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Efficient Differentiation of Cardiomyocytes from Human Pluripotent Stem Cells with Growth Factors

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Abstract

Human pluripotent stem cells have tremendous replicative capacity and demonstrated potential to generate functional cardiomyocytes. These cardiomyocytes represent a promising source for cell replacement therapy to treat heart disease and may serve as a useful tool for drug discovery and disease modeling. Efficient cardiomyocyte differentiation, a prerequisite for the application of stem cell-derived cardiomyocytes, can be achieved with a growth factor-guided method. Undifferentiated cells are sequentially treated with activin A and BMP4 in a serum-free and insulin-free medium and then maintained in a serum-free medium with insulin. This method yields as much as >75% cardiomyocytes in the differentiation culture within 2 weeks, and the beating cardiomyocytes have expected molecular, cellular and electrophysiological characteristics. In this chapter, we describe in detail the differentiation protocol and follow-up characterization focusing on immunocytochemistry, quantitative RT-PCR and flow cytometry analysis.

Keywords

cardiomyocytes; differentiation; flow cytometry analysis; growth factors; immunocytochemical analysis; pluripotent stem cells; qRT-PCR; serum-free medium.

1. Introduction

Human pluripotent stem cells (hPSCs) are a potential cell source for tissue engineering and cellular therapy to treat heart disease, as they have extensive proliferative capacity and can differentiate into functional cardiomyocytes. In addition, hPSC-derived cardiomyocytes can be an excellent system for evaluating cardiotoxicity in drug discovery—the ability to generate large amounts of cardiomyocytes with relevant physiological phenotypes offers considerable advantage over primary or immortalized cell models and could translate to accurate drug evaluation in a cost-effective manner. For these potential applications, controlled lineage-specific differentiation is one of the critical steps. In earlier studies, cardiomyocytes are generated from hPSCs by either embryoid body formation in serum-

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containing medium ([1-3]) or co-culture with mouse END2 cells ([4]). In later and recent studies, more efficient cardiomyocyte differentiation has been achieved with growth factors, small molecules and other specific culture conditions (for reviews, see [5-7]). Members of the TGFβ family (*e.g.* activin A and/or BMP2 or BMP4) and the Wnt regulators (*e.g.* Wnt3a added during early stage of differentiation and DKK1 during late stage of differentiation) have been found to promote cardiomyocyte differentiation from hPSCs ([8-14]). In particular, sequential treatment of hPSCs with two growth factors actvin A and BMP4 ([8]) or with small molecules targeting the Wnt pathway is sufficient to induce efficient cardiomyocyte differentiation ([15, 16]). In addition, extracellular matrix plays an important role in improving efficiency of cardiomyocyte differentiation ([17]), and glucose-depletion from culture medium containing abundant lactate can selectively enrich cardiomyocyte populations due to differential metabolic requirements for cardiomyocytes and non-cardiac cells ([18]).

Cardiomyocyte differentiation from hPSCs can be assessed by several methods. Observation of spontaneously beating cells is the first indication of successful cardiomyocyte differentiation. For further confirmation, examination of cardiomyocyte-associated gene expression is essential. Immunocytochemical analysis is useful not only to evaluate the expression of certain markers but also to obtain information on their subcellular localization. Alternatively, marker expression can be examined by qRT-PCR or Western blotting if antibodies are unavailable or incompatible for immunocytochemical analysis. Flow cytometry detecting cardiac-specific markers can be used to quantitatively analyze cardiac purity in differentiation cultures. Other *in vitro* functional assays such as pharmacological and electrophysiological analyses are also critical to confirm the cardiac phenotype, as described elsewhere ([3, 19-23]).

In this chapter, we provide a detailed protocol for activin A- and BMP4-directed cardiomyocyte differentiation from hPSCs in a serum-free medium as previously described ([8]) with minor modifications. We also describe several *in vitro* assays for the characterization of hPSC-derived cardiomyocytes. These methods have been developed using human embryonic stem cells (hESCs) but are also applicable to human induced pluripotent stem (iPS) cells.

2. Materials

2.1. Growth media

1. Knockout DMEM (Life Technologies, Catalog #10829-018) or DMEM/F12 (Life Technologies, Catalog #11330-057)
2. Conditioned medium from mouse embryonic fibroblasts (MEF-CM): Prepare MEF-CM as previously described ([24]). MEF-CM can be collected every day for 6 days, pooled, aliquoted and stored at −20°C. After thaw, use within a week when stored at 4°C (*see* **Note 1**).

