating the proper number of healthy hESCs should result in an 80-95% confluent monolayer by 24 hours.

7. Until the re-plated hESCs form a confluent monolayer with the compact appearance illustrated by Figure 1 (Day 0), continue to feed the cultures daily with MEF-CM supplemented with 4 ng/mL bFGF. When re-feeding, use MEF-CM plus bFGF at 100 μ L of per well of a 96-well plate well or 1 mL per well of a 24-well plate.

3.2.2 Induction of Cardiac Differentiation with Serial Activin A and BMP-4

1. When the re-plated hESCs assume the compact appearance illustrated by Figure 1 (Day 0), they are ready for induction with serial activin A and BMP-4. At this point (by convention, 'Day 0'), aspirate the MEF-CM and replace with RPMI-B27 medium supplemented with 100 ng/mL activin A, using 100 μ L per well of a 96-well plate well or 1 mL per well of a 24-well plate well.
2. On day 1 post-induction (i.e. 24 hours later), aspirate the activin A-containing medium, and very gently replace it with an equivalent volume of RPMI-B27 medium supplemented with 10 ng/mL BMP-4. Incubate the differentiating cultures for an additional four days without a medium change. (See Note 5.)
3. On day 5 post-induction, gently replace the BMP-containing medium with an equivalent volume of RPMI-B27 medium without exogenous growth factors.
4. Thereafter, feed the cells with RPMI-B27 medium every other day. Spontaneous beating activity typically commences sometime between days 9-11 post-induction and peaks around day 14.

3.2.3 Re-plating hESC-derived Cardiomyocyte Cultures for In Vitro

Experiments—Differentiated hESC-derived cardiomyocyte cultures can be enzymatically dispersed with trypsin for re-plating or direct use in transplantation experiments anytime after day 14 post-induction with minimal loss of viability. Dispersed cardiomyocytes will re-plate particularly well on gelatin-coated tissue culture plastic (coated as described above in section 3.1.1) or glass surfaces coated serially with PEI and gelatin. (To prepare the latter, coat the glass coverslips or glass-bottom dishes with 0.1% PEI at 4 °C overnight, rinse thoroughly with sterile water, and then gelatin-coat as described in section 3.1.1.)

1. To disperse the differentiated hESC-derived cardiomyocytes, remove the RPMI-B27 medium, and rinse the cells with PBS.
2. Remove the PBS, and replace it with 0.05 % trypsin supplemented with 63 U/mL DNase I (using 100 μ L/well of a 96-well plate or 1mL/well of a 24-well plate). Incubate the cells for 3-5 min at 37 °C, and monitoring them periodically under the microscope. Once the cells reach a point where they have rounded up but not yet detached, add an equivalent volume of 1X defined trypsin inhibitor, and gently pipet this solution over the cells to dislodge them.

⁵During this and other medium changes, take care not to dislodge the cell monolayer, which is particularly loosely adherent on days 1-5 post-induction. The best approach is often to aspirate only ~80% of the medium in any given well, leaving behind an undisturbed layer over the cells. Avoid creating turbulence when adding medium or handling the plates.

3. Collect the dispersed cells, and gently triturate them into a single-cell suspension. Remove the enzyme by centrifugation (300g for 5 minutes), and re-suspend the cells in RPMI-B27 medium supplemented with 20 % FBS. Use a hemacytometer to determine the cell count, and then re-plate on gelatin-coated surfaces at density of $1-5 \times 10^4$ cells/cm².
4. Feed the re-plated cells the next day and every other day thereafter, using an appropriate volume of RPMI-B27 medium without FBS or exogenous growth factors. The resultant cultures are typically comprised of ~30-60% cardiomyocytes. (See Note 6.)

3.3 Further Enrichment for Cardiomyocytes by Percoll Gradient Centrifugation

If a greater degree of cardiac purity is required, the directly differentiated cultures can be further enriched by Percoll gradient centrifugation (6, 12). This procedure is best performed on differentiating cultures around day 14-18 post-induction with activin A. Percoll gradient centrifugation of cultures at other timepoints or after re-plating steps may result in reduced yield and/or cardiac purity.

1. Begin by removing the usual RPMI-B27 medium and rinsing the differentiated cell preparation with PBS.
2. Remove the PBS, add Liberase Blendzyme IV (0.56 U/mL) supplemented with DNase I (63 U/mL), and incubate at 37 °C for 30 minutes. Gentle agitation or cell scraping is sometimes required to ensure cell detachment.
3. Collect the dispersed cells, and gently triturate them into a single-cell suspension. Remove the enzyme by centrifugation (300g for 5 minutes), and re-suspend the pelleted cells in 10 mLs of RPMI-B27 medium per starting plate.
4. Transfer each 10 mL cell suspension to a 50 mL tube and add a 12 mL layer of 40.5% Percoll solution to the bottom of the tube. Pipette very slowly (!) to avoid mixing the cells and Percoll solution.
5. Add a layer of 58.5% Percoll solution to the bottom of the tube, again pipetting slowly to avoid mixing of the layers. (A sharp interface should be visible between each of the three layers.)
6. Centrifuge at 1500g for 30 minutes with the brake off. (The tubes should be carefully balanced!)
7. After centrifugation, the cell pellet at the bottom of the tube (i.e. at the bottom of the 58.5% Percoll layer) will be highly enriched for cardiomyocytes (typically 80-95% positive for cardiac markers). Collect these cells, add a vast excess of RPMI, and centrifuge (300g for 5 minutes) to remove the Percoll. These cells can then be re-plated (see section 3.2.3) or cryopreserved as described below.

