Video Article

High Efficiency Differentiation of Human Pluripotent Stem Cells to Cardiomyocytes and Characterization by Flow Cytometry

Subarna Bhattacharya*[1], Paul W. Burridge*[2], Erin M. Kropp[1], Sandra L. Chuppa[1], Wai-Meng Kwok[3], Joseph C. Wu[2], Kenneth R. Boheler[4,5], Rebekah L. Gundry[1,6]

[1]Department of Biochemistry, Medical College of Wisconsin

[2]Stanford Cardiovascular Institute, Stanford University School of Medicine

[3]Department of Anesthesiology, Medical College of Wisconsin

[4]Stem Cell and Regenerative Medicine Consortium, LKS Faculty of Medicine, Hong Kong University

[5]Division of Cardiology, Johns Hopkins University School of Medicine

[6]Cardiovascular Research Center, Biotechnology and Bioengineering Center, Medical College of Wisconsin

*These authors contributed equally

Correspondence to: Rebekah L. Gundry at rgundry@mcw.edu

URL: http://www.jove.com/video/52010
DOI: doi:10.3791/52010

Keywords: Cellular Biology, Issue 91, human induced pluripotent stem cell, flow cytometry, directed differentiation, cardiomyocyte, IRX4, TNNI3, TNNT2, MCL2v, MLC2a

Date Published: 9/23/2014

Citation: Bhattacharya, S., Burridge, P.W., Kropp, E.M., Chuppa, S.L., Kwok, W.M., Wu, J.C., Boheler, K.R., Gundry, R.L. High Efficiency Differentiation of Human Pluripotent Stem Cells to Cardiomyocytes and Characterization by Flow Cytometry. *J. Vis. Exp.* (91), e52010, doi:10.3791/52010 (2014).

Abstract

There is an urgent need to develop approaches for repairing the damaged heart, discovering new therapeutic drugs that do not have toxic effects on the heart, and improving strategies to accurately model heart disease. The potential of exploiting human induced pluripotent stem cell (hiPSC) technology to generate cardiac muscle “in a dish” for these applications continues to generate high enthusiasm. In recent years, the ability to efficiently generate cardiomyogenic cells from human pluripotent stem cells (hPSCs) has greatly improved, offering us new opportunities to model very early stages of human cardiac development not otherwise accessible. In contrast to many previous methods, the cardiomyocyte differentiation protocol described here does not require cell aggregation or the addition of Activin A or BMP4 and robustly generates cultures of cells that are highly positive for cardiac troponin I and T (TNNI3, TNNT2), iroquois-class homeodomain protein IRX-4 (IRX4), myosin regulatory light chain 2, ventricular/cardiac muscle isoform (MLC2v) and myosin regulatory light chain 2, atrial isoform (MLC2a) by day 10 across all human embryonic stem cell (hESC) and hiPSC lines tested to date. Cells can be passaged and maintained for more than 90 days in culture. The strategy is technically simple to implement and cost-effective. Characterization of cardiomyocytes derived from pluripotent cells often includes the analysis of reference markers, both at the mRNA and protein level. For protein analysis, flow cytometry is a powerful analytical tool for assessing quality of cells in culture and determining subpopulation homogeneity. However, technical variation in sample preparation can significantly affect quality of flow cytometry data. Thus, standardization of staining protocols should facilitate comparisons among various differentiation strategies. Accordingly, optimized staining protocols for the analysis of IRX4, MLC2v, MLC2a, TNNI3, and TNNT2 by flow cytometry are described.

Video Link

The video component of this article can be found at http://www.jove.com/video/52010/

Introduction

The generation of cardiomyocytes from hPSCs, including hESC and hiPSC, can function as an *in vitro* model of very early human cardiac developmental processes, providing insight into stages not otherwise accessible for mechanistic studies. This model system provides unique opportunities to study the molecular pathways that control cardiac lineage commitment and cell fate specification. In recent years, the ability to efficiently generate cardiomyogenic cells from hPSCs has greatly improved[1-15]. However, among protocols there is cell line variation with respect to the efficiency in generating cardiomyogenic cells and timing at which the cells express chamber-specific markers (*e.g.*, ventricle and atria). Ideally, for future applications of this model system, more homogeneous populations of functionally defined cells are desired. In contrast to previous methods, the cardiomyocyte differentiation protocol described here does not require cell aggregation or the addition of Activin A or Bone morphogenetic protein 4(BMP4) and robustly generates cultures highly positive for TNNI3, TNNT2, IRX4, MLC2v, and MLC2a by day 10 cells across all hESC and hiPSC lines tested to date. The strategy is technically simple to implement, especially compared to three-dimensional cultures, mass culture, or embryoid body based strategies[4-9], and was recently defined in a study that describes a small molecule with selective toxicity to hPSCs (Boheler *et al.*)[65]. Features of this protocol include differentiation of hPSCs in monolayer culture using a single layer of a hESC qualified matrix (Matrigel), fully defined media using small molecules to modulate Wnt signaling (similar, yet distinct from[1,2,7,13]), and optimized flow cytometry staining methods for evaluation of differentiation efficiency and cell identity. In summary, advantages of this protocol compared to

previous reports include its cost-effectiveness, reproducibility, and its high efficiency for generating cardiomyocytes among multiple hPSC lines, including hESC and hiPSC lines.

Flow cytometry is a powerful analytical tool for assessing the quality of cells in culture and determining subpopulation homogeneity, and with proper experimental design, can provide quantitative measurements. As with all antibody-based strategies, accurate interpretation of experimental results requires that elements of the assay design including antibody concentration and fixation and permeabilization conditions (when targeting intracellular antigens) are carefully tested for each antibody as sub-optimal conditions significantly affect efficiency of antibody binding, and therefore, interpretation of results. Importantly, if quantitation is required, monoclonal antibodies are essential, as polyclonal antibodies can recognize multiple epitopes and are prone to batch-to-batch variation. Currently, a variety of antibodies (polyclonal and monoclonal) and staining protocols have been described for the assessment of *in vitro* differentiation, making it difficult to compare efficiency of cardiomyogenesis among protocols[1,2,9,11]. For that reason, monoclonal antibodies are used when available for all flow cytometry analyses. Going forward, it is expected that standardization of these staining protocols, especially with regards to quantitation, should better permit comparison among differentiation strategies.