[1]Materials such as medium components and growth factors require appropriate storage. Store aliquots at −20°C or −80°C in a manual defrost freezer. Avoid repeated freeze-thaw cycles. To prevent contamination, it is optional to supplement with 1% penicillin-streptomycin (Life Science Tech, Catalog # 15140-122) to media for growth of undifferentiated cells and differentiation.

3. RPMI/B27 insulin-free medium (500 ml): Mix 490 ml RPMI 1640 (Life Technologies, Catalog #11875) with 10 ml B27 insulin-free supplement (Life Technologies, Catalog #0050129SA) and pour into a 500 ml filter unit (0.22 μM, Corning, cellulose acetate, low protein-binding) and filter. Store the medium at 4°C and use within 2 weeks. Warm the desired aliquot of the medium at 37°C before use (*see* **Note 2**).
4. RPMI/B27 medium (500 ml): Similarly to RPMI/B27insulin-free medium, replace B27 insulin-free supplement with B27 supplement (Life Technologies, Catalog #17504-044) (*see* **Note 2**).
5. 10% FBS medium: DMEM (Life Technologies, Catalog #11965118) supplemented with 10% FBS.

2.2. Matrigel-coated plates

1. 1:4 Matrigel stock: Growth factor-reduced Matrigel (Becton Dickinson, Catalog #356231) is used for coating plates. To prepare Matrigel stock aliquots, slowly thaw Matrigel at 4°C overnight to avoid the formation of gel. Add 10 ml of cold knockout DMEM to the bottle containing 10 ml Matrigel, and transfer Matrigel to a 50-ml tube. Wash the Matrigel bottle with 20 ml cold knockout DMEM and transfer the medium to the Matrigel tube (total volume is now 40 ml; 1:4 diluted). Mix well and aliquot 1 or 2 ml into each pre-chilled tube; store at −20°C immediately.
2. Tissue culture plates and flasks: 6-well plates (Falcon, Catalog #3046) and T75 flasks (Corning, Catalog #430641) are used for hPSC culture. T225 flasks (Corning, Catalog #431082) are used for MEF culture.
3. To coat plates/flasks with Matrigel, slowly thaw 1:4 Matrigel aliquots at 4°C for at least 2 hrs to avoid the formation of a gel. Alternatively, add 28 ml cold knockout DMEM to each of the 2-ml Matrigel aliquot and pipet several times until the Matrigel dissolves into the solution. Knockout DMEM can be replaced with DMEM/F12.
4. Dilute the thawed Matrigel aliquots 1:15 in cold knockout DMEM (for a final dilution of 1:60).
5. Add diluted Matrigel to plates or flasks (0.5 ml/well for 24-well plates, 1 ml/well for 6-well plates and 7.5 ml/T75).
6. Incubate the plates or flasks at room temperature for at least 1 hr. The Matrigel-coated plates or flasks can be stored in a sealed container at 4°C for up to 1 week. Remove Matrigel solution immediately before use.

2.3. Cardiomyocyte differentiation

1. Cells: H7 hESCs, iPS(IMR90)-1 cells (WiCell Research Institute)

[2]For small-scale experiments, prepare less amount of the differentiation medium that can be used within 2 weeks. B27 supplements or B27 without insulin supplements can be aliquoted and stored at −20°C.

2. Collagenase IV solution (200 units/ml): Dissolve 20,000 units of collagenase IV (Life Technologies, Catalog #17104-019) in 100 ml knockout DMEM. Add all components to a 250 ml filter unit (0.22 μM, Corning, cellulose acetate, low protein-binding) and filter. Aliquot and store at −20°C until use.
3. Versene (EDTA) (Life Technologies, Catalog #15040-066)
4. Trypan blue (Life Technologies, Catalog #15250061)
5. Recombinant human basic fibroblast growth factor (bFGF) (10 μg/ml): Dissolve 10 μg bFGF (Life Technologies, Catalog #13256-029) in 1 ml D-PBS with 0.2% bovine serum albumin (BSA, Sigma, Catalog #A2153). Filter the solution using a 0.22 μM Corning cellulose acetate, low protein-binding filter. When handling bFGF, prewet all pipette tips, tubes and the filter with D-PBS + 0.2% BSA (bFGF can bind to pipettes, tubes and filters, and this will prevent some loss of the factor). Store stocks at −20°C or −80°C for long-term storage. Store thawed aliquots at 4°C for up to 1 month.
6. D-PBS without $Ca^{2+}Mg^{2+}$ (Life Technologies, Catalog #14190-144).
7. 0.25% trypsin with 0.53 mM EDTA (Life Technologies, Catalog #25200-056). Store aliquots at −20°C.
8. Recombinant human activin A (R&D Systems, Catalog #338-AC): Reconstitute in sterile D-PBS containing 0.2% BSA to prepare a stock solution of 100 μg/ml. Store aliquots at −20°C.
9. Recombinant human bone morphogenetic protein-4 (BMP4, R&D Systems, Catalog #314-BP): Reconstitute in sterile 4 mM HCl containing 0.2% BSA to prepare a stock solution of 10 μg/ml. Store aliquots at −20°C.
10. Defined trypsin inhibitor (Cascade Biologicals, Catalog #R-007-100).
11. Incubators: all cell culture conditions are performed in humidified incubators in a 5% CO_2-95% air atmosphere at 37°C.