⁶The cardiac purity of the resultant cell preparations can be assessed by immunocytochemistry or fluorescent-activated cell sorting (FACS), using antibodies against any of a number of cardiac or muscle markers (e.g. Nkx2.5, sarcomeric actins, cardiac troponin, etc). We routinely use a commercially-available monoclonal antibody (clone A4.951, which can be purchased as hybridoma from American Type Culture Collection, Manassas, VA, or as purified antibody from multiple vendors) against β -myosin heavy chain, a striated muscle marker which is strongly expressed by human cardiomyocytes.

3.4 Cryostorage of hESC-derived Cardiomyocyte Preparations

3.4.1 Cryopreservation of hESC-derived Cardiomyocytes

1. Prepare a single-cell suspension of differentiated hESC-derived cardiomyocytes (as described in sections 3.2.3 or 3.3). Wash thoroughly with RPMI-B27 medium to ensure removal of enzymes, Percoll, etc., and determine the total cell number by hemacytometer.
2. Centrifuge the cells at 300g for 5 minutes, aspirate the supernatant, and gently resuspend the pellet in 250 μ L Cryostor CS10 per 10×10^6 cells, while slowly swirling in an ice-water bath.
3. Aliquot the resuspended cells into cryovials, using volumes <500 μ L to promote more uniform freezing and thawing. Transfer the cryovials to a controlled-rate freezer, previously chilled to 0 °C. Cool the samples from 0 to -7 °C at a rate of 1 °C/min, from -7 to -10 °C at 0.75 °C/min, and then finally from -10 to -80 °C at 1 °C/min. The frozen cardiomyocytes can then be transferred to liquid nitrogen for long-term storage (>1 year).

3.4.2 Thawing Cryopreserved hESC-derived Cardiomyocytes

1. Transfer the cryovial to a bucket of dry ice until ready for use. Pre-heat a 50 mL tube of RPMI-B27 medium to 37 °C.
2. Thaw the vial in a 37 °C water bath with gently agitation until the pellet is completely melted.
3. Add 1 mL of the pre-heated RPMI-B-27 medium to the cryovial and mix by gently shaking. Slowly transfer the resultant cell suspension in a drop-wise fashion to the remaining medium in the 50 mL tube.
4. Remove the cryopreservative by centrifugation (300g for 5 minutes), and re-suspend in an appropriate volume of RPMI-B27 medium. The thawed cardiomyocytes can then be re-plated on gelatin-coated surfaces as described in section 3.2.3.

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Figure 1. Directed cardiac differentiation protocol

A: Timeline for the protocol used to generate cardiomyocytes from human pluripotent stem cells. In brief, after growth to confluence in the undifferentiated state in MEF-CM plus bFGF, cultures are switched to differentiating conditions in RPMI-B27 medium and are serially pulsed with two growth factors, activin A (day 0) and BMP-4 (day 1). After day 5 post-induction with activin A, the differentiating cultures are grown in RPMI-B27 medium in the absence of exogenous cultures. Spontaneous beating activity commences on ~day 9-11 post-induction, and cultures may be harvested after day 14. **B: Photomicrographs illustrating morphological changes in cultures during the protocol.** Prior to treatment with activin A (day 0), the cultures should have formed a compact, 100% confluent monolayer that is composed of cells with a high nuclear-to-cytoplasmic ratio but greater irregularity in cell shape than do hESCs in a usual undifferentiated colony. By day 5, cells in

the monolayer are phase-bright, more loosely attached, and have a rounded morphology. Some cell death is to be expected at this stage, but the monolayer should still occupy >90% of the well surface area. By day 14, the cultures are far more heterogeneous and include widespread areas with spontaneous beating activity. After dispersion and re-plating at low density (day 25), the resultant differentiated cultures are comprised of individual and small clusters of contractile cardiomyocytes with either a spindled- or triangular morphology.

Scale bar = 50 μm .

Table 1

Preparation of Percoll Gradient Solutions (for 100 mL final volumes).

	40.5 % Percoll solution	58.5 % Percoll solution
Percoll (GE Healthcare/Amersham, Piscataway, NJ; cat. no. 17-0891-02)	40.5 mL	58.5 mL
1.5 M NaCl (sterile-filtered)	10 mL	10 mL
1 M HEPES (Invitrogen, Carlsbad, CA; cat. no. 15630-080)	1 mL	1 mL
H ₂ O (WFI-quality, Mediatech, Manassas, VA; cat. no. 25-055-CV)	48.5 mL	30.5 mL