The choice of markers, and their corresponding antibodies, used to assess purity of *in vitro* cardiomyogenesis varies among reports. TNNT2 has been considered an indicator of cells committed to the cardiomyogenic fate and is routinely used to assess efficiency of cardiac differentiation protocols. However, TNNT2 is also expressed in skeletal muscle during early chick and rat development[16,17] and it is present in human smooth muscle[18]. Thus, TNNT2 is not necessarily a specific marker of human cardiomyogenesis *in vitro*. MLC2v and MLC2a are routinely used as surrogate markers of ventricular and atrial subtypes, respectively. However, challenges with relying on MLC2v and MLC2a to determine cardiomyocyte subtype in the context of *in vitro* differentiation arise from the fact that these gene products may not be restricted to a specific chamber throughout cardiac development, from heart tube through adult. In the rodent looped heart, MLC2a mRNA is predominant in the atrial/inflow tract area and MLC2v mRNA is predominant in the ventricular/outflow tract regions. In the looped heart, co-expression of MLC2a and MLC2v mRNAs are observed in the inflow tract, atrioventricular canal, and the outflow tract[19,20]. By 3 days after birth, MLC2v mRNA is restricted to the ventricle and by 10 days after birth, MLC2a is restricted to the atria in the neonatal rat heart[19]. Therefore, interpretation of data regarding cardiomyogenesis efficiency and subtype identity must not only consider the presence and quantity of reference marker levels, but must consider the developmental stage(s) to which the timepoints of differentiation that are analyzed correspond. This is especially important considering that the maturation stage of cardiomyogenic cells generated by *in vitro* differentiation of hPSCs resembles most closely those of embryonic/fetal development[21-25]. Thus, relying on a marker's spatial expression in the postnatal heart may not be appropriate for the assessment of hPSC-derived cells, at least in some cases.

In an effort to facilitate the development of more specific criteria for defining cardiomyocyte identity *in vitro*, TNNI3 is considered to be a valuable marker for evaluating cardiomyogenesis *in vitro* as it is restricted to cardiac muscle throughout embryogenesis in chick and zebrafish[15,20] and is absent in human fetal skeletal muscle[26]. While TNNI1 is present in human fetal heart, TNNI3 is the only TNNI isoform present in normal adult heart[27,28]. Regarding cardiomyocyte subtype identity, IRX4[29-31] is an informative marker of cells with a ventricular fate. At the protein level, IRX4 has recently been shown to be restricted to the ventricle from linear heart tube through neonatal stages in the mouse[32]. Accordingly, optimized staining protocols for the analysis of TNNI3 and IRX4 by flow cytometry are described. To our knowledge, this is the first description of a method for efficient antibody-based staining and analysis of IRX4 levels in human cardiomyocytes by flow cytometry.

Protocol

1. Solution and Media Preparation

1. hESC Qualified Matrix Coating Stock Solution
    1. Slowly thaw hESC qualified matrix (5 ml) on ice at 4 ºC overnight. Dispense aliquots into pre-chilled, 1.5 ml sterile microcentrifuge tubes and immediately store at -20 ºC.
    NOTE: The volume of the aliquot will vary based on lot and typically ranges 270-350 µl. Manufacturer provides details regarding volume of aliquot required to achieve a 1x concentration upon dilution into 25 ml as described in step 2.1.

2. hPSC Media Stock Solutions
    1. Use ultrapure water as a diluent unless otherwise indicated. Sterilize all components using a 0.22 µm filter. Store the following as bulk solutions at 4 ºC: sodium bicarbonate (75 mg/ml); citric acid (10 mM, pH = 3).
    2. Sterilize all components using 0.2 µm syringe filter and store each as aliquots at -20 ºC: Rho kinase (ROCK) inhibitor Y-27632 (10 mM in DPBS, 100 µl aliquot); L-ascorbic acid 2-phosphate (64 mg/ml, 500 µl aliquot); sodium selenite (70 µg/ml, 100 µl aliquot); transferrin (50 mg/ml, 107 µl aliquot); fibroblast growth factor 2 (FGF2; 200 ng/µl in DPBS, 250 µl aliquot); transforming growth factor beta 1 (TGFβ1, 100 ng/µl in cold 10 mM citric acid,10 µl aliquot).

3. hPSC Media E8 Solution Composition
    1. Prepare media using aliquots prepared in Step 1.2: DMEM/F12 (with L-Glutamine and HEPES; 500 ml), sodium bicarbonate (3.62 ml; final: 543 µg/ml), L-ascorbic acid 2-phosphate (500 µl; final: 64 µg/ml), sodium selenite (100 µl; final: 140 ng/ml), transferrin (107 µl; final: 10.7 µg/ml), insulin (1 ml; final: 20 µg/ml), FGF2 (250 µl; final: 100 ng/ml), TGFβ1 (10 µl; final: 2 ng/ml).
    2. Combine all components, filter sterilize and store at 4 ºC for up to 2 weeks.

4. hPSC Media E8 with ROCK Inhibitor
    1. Add 15 µl ROCK inhibitor to 12 ml of E8 media prepared as above and mix well. Ensure that the final concentration is 10 µM after addition of cells/media in step 3.7.

5. Stock Solutions of Wnt Modulators

1. Store the following aliquots at -20 ºC. CHIR 99021 (6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1*H*-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile; 10 mM in DMSO, 15 µl aliquot). IWR-1 (4-(1,3,3a,4,7,7a-Hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-Benzamide; 10 mM in DMSO, 6 µl aliquot).

6. Differentiation Media
   1. Prepare differentiation media #1 with RPMI 1640 (with L-glutamine) with 2% B27 minus insulin supplement.
   2. Prepare differentiation media #2 with RPMI 1640 (with L-glutamine) with 2% B27 plus insulin supplement and 2% FBS.

7. Cell Wash Solution for Cell Culture
   1. Use Dulbecco's phosphate buffered saline (DPBS), without calcium or magnesium.

8. Cell Wash Solution for Flow Cytometry
   1. Use phosphate buffered saline (PBS, without calcium or magnesium) with 1% FBS. Store at 4 ºC.

9. Cell Maintenance Solution for Flow Cytometry
   1. Use Hank's Balanced Salt Solution (HBSS; without calcium or magnesium) with 5% FBS. Store at 4 ºC.

10. Fixation Solutions for Flow Cytometry
    1. Use Cytofix buffer as fixation solution for TNNI3 and IRX4 antibodies.
    2. Use 4% paraformaldehyde in 1x PBS (make fresh) as fixation solution for TNNT2 and MLC2a antibodies.
    3. Use 70% methanol/30% acetone as fixation solution for MLC2v antibody.
    4. Store all solutions at 4 ºC.

11. Permeabilization Solutions for Flow Cytometry
    1. Use Phosflow Perm Buffer III as permeabilization solution for TNNI3 and IRX4 antibodies. Store at room temperature (25 °C).
    2. Use 0.2% Triton X-100 in 1x PBS as permeabilization solution for TNNT2, MLC2v, and MLC2a antibodies. Store at 4 ºC.