2.4. *In vitro* characterization

1. Paraformaldehyde (PFA): Prepare fresh 2 or 4% PFA solution by diluting 16% PFA (Electron Microscopy Science, Catalog #15710) in D-PBS. The solution can be stored at 4°C in a tube covered with foil and used within a week. Perform under a chemical hood when using PFA solution.
2. Ethanol, 200 proof (Sigma, Catalog # E7023)
3. Normal goat serum (NGS) (Life Technologies, Catalog #16210): Heat-inactivate NGS by incubating the serum in a 56°C water bath for 30 min and gently swirl the bottle every 10 min during incubation. Store the heat-inactivated serum in small aliquots at −20°C. Prepare a 5% or 1% NGS solution in D-PBS. Store at 4°C and use within 2 weeks.
4. Primary antibody for immunocytochemical analysis: Mouse IgG1 against α-actinin (1:200, Sigma) and rabbit antibodies against NKX2-5 (1:200, Santa Cruz Biotech).

For each new lot of primary antibody, it is highly recommended to titrate the antibody.

5. Secondary antibodies for immunocytochemical analysis: FITC-conjugated goat anti-mouse IgG (Sigma, Catalog #F2012), goat anti-mouse IgG1 conjugated with Alexa 488 (Life Technologies, Catalog # A-21121), or goat anti-rabbit IgG conjugated with Alexa 594 (Life Technologies, Catalog #A-11012)
6. Vectashield® mounting media containing DAPI (4', 6-diamidino-2-phenylindole) (Vector Laboratories, Catalog #H-1200)
7. Qiagen RNeasy kit (Qiagen, Catalog #74104) or Aurum total RNA mini Kit (Bio-Rad, Catalog #732-6820)
8. RNaseZap (Ambion, Catalog #AM9780)
9. Nuclease-free water (Ambion, Catalog #AM9939)
10. Qiashredder column (Qiagen, Catalog #79656)
11. Bench-top centrifuge (Eppendorf centrifuge 5424)
12. Nanodrop spectrophotometer (Thermo Scientific)
13. DNase I (Ambion, Catalog # 18047-019)
14. Superscript VILO cDNA synthesis kit (Life Technologies, Catalog #11754-250)
15. Thermal cycler (Bio-Rad, C1000 touch)
16. TaqMan gene expression assays (Applied Biosystems)
17. TaqMan master mix (Applied Biosystems, Catalog #4369016)
18. iTaq SyBr green master mix (Bio-Rad, Catalog #172-5121)
19. Forward and reverse primers (100 μM, Integrated DNA Technology)
20. Optical 96-well thermal cycling plates (Gene Mate, Catalog #T-3107-1)
21. Polyolefin sealing film (Gene Mate, Catalog #T-2450-1)
22. 7500 or 7700 Sequence Detection System (Applied Biosystems)
23. 10% FBS medium or defined trypsin inhibitor (Cascade Biologicals, Catalog # R-007)
24. Staining buffer: D-PBS with 2% FBS (Life Technologies, Catalog #10439-024)
25. Methanol (Sigma, Catalog #34860-IL-R): Pre-chill aliquots by storing at −20°C.
26. Blocking buffer: Staining buffer supplemented with 20% heat-inactivated NGS
27. Primary antibodies for flow cytometry analysis: Mouse IgG1 against α-actinin (Sigma, Catalog #A7811; use at 0.5 μg/5×10^5 cells/100 μl), mouse IgG2b against cardiac troponin I (cTnI) (Millipore, Catalog #MAB1691; use at 0.05 μg/5χ10^5 cells/100 μl). Isotype controls: mouse IgG1 (Becton Dickinson Biosciences, Catalog #554121), mouse IgG2b (BD Bioscience, Catalog #557351)

28. Secondary antibodies for flow cytometry analysis: Alexa 488 goat anti-mouse IgG1 (Life Technologies, Catalog #A-21121) or Alexa 647 goat anti-mouse IgG2b (Life Technologies, Catalog #A-21242)
29. Ethidium bromide monoazide (EMA, Sigma, Catalog #E2028 or Life Technologies, Catalog #E1374): Prepare a stock solution as 5 mg/ml (5000x) in DMSO under a chemical hood and store as single-use aliquots at −20°C. Minimize exposure to light when making the stock since EMA is extremely light-sensitive.
30. FACS tubes (Becton Dickinson Biosciences, Catalog # 352052)
31. FACSCanto™ II Flow Cytometer (BD Biosciences)

3. Methods

3.1. Growth factor-guided cardiomyocyte differentiation

Culture of undifferentiated hPSCs—Stock cultures of undifferentiated hPSCs are maintained in feeder-free culture conditions and passaged every 5 to 7 days using collagenase IV or Versene. Examples are given using cells maintained on Matrigel in MEF-CM ([24]). Similar method can be used for cells maintained in serum-free medium supplemented with growth factors ([14]). Detailed methods for culture and characterization of undifferentiated hPSCs are described elsewhere ([25]). Note that successful cardiomyocyte differentiation is highly dependent upon the quality of undifferentiated cells (*see* **Note 3**).

Cardiomyocyte differentiation can be achieved through sequential treatment of activin A and BMP4 in RPMI/B27 medium ([8]). As illustrated in Fig. 1A, to induce cardiomyocyte differentiation, undifferentiated cells are first cultured on Matrigel in MEF-CM for a few days and then treated with activin A for 1 day followed by BMP4 for 4 days in RPMI/B27 insulin-free medium. Insulin-free B27 is expected to improve differentiation efficiency because insulin negatively affects cardiomyocyte differentiation ([26, 27]). Subsequently, the growth factors are removed, and the cells are maintained in RPMI/27 for 10 to 20 days. Cells are harvested for *in vitro* characterization at the end of differentiation or earlier during differentiation when characterizing progenitors.

3.1.1 Setup differentiation cultures

1. After stock culture of undifferentiated cells has been maintained for 4 to 6 day or until colonies occupy approximately 80% of the well surface area, cells are ready to be passaged and set up for cardiac differentiation. Example here is stock culture maintained in 6-well plates.
2. Warm up required amount of MEF-CM for setting up differentiation cultures and supplement the MEF-CM with 8 ng/ml bFGF (*see* **Note 4**).

[3]In early passage cultures, spontaneously differentiated cells in between colonies of undifferentiated cells appear fibroblast-like. When the cells reach higher passages, the amount of fibroblast-like cells typically is reduced while the percentage of undifferentiated cells increases in cultures ([14]). To passage stock cultures using collagenase, incubation time varies among different passages of the cells and different batches of collagenase; therefore, it is advised to determine the appropriate incubation time by examining the colonies. Stop incubation when the edges of the colonies start to pull away from the plate. Typically, cells at higher passages require shorter collagenase incubation time.

3. Remove medium from each well of the stock culture and rinse cells with 2 ml D-PBS/well.
4. Aspirate D-PBS and add 2 ml Versene to each well and incubate at 37°C for 5 min (*see* **Note 5**).
5. Remove Versene and add 1 ml MEF-CM into each well.
6. Dislodge cells by gently adding 1 ml MEF-CM to the wells and then triturating (approximately 10 times) with 1 ml pipet tip to make it single cell suspension (*see* **Note 6)**. Transfer and pool the cells into a 50-ml conical tube.
7. Further, take 1 ml MEF-CM to wash each well by transferring MEF-CM from one well to another and finally pool into the conical tube.
8. Count cells using trypan blue with a hemacytometer and make dilution of required cells in a 50-ml tube (which works better than a 15-ml tube for properly mixing and evenly distributing cells into wells).
9. Seed 2×10^5 to 4×10^5 cells in 1 ml of MEF-CM for each well of a 24-well Matrigel-coated plate. For other culture formats, seed cells at 1×10^5 to 2×10^5 cells/cm^2. Feed cells daily by replacing MEF-CM supplemented with bFGF (8 ng/ml) until cells compactly cover the wells (~100% confluence) (*see* **Note 7**).
10. Passage the rest of the culture as stock culture using 200 units/ml collagenase IV or Versene. Detach the cells from surface using a cell scraper and triturate the cells less than 10 times using a 5-ml pipet and culture the cells on Matrigel-coated 6-well plates in MEF-CM supplemented with 8 ng/ml bFGF.