12. Blocking Solution
    1. Use 10% goat serum in 1x PBS. Make fresh and store at 4 ºC until use.

2. Plate Coating

1. Slowly thaw 1 aliquot of hESC qualified matrix at 4 ºC for 30 min, add to 25 ml of chilled DMEM/F12 and mix well in sterilized tissue culture hood immediately before coating 6-well plates.
2. Add 1 ml per well of a 6-well plate under sterilized tissue culture hood (1 aliquot of the hESC qualified matrix is sufficient to coat four 6-well plates).
3. Allow hESC qualified matrix to set for 30 min at room temperature under the hood. Aspirate the excess hESC qualified matrix and add 2 ml of E8 media with ROCK inhibitor to each well. Use plates immediately, or if desired, store at 4 °C immediately after plating for up to 1 week and equilibrate to room temperature for 30 min prior to use.

3. Passaging and Maintenance of Undifferentiated hPSCs in Monolayer Culture

1. Maintain hPSCs in monolayer culture in 6-well plates and perform all steps under a sterile tissue culture hood.
2. Use hPSC lines that are well established (>p20) and exhibit a homogeneous morphology without the presence of cells with a neural, epithelial- or fibroblast-like morphology. Ensure cells proliferate robustly, and on average, passage every three days at 75% confluence when seeded at 0.75 x 10^6 per well.
3. Passage hPSCs with dissociation to the single cell level at least 5x prior to start of differentiation to ensure maximum differentiation efficiency. To passage hPSCs, aspirate E8 media and wash cells twice with 4 ml of 1x DPBS (pre-warmed to room temperature). Add 1 ml of the cell detachment solution (pre-warmed to room temperature) to each well and leave the well undisturbed for 3-7 min, until cell boundaries begin to round-up.
4. Use a cotton-plugged glass pipette with bulb to dislodge cells and transfer into a 15 ml conical tube containing 1 ml of DMEM/F12 media (to inactivate the cell detachment solution).
5. Remove 10 µl aliquot of cell solution and mix with 10 µl of trypan blue in a microcentrifuge tube. Count cells (*e.g.* automated or manual hemocytometer) while remainder of cells are collected by centrifugation at 130 x g for 5 min at room temperature. Using this protocol, expect 70-80% viability using an automated hemocytometer and going forward, base all cell numbers on total cell counts.
6. Aspirate media and resuspend the cell pellet in DMEM/F12 to a final concentration of 0.75 x 10^6 cells per 500 µl.
7. Add 500 µl of the cell suspension to each well of a 6-well plate where each well contains 2 ml E8 media with ROCK inhibitor, prepared in step 2.3. Leaving plate on the worktop, gently move in a front-to-back and side-to-side motion to uniformly disperse cells across the well. Return cells to incubator at 5% CO_2, 37 ºC.
8. Beginning 24 hr after passaging, replace media daily using 2 ml/well E8 without ROCK inhibitor. Optimize seeding density to achieve 100% confluence prior to start of differentiation. Seed 0.75 x 10^6 cells/well four days prior to start of differentiation, but optimize for each cell line as needed.

4. Cardiomyocyte Induction of hPSCs by Selective Modulation of the Wnt Pathway

1. Refresh media (2 ml/well) daily during days 0-7, and every other day after day 8.
2. On day 0, begin differentiation process by replacing E8 media with differentiation media #1. Add 1.2 µl CHIR (6 µM final) to each well. Repeat on day 1.

3. On days 2-3, replace with fresh differentiation media #1.
4. On day 4, replace with fresh differentiation media #1. Add 1 µl IWR-1 (5 µM final) to each well. Repeat on day 5.
5. On days 6-7, replace with fresh differentiation media #1.
6. On day 8, replace with fresh differentiation media #2.
7. Continue to replace with differentiation media #2 every other day for desired time of culture.
8. If desired, passage cardiomyocytes using the cell detachment solution following steps outlined in 3.3-3.6, but substituting media with differentiation media #2. During passaging, dissociate cardiomyocytes into small clusters of ~3-10 cells. Seed at ~6 x 10^5 cells per well using hESC qualified matrix coated wells and differentiation media #2.

5. Collection of Cells for Flow Cytometry

1. Perform all remaining aspects of steps 5-9 on the lab bench (*i.e.*, not sterile).
2. Aspirate growth media and wash cells twice with 1x PBS. Add 1 ml of the cell detachment solution (pre-warmed to room temp) to each well and leave undisturbed for 3-7 min, until cell boundaries begin to round-up. Use a cotton-plugged borosilicate glass disposable 9" pipette with bulb to dislodge cells and transfer into a 15 ml conical tube on ice.
3. Throughout remainder of protocol, maintain cells on ice and perform centrifugations at 200 x g for 5 min at 4 ºC.
4. Collect cells by centrifugation and aspirate solution. Resuspend cells in 10 ml cell wash solution using a 10 ml serological pipette with repeated trituration to ensure cell clumps are dispersed into single cells. Count cells as in step 3.5 while remainder of cells are collected by centrifugation.
5. Resuspend cells in cell wash solution taking care to completely disaggregate cell pellet and aliquot 1 x 10^6 cells per round bottom tube. Collect cells by centrifugation and aspirate solution.

6. Fixation and Permeabilization of Cells for Intracellular Antigen Staining

1. Add 100 µl fixation solution to cell pellet. Add solution drop-wise with continuous gentle vortexing and then set on ice for 15 min.
2. Add 3 ml cell wash solution. Collect cells by centrifugation and aspirate solution.
3. Add 100 µl permeabilization solution to cell pellet. Add solution drop-wise with continuous gentle vortexing then set on ice for 30 min. Use fixation and permeabilization conditions as outlined for each antibody in **Table 1**.
4. Add 3 ml cell wash solution. Collect cells by centrifugation and aspirate solution. Repeat for a total of two washes after permeabilization.