3.1.2 Growth factor-induced differentiation—When undifferentiated cells reach full confluence, typically 2 to 4 days after the seeding, the cultures are sequentially treated with activin A for 1 day followed by BMP4 for 4 days (*see* **Note 8**). The day when activin A treatment starts is designated as differentiation day 0.

1. Aspirate medium and add 1 ml/well of RPMI/B27 insulin-free medium supplemented with 100 ng/ml activin A onto a 24-well plate. Adjust medium volume based on culture vessel surface areas, if other culture formats are used (We have obtained successful differentiation in 96-well plates and T75 or T225 flasks).
2. 24 hrs later (on differentiation day 1), aspirate the medium to remove activin A.

[4]Before feeding or passaging cells, aliquot the amount of medium needed each time and warm up only the aliquot rather than the entire bottle of medium. Avoid repeated warming and cooling or overheating the medium. Add the appropriate amount of growth factors right before feeding.

[5]Observe cells under a microscope during treatment of Versene. Stop incubation with Versene if the edge of colonies curls up and cells become rounded up. If not, incubate cells for another 3 min, but not more than 10 min in total. Typically, cells at early passages require longer time incubation with Versene while cells at late passages need shorter treatment with Versene.

[6]Do not over triturate the cells; some of the cells may still stay as small clumps of cells after the dissociation.

[7]The doses of growth factors are critical for achieving efficient differentiation of cardiomyocytes. For example, H7 cells respond to activin A in a dose-dependent manner—a high dose of activin A (100 ng/ml) induces differentiation reaching higher purity of cardiomyocytes compared with lower doses of activin A (25 or 12 ng/ml) (data not shown). Therefore, it is important to make sure growth factor aliquots are stored and handled appropriately to maintain their biological activity.

[8]Cell density at the time of growth factor induction plays an important role in cardiomyocyte differentiation. It is important to make sure that cells are seeded evenly and reach full confluence before induction (Fig. 1B). It is advisable to establish the most efficacious cell seeding density and determine the optimal timing of induction in pilot experiments.

3. Add RPMI/B27 insulin-free supplemented with BMP4 at 10 ng/ml (1 ml/well for a 24-well plate).
4. Cells are maintained in the BMP4-containing medium for 4 days. No medium exchanges are performed until day 5. During this treatment, some cell death is observed, and surviving cells will continue to differentiate and proliferate.
5. On differentiation day 5, aspirate the medium to remove BMP4, and add RPMI/B27 (1 ml/well for a 24-well plate).
6. Change medium every other day. Beating cells usually appear from day 9 onwards.
7. Harvest cells at day 14 to 20 for flow cytometry analysis to determine the percentage of cells expressing cardiac markers, such as α-actinin and/or cardiac troponin I, or for further enrichment and characterization (see following section). The cells can also be cryopreservated for later use in cell transplantation and other applications. The cryopreservation procedure is described in detail elsewhere ([14]).

3.1.3 Cell harvesting

1. Feed cells a day before harvesting.
2. Remove medium and add D-PBS (0.5 ml/well of a 24-well plate) to wash the cells.
3. Remove D-PBS, add 0.25% Trypsin/EDTA (0.5 ml/well of a 24-well plate) and incubate the cells at 37°C for 5 to 7 min.
4. During incubation, add 10% FBS medium or trypsin inhibitor to 15 ml centrifuge tubes (1.5 ml/tube for a well of cells in a 24-well plate).
5. Observe cells under a microscope. When cells are rounded up, gently pipet up and down a few times with a 5-ml pipet to dislodge cells from surface.
6. Incubate partially dissociated cells at 37°C for additional 1 to 2 min. Skip this step if dissociation is complete.
7. Pipet up and down 5 to 7 times with a 5-ml pipet. Observe cells under a microscope to ensure complete dissociation.
8. Transfer the cell suspension to a tube containing 10% FBS medium to neutralize trypsin. If >15 samples are processed, add 10% FBS medium (0.5 ml/well of a 24-well plate) to each well before performing the dissociation and transfer. 10% FBS can be replaced with defined trypsin inhibitor if serum-free is required for the procedure.
9. Wash the plates with 10% FBS medium (1 ml/well of a 24-well plate). Observe the plate under a microscope to make sure the harvesting is complete. Mix the cell suspension well.
10. Centrifuge at 300g for 5 min and resuspend cells in 10% FBS medium. Skip this step if cell counting can be done within 30 min.
11. Perform cell counting using trypan blue and aliquot cells for flow cytometry assays.