Primary Antibody (Clone)	Immunogen/ Epitope Recognized	Istotype Control	Amount of primary antibody per 1 x 10^6 cells in 100 µl		Fixation Solution	Permeabilization Solution	Secondary Antibody	Amount of secondary antibody/1 x 10^6 cells in 100 µl
			For percent positive measurements	For Antigen Quantitation				
TNNI3 (284 (19C7)	ISASRKLQL (human)	Mouse IgG2b	1.0 µg	3.0 µg	BD Cytofix	BD Phosflow perm III	Goat anti-mouse IgG2b-Alexa488	600 ng
TNNT2 (1C11)	Full length purified native human troponin T protein.	Mouse IgG1	1.0 µg	2.0 µg	4% PFA in 1% PBS	0.2% Triton X-100 in 1% PBS	Goat anti-mouse IgG1 - Alexa 488	600 ng
MLC2v (330G5)	FDPEGKG	Mouse IgG2a	2.0 µg	3.0 µg	70% methanol/30% acetone	0.2% Triton X-100 in 1% PBS	Goat anti-mouse IgG2a - Alexa 647	600 ng
MLC2a (4E7)	full length human recombinant protein of human MYL7 produced in *E. coli*	Mouse IgG1	0.5 µg	3.0 µg	4% PFA in 1% PBS	0.2% Triton X-100 in 1% PBS	Goat anti-mouse IgG1 - Alexa 488	600 ng
IRX4	LQEHRKNP YPTKGEKI MLAIITKM TLTQVST	Rabbit IgG	0.5 µg	0.5 µg	BD Cytofix	BD Phosflow perm III	Goat anti-rabbit IgG-PE	600 ng

Table 1. Antibody Concentrations. Listed are the primary antibody, clone (if monoclonal), optimized concentrations and fixation and permeabilization conditions for each primary and secondary antibody used for flow cytometry analyses. As antibody stock concentrations can vary among vendors of the same clone, final concentrations of each antibody per 1 x 10^6 cells in a fixed assay volume, rather than dilutions, are provided. The immunogen used to generate the antibody, or epitope recognized by antibody, as provided by manufacturer, is listed but was not experimentally verified here.

7. Antibody Staining

1. For every experiment, include one unstained control per fixation/permeabilization condition and the appropriate isotype control for each primary antibody used. Include non-cardiomyocyte cell types as negative controls (*e.g.*, pluripotent stem cells or fibroblasts). Use isotype controls at the same concentration as corresponding primary antibody (see **Table 1**).
2. Resuspend cells in 100 µl blocking solution using a P200 pipette to disaggregate cells. Set samples on ice for 25 min with gentle rocking.
3. Without removing blocking solution, add primary antibody or isotype control per guidelines in **Table 1**. Incubate 45 min on ice with gentle rocking.
4. Add 3 ml cell wash solution. Collect cells by centrifugation and aspirate solution. Repeat for a total of two washes after primary antibody labeling.
5. If a fluorophore-conjugated primary antibody is used, proceed to 8.1. When an unconjugated primary antibody is used (such as for all markers described here), perform labeling with secondary antibody that is conjugated to a fluorophore as follows.
6. Resuspend cells in 100 µl blocking solution using a P200 pipette to disaggregate cells.
7. Add secondary antibody per guidelines in **Table 1**. Incubate 30 min on ice with gentle rocking.
8. Add 3 ml cell wash solution. Collect cells by centrifugation and aspirate solution. Repeat for a total of two washes after secondary antibody labeling.

8. Preparation of Cells for Flow Cytometry

1. Resuspend cells in 400 µl cell maintenance solution using a P1000 pipette to disaggregate cells.
2. Prepare a cell strainer cap on a round bottom tube by pre-wetting with 50 µl cell maintenance solution. Set tube on ice. Use the cell strainer cap to prevent cell aggregates from clogging the flow cytometer.
3. Transfer cell solution to cell strainer cap and allow cell suspension to drain by gravity. Tap bottom of tube gently on bench top as necessary so that cells are collected into tube and set back on ice as quickly as possible. Rinse strainer with 250 µl cell maintenance solution to ensure maximal recovery of cells.
4. Maintain cells on ice and protect from light until analyzed by flow cytometry.

9. Flow Cytometry Analysis

Detailed acquisition settings will vary among instruments. Fundamental parameters to consider for optimal data collection are described below.

1. For optimal stream stability, ensure that the nozzle size exceeds 5-6x the diameter of the cell being analyzed.
   NOTE: This may vary among instruments but is an important consideration both for analyzers and selecting appropriate nozzle size for cell sorting. The average diameter of day 10 cardiomyocytes is 11 µm (ranging 8-14 µm); thus, a standard 150 µm sample injection tube on an analyzer works well.
2. Immediately prior to data analysis, vortex each tube briefly to disperse cell aggregates.
3. Optimize forward and side scatter voltage settings for each cell type based on unstained control for each fixation/permeabilization condition used in the experiment such that the populations of interest are on scale and centered (**Figure 2A**).
   1. Maintain these settings throughout a single experiment and be consistent from experiment to experiment on the same instrument, as significant shifts may indicate potential problems with the flow cytometer or sample preparation.
4. For each fluorophore, analyze the isotype control and adjust appropriate laser voltage to determine the minimum intensity required to obtain a fluorescence histogram that displays both left and right edges of the peak. Maintain the laser settings for each isotype control when acquiring data on corresponding antibody-stained samples.
   NOTE: Signal drift often occurs over time on the same instrument. It is critical to adjust laser settings at the beginning of each experiment based on the appropriate isotype control.
5. Collect a minimum of 10,000 events. 50,000 events are preferred.
6. For statistical analyses, gate the live cell population to exclude debris (**Figure 2A**). Determine percent positive cells within the gated population based on marker placement that allows ≤2% contribution from isotype control, as shown in **Figure 2B,C**.

Representative Results

On day 0, cells are 100% confluent with compact morphology and minimal cell debris. On days 1-2, it is common to observe significant cell death (40-50%), but attached cells will retain compact morphology (**Figure 1A**). During this time, media is orange and turbid. Pink media indicates excessive cell death, and in this case, confirm with trypan blue and discontinue if cell death exceeds 70%. Cells will recover during days 3-4 and density will increase. During days 5-6, minimal cell death occurs and dense patches may begin to appear. By days 7-8, a confluent monolayer is achieved with compact morphology interspersed with dense patches. Cells begin spontaneously contracting by day 8 and the first contractions will often be visible in the dense patches. By day 10, more robust contraction is observed and will continue to spread throughout the culture in subsequent days. Immunocytochemistry staining for α-actinin and TNNT2 show sarcomere structure (**Figure 1A**). As measured by quantitative real time polymerase chain reaction (qRT-PCR), mRNA levels of reference markers of mesoderm and cardiomyogenic commitment display temporal profiles as expected (**Figure 1B**), with robust expression of cardiac markers Homeobox protein Nkx-2.5 (NKX2.5), TNNI3, MLC2a, MLC2v, and myosin-6 (MYH6). Very low to non-detectable levels of smooth muscle (Myosin heavy chain 11, MYH11) and skeletal muscle (myogenin, MYOG) markers are routine. Consistent with early development where expression is observed in both cardiac and skeletal muscle before being restricted to skeletal muscle in the adult[20,27,28], troponin isoforms TNNI1 and TNNI2 are robustly detected during days 7-8.