12. For immunocytochemistry or other assays, centrifuge, resuspend the cells in RPMI/B27 medium and seed the cells onto Matrigel or gelatin-coated 96-well plates or chamber slides.

3.2. *In vitro* characterization

3.2.1. Immunocytochemistry analysis—Immunocytochemistry analysis allows detection of expression and cellular location of proteins/antigens of interest.

1. Plate dissociated differentiation cultures in RPMI/B27 onto Matrigel-coated chamber slides (*see* **Note 9**) and culture for 2 to 7 days. Change medium every 2 to 3 days.
2. Fix cells with 2% PFA in D-PBS at room temperature for 15 min.
3. After washing with D-PBS, permeabilize cells in 100% ethanol for 2 min. After another wash, incubate cells in 5% NGS in D-PBS at room temperature for 2 hrs or at 4°C overnight.
4. Incubate the cells at room temperature for 2 hrs with primary antibodies, e.g., antibodies against cTnI, α-actinin, or NKX2-5, diluted appropriately in 1% NGS in D-PBS (*see* **Notes 10 and 11**).
5. After washing, incubate the cells with corresponding secondary antibodies diluted in D-PBS containing 1% NGS at room temperature for 30 min to 1 hr in the dark. For single staining of cTnI, use FITC-conjugated goat anti-mouse IgG (1:120). For double staining of α-actinin and NKX2-5, use goat anti-mouse IgG1 conjugated with Alexa 488 (1:1000) together with goat anti-rabbit IgG conjugated with Alexa 594 (1:1000).
6. Wash the cells 3 times with D-PBS (5 to 10 min/washing) and mount the slides with Vectashield® mounting media containing DAPI for examination with a UV microscope. Images can be merged as in Fig. 1C.

3.2.2 Quantitative RT-PCR analysis—qRT-PCR analysis is an alternative method to determine relative gene expression, particularly when specific antibodies are lacking.

1. Remove culture medium from cells maintained in 24 or 6-well plates and wash with PBS. Harvest the cells by adding 350 to 700 μl lysis buffer to each well of cell culture.
2. Isolate RNA using a Qiagen RNeasy kit or BioRad Aurum total RNA mini kit as per manufacturer's recommendations following the tissue isolation procedure recommended for the QiaShredder (*see* **Note 12**).

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[9]Chamber slides can be coated with 1:30 Matrigel at room temperature for 2 hrs since overnight Matrigel-coated surface tends to produce nonspecific background staining.

[10]To detect multiple markers at the same time, incubate cells with a mixture of the first antibodies, wash, and then incubated with a mixture of the corresponding secondary antibodies conjugated with dyes that can be detected through different filters.

[11]Due to lot-to-lot variations, it is recommended to work out the optimal concentrations for each specific lot of antibodies when performing immunocytochemical analysis or flow cytometry analysis.

[12]Great care should be taken to prevent RNase contamination during or after the isolation of RNA, therefore wipe RNA work area with RNAse Zap and use only RNase-free materials.

3. Prior to RT-PCR analysis, treat RNA samples with DNase I to remove contaminating genomic DNA.

4. Convert 1 μg RNA into cDNA in a 20 μl reaction using SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies). Set up the RT reaction in BioRad C1000 Touch Thermal Cycler as follows: 25°C for 10 min; 42°C for 120 min; 85°C for 5 min and then keep in 4°C.

5. Dilute cDNA 15-times by adding 280 μl of nuclease-free water and then take 2 μl for each reaction real-time PCR run.

6. Examine relative gene expression by real-time PCR using TaqMan primer-probe or SyBr Green reaction.

   For TaqMan PCR reaction, use specific primers and probes for cardiac markers (examples listed in Table 1). Each reaction mixture contains 1x RT-master mix, 1x TaqMan gene specific primer-probe (300 nM of each primer, and 80 nM of probe), and 2 μl diluted cDNA in a final volume of 20 μl. As a control, the samples are also subjected to the analysis of 18S ribosomal RNA by real-time RT-PCR.

   For SyBr Green reaction master mix, each reaction mixture contains 1x iTaq SyBr green master mix, 4 nM forward primer, 4 nM reverse primer, and 2 μl diluted cDNA in a final volume of 20 μl. As a control, the samples are also subjected to the analysis of house-keeping genes such as GAPDH.

7. Set up PCR reaction master mix without cDNA, mix well and distribute 18 μl of the master mix to each well of a 96-well PCR plate.