Consistent with other reports, this *in vitro* differentiation strategy generates cardiomyocytes with characteristics of early cardiac progenitors (rounded morphology, co-expression of MLC2a, MLC2v)[19], and based on the high percentage of IRX4-positive cells, a significant majority of

the cells generated by this protocol appear to be committed to the ventricular phenotype[30,32]. In flow cytometry, the light scatter profiles will vary among cell types and change considerably among fixation/permeabilization conditions (**Figure 2A**). Under these various cell preparation conditions, flow cytometry analysis reveals isotype controls with single peaks with a clear distinction between isotype control and antibody (**Figure 2B**). Pluripotent cells or other non-cardiomyocyte cells are negative for the markers used here.

Using this differentiation and staining protocol, the resulting cell population is typically 99% TNNI3, 96% IRX4+, 91% MLC2v+, and 96% MLC2a+ by day 10 of differentiation as measured by flow cytometry. 99% TNNT2+ cells are observed (**Figures 2C, 2D**), but as described above, TNNT2 is not unique to cardiomyocytes during development and thus the assertion that the cells are 99% authentic cardiomyocytes by day 10 is based on TNNI3 protein. It is noted that with continued culturing beyond day 10, protein levels of structural proteins will remain high although relative gene expression levels will decrease relative to peak expression as monitored by qRT-PCR, consistent with protein stability and turnover rates of 3.2 and 3.5 days, respectively, for TNNI and TNNT[33]. An example of this observation is shown for TNNT2 (peak mRNA expression at day 11 (**Figure 1B**), robust protein levels at day 20+ (**Figure 2E**).

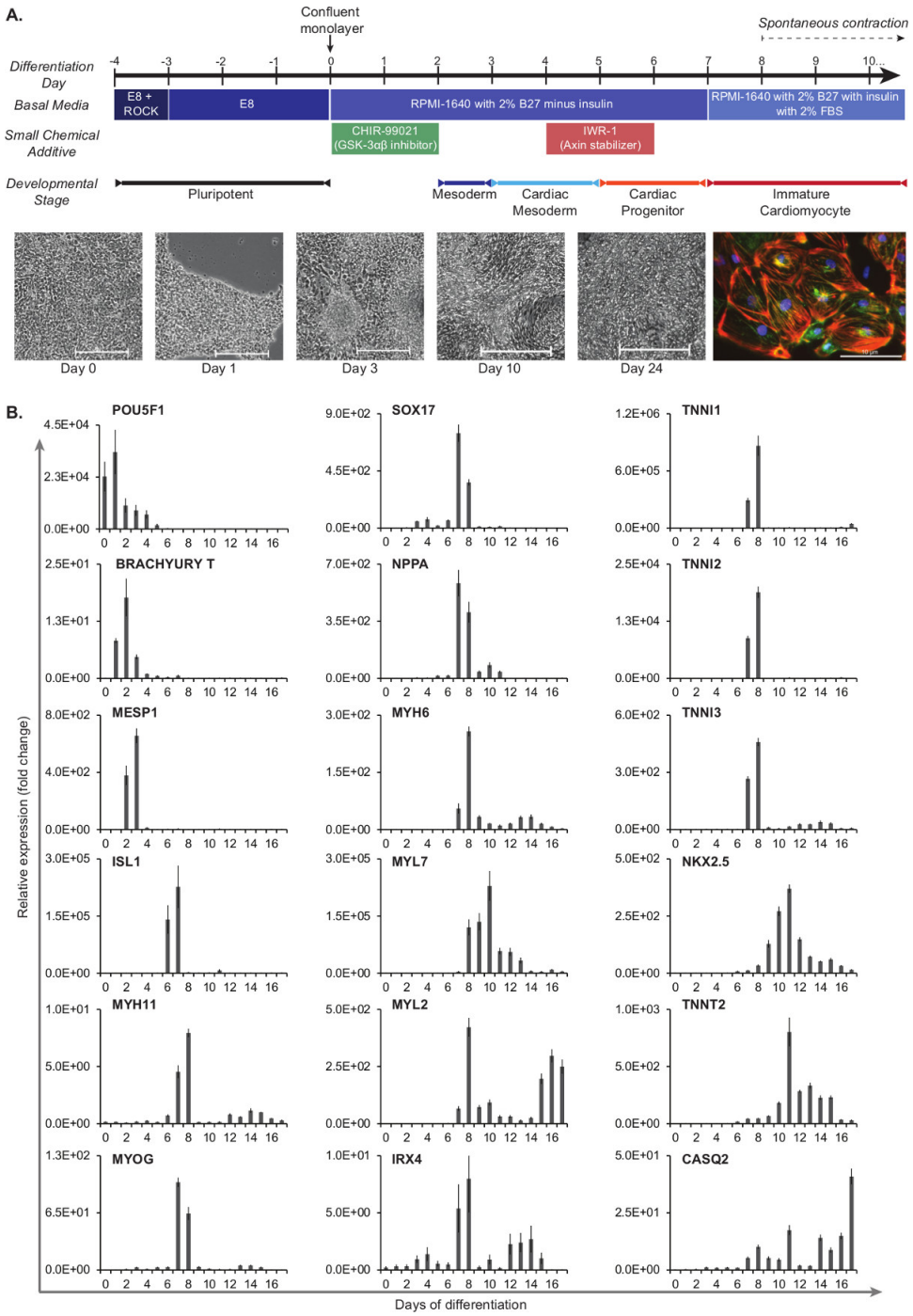

Figure 1. Overall schematic of cardiomyocyte differentiation from hPSCs and representative data. (A) Schematic of cardiomyocyte differentiation protocol annotated with corresponding stage of lineage and/or cell fate commitment. Representative phase contrast images of cells at major stages of the differentiation process. Scale bar = 10 μm. Far right = immunocytochemistry image of α-actinin (green) overlaid with TNNT2 (red) and nuclei (blue). **(B)** qRT-PCR analysis (for probe set, see the Materials table) of 18 reference mesoderm and cardiac development markers during the first 17 days of differentiation. All data are an average of three biological replicates each analyzed in triplicate, where error bars represent standard error of the mean, and are normalized to actin (ACTB). Message levels for all time points are relative to the first day of differentiation where the Ct values are detectable (*i.e.* Ct <35). Please click here to view a larger version of this figure.

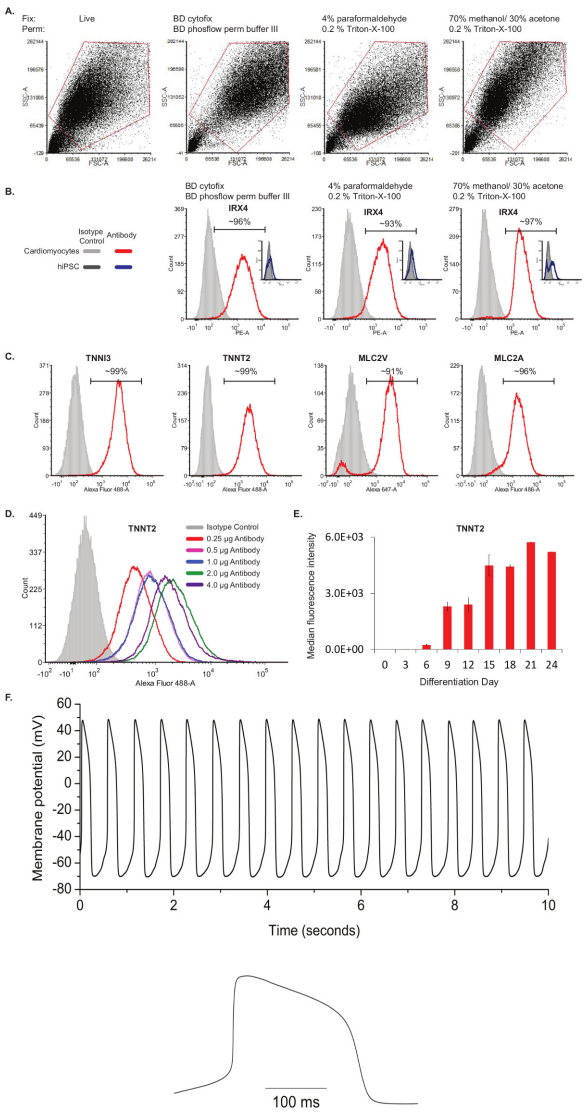

Figure 2. Characterization of cardiomyocytes by flow cytometry and electrophysiology. (A) Light scatter profiles of day 10 hiPSC-derived cardiomyocytes under various fixation and permeabilization conditions. 50,000 events were collected and the gated population (*i.e.* excluding debris) used for statistical analyses is shown in red. **(B)** Histograms of IRX4 on day 10 of differentiation using three different fixation/ permeabilization conditions, illustrating differences in staining efficiency among the methods. Insets show differences in staining of IRX4 on hiPSCs using each condition. These data illustrate why methanol/acetone is avoided for IRX4 due to low, but detectable, staining on hiPSCs. **(C)** Histograms of TNNI3, TNNT2, MLC2v, MLC2a on day 10 of differentiation using optimized staining conditions for maximum percent positive cells detailed in **Table 1**. **(D)** Histograms of TNNT2 from antibody titration experiments, illustrating that 0.5 µg is the minimum amount required for measuring 99% TNNT2+ cells, but that 2 µg is the saturation point required for quantitation. Only a single isotype control is shown, but titration experiments are calculated based on comparing each antibody to its corresponding titration of isotype control. **(E)** Quantitation of TNNT2 protein on cardiomyocytes on days 0-24 of differentiation, represented as the median fluorescence intensity difference between antibody and isotype control for each time point. **(F)** Action potential recorded from a single spontaneously contracting ventricular-like cardiomyocyte on day 46 of differentiation using the whole-cell current clamp configuration of the patch clamp technique. Please click here to view a larger version of this figure.

Discussion

Critical to the success of the differentiation protocol is the use of high quality cultures of hPSCs that have been passaged at the single cell level for at least five passages prior to the start of differentiation. Similar differentiation efficiencies are routinely observed among various hPSC lines if they are 100% confluent at the start of differentiation, independent of cell line. Suboptimal efficiency is observed if the confluence of cells at the start of differentiation is ≤95% or >100%. Therefore, seeding density and timing of passaging should be optimized carefully for each line to achieve 100% confluent monolayers at the start of differentiation. In this regard, plating an explicit number of cells/well, rather than using a split ratio, rapidly facilitates successful implementation of the protocol regardless of the user's experience because it eliminates the subjective nature of estimating confluence macroscopically. Commercial preparations of E8 media can also be used, although the formulation detailed here offers significant cost-savings. As an alternative to the hESC qualified matrix, a growth factor-reduced LDEV-Free hESC-qualified matrix has been used successfully. Routine passaging can also be performed with EDTA-based cell detachment solutions. While it is possible to maintain cells

in differentiation media #2 after day 8 without the addition of serum, enhanced survivability is observed when 2% FBS is included, but it should be noted that serum can cause hypertrophy of cardiomyocytes *in vitro*. When clinical applications are required, it should be possible to optimize the protocol to work without the addition of FBS and on an alternative matrix that is xeno-free and fully defined, though these have not been evaluated here. Cells can be successfully maintained for more than 90 days in culture when using serum-free RPMI-B27 plus L-glutamine on 0.1% polyethyleneimine coated plates as described[34].

With regards to flow cytometry, blocking non-specific interactions is critical to minimizing background signals. Importantly, consider that IgG concentrations in serum vary among species[35,36]. For this reason, goat serum is used routinely as it has higher concentrations of IgGs compared to rabbit and fetal bovine serum. Fc receptor blocking reagents can also be employed with some primary antibodies, but at higher cost. Implementing this flow cytometry preparation protocol for other protein targets should therefore consider the source and subtype (*e.g.*, IgM) of primary antibody and if the primary is goat, donkey serum can serve as a suitable alternative as it has similarly high IgG levels, but this step should be optimized for each isotype to minimize background regardless of serum source.