8. Add 2 μl of diluted cDNA to each well. Seal plate from all sides with RT-grade sealing film. Centrifuge plate at 1500 g for 2 min.

9. Perform real-time RT-PCR on the ABI Prism 7700 Sequence Detection System using following conditions: denaturation and AmpliTaq gold activation at 95°C for 10 min; and amplification for 40 cycles at 95°C for 15 sec and 60°C for 1 min.

10. Analyze the reactions using the software from the ABI Prism 7700 Sequence Detection System. The relative quantitation of gene expression can be obtained by normalization against endogenous 18S ribosomal RNA using the $\Delta\Delta C_T$ method described in the ABI User Bulletin, Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR, 2008 (Fig. 1D, *see* **Note 13**).

3.2.3 Flow cytometry analysis—Flow cytometry analysis permits quantitative analysis of purity of cardiomyocytes in differentiation cultures. Here we provide a procedure for intercellular staining of cardiomyocyte-associated protein α-actinin. Other intercellular proteins can be detected using similar method. Typically, dissociated cells are first labeled with EMA to allow distinguishing live and dead cells, fixed and permeabilized before detection of proteins/antigens with antibodies.

[13]It is recommended for each sample to run PCR reactions in triplicate along with minus RT and non-template controls. Triplicate biological cultures are used to derive relative levels of gene expression in mean ± standard deviation.

1. Harvest cells as described in Section 3.1. After cells are dissociated and counted, aliquot cells (0.5×10^6 to 1×10^6 trypan blue negative cells/test) into 15-ml tubes. For each culture, prepare 2 tests in one 15-ml tube, which will be separated into 1 test for isotype control/EMA staining, and 1 test for α-actinin/EMA staining after or during the blocking step.
2. Prepare 1 extra tube (0.5×10^6 cells/test) for unstained cell control and 2 extra tubes (0.5×10^6 cells/test) for single color staining compensation controls: one for EMA only and another for α-actinin staining only using the culture containing high amount of beating cells.
3. Wash cells with D-PBS (5 ml/tube).
4. Prepare EMA working solution: 1 μg/ml (dilute EMA stock solution 1:5 and add 1 μl of the diluted EMA per 1 ml staining buffer), used within the day of staining (*see* **Note 14**).
5. Centrifuge, remove D-PBS, resuspend the cells in staining buffer containing EMA at 1 μg/ml (0.5 ml/test) and incubate the cells on ice in the dark for 15 min. All centrifuge steps for flow cytometry analysis are performed at 300 g for 5 min.
6. Centrifuge, remove EMA and resuspend the cells in 1 ml D-PBS.
7. Place the tubes horizontally on ice and expose them to bright light (a reading lamp with a fluorescent light bulb) at a distance of 3 to 4 inches for 10 min.
8. Add D-PBS (2 ml/test), spin, remove EMA and resuspend the cells in 500 μl of D-PBS/sample.
9. Fix the cells by adding equal volume (0.5 ml/test) of 4% PFA under a chemical hood. Incubate the cells at room temperature for 15 min in the dark.
10. Centrifuge, remove PFA into a PFA waste container and resuspend the cells in 2 ml D-PBS/test to wash the cells under a chemical hood.
11. Centrifuge and resuspend the cells in the staining buffer. The fixed cells can be stored at 4°C overnight before continuing following steps.
12. Centrifuge, remove staining buffer and resuspend the cells in D-PBS (100 μl/sample).
13. Permeabilize the cells by adding 9 volumes (900 μl/sample) of cold methanol.
14. Mix well and incubate the cells on ice for 30 min in the dark.
15. Add 1 ml D-PBS/sample, mix, and centrifuge.
16. Remove the supernatant and resuspend the cells in blocking buffer (100 μl/test). Incubate cells with the blocking buffer at room temperature for 30 min.

[14]For safety, EMA liquid waste and all tips and tubes used for EMA need to be collected into separate liquid and solid containers, respectively, and handled appropriately according to safety guidelines.