Three different fixation and permeabilization conditions were tested for each antibody used here, and the conditions that reproducibly generated the highest percent positive cells are reported for each antibody while at the same time provided for negative staining in pluripotent stem cells and fibroblasts. As shown for IRX4, Cytofix/PhosFlow Perm III was selected because methanol/acetone generated low level positive staining in hiPSCs, likely a result of non-specific binding of the antibody to epitopes exposed under these fixation conditions. For antibodies other than those described here, conditions should be optimized individually for each new target as different fixation and permeabilization conditions will render different epitopes accessible. Thus, different antibodies against the same target protein, if recognizing a different epitope (or epitopes in the case of polyclonal antibodies), will each require testing. If cell surface antigens are targeted, the cell dissociation method used here is equally applicable as extracellular epitopes remain intact and the procedure can be performed as described, minus the fixation/permeabilization steps. The amount of each antibody was titrated, ranging 0.25-4.0 µg, to determine the minimum levels needed for efficient and reproducible staining of 1 x 10^6 cells in a defined volume (100 µl), and this is scalable if more/fewer cells are used. Importantly, two values for antibody amounts are included in **Table 1**. First, the minimum amount of antibody required for measuring the maximum percent positive cells as shown in **Figure 2B-C** is provided. This is distinct from the minimum amount of antibody required for quantitation of the protein levels within a cell population. For reliable quantitation, the amount of antibody must reach saturation levels without a significant increase in non-specific binding (*i.e.* shift in fluorescence for isotype and negative controls) and such that any small variation in the number of cells stained will not have a significant effect on the median fluorescence intensity, which is the value used in quantitative comparisons. An example of this is shown for TNNT2 in **Figure 2D**, where 0.5 µg is sufficient to stain 99% TNNT2+ cells, but saturation is reached around 2 µg and is determined by titrating the antibody amounts for a fixed number of cells and measuring the difference in median fluorescence intensity between antibody and corresponding isotype control. As the amount of antibody required to reach saturation levels is typically higher than that needed to determine percent positive, both values are provided so that the most appropriate, yet cost-effective, conditions can be implemented for routine analyses.

Fluorophores other than those listed here can also be used successfully. However, it is helpful to pair the emission characteristics of each with expected levels of each target (*e.g.*, bright fluorophores are used with lower abundance targets). As binding affinities vary among antibodies, flow cytometry cannot be used to quantitatively compare among different antigens unless methods for absolute quantitation are used (*e.g.*, quantitation beads). Thus, comparisons here regarding percent positive populations and median fluorescence intensity are restricted to comparing the same antigen across multiple time points of differentiation, with the realization that post-translational modifications or structural changes that affect antibody binding may also affect the quantitative assessments of specific proteins among samples. It is acknowledged that by virtue of the polyclonal antibody used for IRX4, this is not suitable for quantitative comparisons; however, the percent positive data are reliable and this protocol is valuable until suitable monoclonal antibodies become available. Of course, as with any antibody-based technique, data interpretation should consider the specificity of the antibody used (*i.e.* uniqueness of the epitope). Especially in the case of cardiac development, the distinct temporal and spatial distribution of the various embryonic/adult isoforms for informative markers during embryogenesis should be carefully considered when selecting targets, selecting antibodies that recognize the appropriate epitope, and interpreting data. Numerous comprehensive studies and reviews on this topic are available[19,20,29,37]. When considering the mRNA and protein levels over the time course of differentiation, protein abundance for markers including MYL2, TNNT2, and TNNI3 are robust after peak mRNA expression observed via qRT-PCR. mRNA levels in later stages (*e.g.* after day 12 for TNNT2) are detectable, but lower than the time point of peak expression (*i.e.* day 11 for TNNT2). Therefore, while protein levels for TNNT2 increase over time and stabilize, which at first glance may appear to be at odds with the mRNA expression pattern, once cells are committed to the cardiomyocyte fate (~day 10), stable proteins with slow turnover, like TNNT2, are maintained even though mRNA levels are relatively low compared to earlier in the differentiation process.

In flow cytometry, as with any antibody-based staining method, technical variation among staining approaches can be misinterpreted as biological variation. Therefore, it is expected that implementing standardized protocols for antibody labeling and analysis should better permit reliable comparison of the efficiency of cardiomyogenesis and subtype identity among protocols. As the field continues to establish robust protocols for efficient cardiomyogenesis, future efforts to more fully define the cells generated from such protocols will include the analysis of maturation stage and chamber-restricted markers such as hairy-related transcription factors 1 and 2 (HRT1, HRT2[38,39]), gap junction alpha-5 protein (GJA5, CX40[40]), sarcolipin (SLN[41]) natriuretic peptides A (NPPA[42,43]), potassium voltage-gated channel subfamily E member 1 (KCNE1[44]), T-box transcription factor TBX3 (TBX3[45]),heart- and neural crest derivatives-expressed protein(HAND1, HAND2[46-48]), and potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4[49-51]), among others. For marker analysis, it is recommended that both mRNA and protein levels be measured when possible, as protein modifications, stability and turnover can affect the relationship between gene expression and protein abundance (*e.g.,* TNNT2 in **Figures 1B, 2E**) and, during development, patterns of expression differ between mRNA and protein for some markers (reviewed in Franco *et al.*[20]). In summary, careful consideration of the complex temporal and spatial restriction for each marker during early embryogenesis should better inform marker selection for *in vitro* analysis. Moreover, morphological and structural characterizations (*e.g.*, sarcomere) and functional properties (*e.g.*, action potentials, calcium transients) will also be important for fully defining the functional potential of cells generated by *in vitro* differentiation protocols. Of course, as with any antibody-based strategy, interpretation of data ultimately relies on the specificity of the antibody used, and, as illustrated here, can be drastically affected by sample handling conditions.

Future applications of the cells generated by this protocol include studying the molecular pathways that control early cardiac lineage commitment and cell fate specification. However, before use, the functional identity of the cells generated by this protocol should be carefully considered as

to whether they provide the appropriate model for the question at hand. Consistent with other published protocols, this strategy generates cells that spontaneous contract and resemble cells at embryonic and fetal stages of heart development[3] in terms of electrophysiological signals[52,53] and gene expression patterns[21,23]. Because the highly complex process of heart development may not be entirely mimicked *in vitro*, terminal differentiation of chamber-specific cells may not occur in the absence of the three-dimensional positional cues found *in vivo,* although a recent report does present progress towards the generation of cells with more adult-like elongated morphology[34]. In contrast to previous reports that observed little to no MLC2v by days 15-20[1,14] as measured by flow cytometry, the protocol detailed here produces ~96% IRX4+, ~91% MLC2v+ cells by day 10 (**Figures 2B, 2C**). While multiple protocols will likely be valuable in future endeavors, the distinctions in timing of these signature markers should be considered when comparing protocols and selecting the most appropriate strategy for addressing a specific biological question. Considering IRX4 and electrophysiological analyses of older cells (**Figure 2B**), the majority of cells generated by the protocol described here appear to be committed to the ventricular fate, thus, alternative strategies would be required for the generation of cultures highly enriched for atrial-like or nodal-like cells.