17. Separate each sample into 2 FACS tubes —one for isotype control and one for α-actinin staining.
18. Prepare the primary antibodies and corresponding isotypes in 50 to 100 μl blocking buffer/test.
19. After blocking, incubate the cells for 20 to 30 min at room temperature with primary antibodies in the blocking solution. To titrate a new lot of antibody, for example, use sarcomeric α-actinin at 0.5 to 5 μg/test in 100 μl.
20. Wash the cells twice with staining buffer (2 ml/test).
21. Prepare the secondary antibody, for example, Alexa 488-conjugated goat anti-mouse IgG1 (0.5 μg in 100 μl blocking buffer/test).
22. Remove the supernatant, and add 100 μl the secondary antibody to each tube, except for the EMA only tube, add blocking buffer instead.
23. Incubate the cells for 15 min at room temperature in the dark.
24. Wash the cells 3 times with staining buffer (2 ml/test). Resuspend the cells in 200 μl staining buffer/tube.
25. Use a FACSCanto™ II Flow Cytometer to acquire the data. Set PMT voltage using unstained controls and compensation using single color staining controls. Set quadrant markers based on isotype controls using the appropriate excitation and detection channels (FITC and PerCP Cy5.5 for α-actinin and EMA respectively). Acquire at least 10,000 EMA negative events.
26. Dot plots or histograms are generated upon data analysis using FlowJo software to display the frequency of α-actinin or isotype positive cells versus forward scatter in differentiated culture samples (Fig. 1E).

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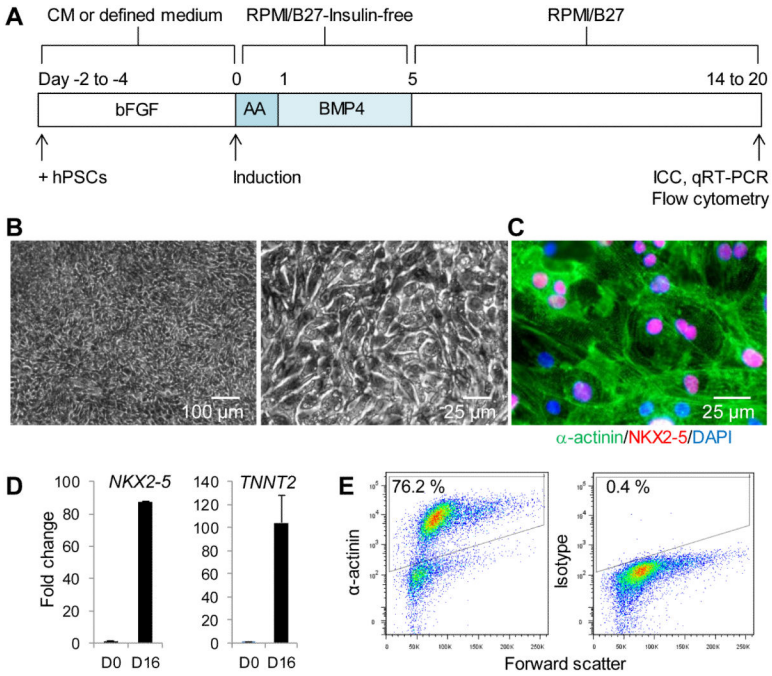

Fig. 1.

Cardiomyocyte differentiation and characterization. Differentiation procedure is shown in **A**. When undifferentiated cells became fully confluent after cultured on Matrigel in MEF-CM as shown in **B**, cells were induced to differentiate by treatment with activin A (AA) for 1 day followed by BMP4 for 4 days in RPMI/B27 insulin-free medium. Cells were maintained in RPMI/B27 for 10 to 15 days after the treatment of growth factors and were harvested for *in vitro* characterization, such as immunocytochemical analysis, qRT-PCR, and flow cytometry, as shown in **C, D** and **E**, respectively. The day of adding activin A is designated as day 0.

Table 1

Primers and probes for real-time RT-PCR assays

Genes	Sequences	
TaqMan assays:		
NKX2-5	Forward	ACCCAGCCAAGGACCCTAGA
	Probe	FAM-CGAAAAGAAAGAGCTGTGC-MGB
	Reverse	CTCCACCGCCTTCTGCAG
TNNT2	Primers and probe	Purchased from Applied Biosystems (Assay number Hs00165960_m1)
18S	Primers and probe	Purchased from Applied Biosystems (Assay number Hs03003631_g1)
SyBr Green reactions:		
NKX2-5	Forward	CTGTCTTCTCCAGCTCCACC
	Reverse	TTCTATCCACGTGCCTACAGC
	(http://primerdepot.nci.nih.gov/)	
TNNT2	Forward	GCGGGTCTTGGAGACTTTCT
	Reverse	TTCGACCTGCAGGAGAAGTT
	(http://primerdepot.nci.nih.gov/)	
GAPDH	Forward	CTGGGCTACACTGAGCACC
	Reverse	AAGTGGTCGTTGAGGGCAATG
	(http://pga.mgh.harvard.edu/primerbank/)	