This protocol works robustly across many different hESC and hiPSC lines (>15 tested to date). Several common challenges encountered during routine culturing can significantly affect differentiation efficiency. Pluripotent cells must be growing exponentially, ideally where plating 0.75 x 10^6 cells/well every four days results in 70-80% confluence. Freshly thawed cells or cells cultured in other media such as mTeSR1 or StemPro hESC require adaptation. For the latter, more than five passages may be required for cells to reach suitable growth rates and to select for the most robustly growing cells. E8 media quickly becomes acidic and cell death will occur once the cells become 100% confluent. Timing and monitoring of the culture is critical, as differentiation should commence just as the cells reach confluence (*i.e.* not started once the cells have been confluent for a day). The dose of 6 µM CHIR99021 for 48 hr is optimal for a large range of hiPSC and hESC lines, although treatment dose (5 µM, 6 µM, 7 µM) and timing (24 hr or 48 hr) can be varied. Significant cell death is observed after the initial dose of CHIR99021, but it should not exceed 60-70%. The exact cause of cell death post-treatment with CHIR99021 is currently unclear; however, the most common cause of excessive cell death upon treatment with CHIR99021, as well as low overall efficiency of differentiation, is the use of pluripotent cells at low passage (<p20) or those that exhibit poor pluripotent growth rates. If necessary, testing various seeding densities or waiting until the cells are at a later passage and growing more quickly will likely yield success faster than attempting to alter CHIR99021 dose. Regarding troubleshooting of flow cytometry, single cells are required. Incomplete digestion results in aggregates that are not suitable for flow cytometry, while over-digestion can produce excess debris that can affect antibody binding. Fixation and permeabilization steps are often culprits for low efficiency of antibody staining. Vortex cells gently during fixation to avoid aggregates. The choice of solution and treatment time should be tested if alternative antibodies to the same targets are used. As with any cardiomyocyte differentiation protocol, there are limitations. Consistent with other reports, cardiomyocytes generated with this protocol are fetal/embryonic-like. Further work will be required to optimize strategies for driving developmental maturation to the adult-like phenotype. Moreover, this staining protocol does not include important steps required for efficient co-staining for multiple intracellular antigens simultaneously. Appropriate methods for co-staining require that isotypes are distinct and secondary antibodies do not cross-react unless fluorophore-conjugated primary antibodies are used. This requires careful selection of fluorophores to minimize spectrum overlap, the possible use of compensation during flow cytometry data acquisition, and optimization of fixation/permeabilization conditions that provide optimal staining results for multiple antibodies – which can be a challenge depending on the antibodies. Finally, this protocol is designed for efficient flow cytometry analysis, but is not intended to include all necessary aspects required to perform fluorescence activated cell sorting, which has been described previously[54].

Long-term, mechanistic and developmental studies, personalized disease modeling, drug discovery, and eventually, regenerative medicine should benefit from the development of strategies to efficiently generate cardiomyogenic cells from pluripotent stem cells. As the success of each of these applications will ultimately require the ability to efficiently and reproducibly select and track pluripotent cell derivatives at the appropriate developmental stage and subtype, new non-transgene based strategies for cell identification and selection will likely be required[55]. In this respect, proteomic technologies are predicted to a play major role in defining new cell surface markers to facilitate the identification and selection of cells[56-60], similar to what is routine in the hematopoietic hierarchy[61-64]. However, proteomic technologies currently provide a snapshot averaged across the population of cells sampled. Thus, the ability to reproducibly and efficiently generate highly pure populations of cells committed to the cardiomyogenic fate afforded by this protocol, albeit with some degree of heterogeneity regards to subtype, will greatly benefit these proteomic efforts.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This research was supported by NIH 4R00HL094708-03, MCW Research Affairs Committee New Faculty Award, and the Kern foundation (startup funds) at the Medical College of Wisconsin (RLG); Research Grants Council of Hong Kong Theme-based Research Scheme T13-706/11 (KRB); U01 HL099776, CIRM TR3-05556, CIRM DR2A-05394, and AHA Established Investigator Award (JCW); AHA Postdoctoral Fellowship 12POST12050254 (PWB). We thank Hope Campbell at the Flow Cytometry Core of the Blood Research Institute of Wisconsin for assistance with data collection and careful review of the manuscript.

References

1. Lian, X. *et al.* Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proc Natl Acad Sci U S A.* **109**, E1848-1857, doi:10.1073/pnas.1200250109 1200250109 [pii] (2012).
2. Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-catenin signaling under fully defined conditions. *Nat Protoc.* **8**, 162-175, doi:10.1038/nprot.2012.150 nprot.2012.150 [pii] (2013).
3. Burridge, P. W., Keller, G., Gold, J. D., & Wu, J. C. Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. *Cell Stem Cell.* **10**, 16-28, doi:S1934-5909(11)00594-7 [pii] 10.1016/j.stem.2011.12.013 (2012).

4. Burridge, P. W. *et al.* A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. *PLoS One.* **6**, e18293, doi:10.1371/journal.pone.0018293 (2011).
5. Yang, L. *et al.* Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature.* **453**, 524-528, doi:nature06894 [pii] 10.1038/nature06894 (2008).
6. Kattman, S. J. *et al.* Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell.* **8**, 228-240, doi:S1934-5909(10)00703-4 [pii] 10.1016/j.stem.2010.12.008 (2011).
7. Willems, E. *et al.* Small-molecule inhibitors of the Wnt pathway potently promote cardiomyocytes from human embryonic stem cell-derived mesoderm. *Circ Res.* **109**, 360-364, doi:CIRCRESAHA.111.249540 [pii] 10.1161/CIRCRESAHA.111.249540 (2011).
8. Burridge, P. W. *et al.* Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability. *Stem Cells.* **25**, 929-938, doi:2006-0598 [pii] 10.1634/stemcells.2006-0598 (2007).
9. Elliott, D. A. *et al.* NKX2-5(eGFP/w) hESCs for isolation of human cardiac progenitors and cardiomyocytes. *Nat Methods.* **8**, 1037-1040, doi:10.1038/nmeth.1740 nmeth.1740 [pii] (2011).
10. Laflamme, M. A. *et al.* Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol.* **25**, 1015-1024, doi:nbt1327 [pii] 10.1038/nbt1327 (2007).
11. Zhang, Q. *et al.* Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals. *Cell Res.* **21**, 579-587, doi:cr2010163 [pii] 10.1038/cr.2010.163 (2011).
12. Uosaki, H. *et al.* Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression. *PLoS One.* **6**, e23657, doi:10.1371/journal.pone.0023657 PONE-D-11-05980 [pii] (2011).
13. Hudson, J., Titmarsh, D., Hidalgo, A., Wolvetang, E., & Cooper-White, J. Primitive Cardiac Cells from Human Embryonic Stem Cells. *Stem Cells Dev.* **21**, 1513-1523, doi:10.1089/scd.2011.0254 (2011).
14. Zhang, J. *et al.* Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method. *Circ Res.* **111**, 1125-1136, doi:10.1161/CIRCRESAHA.112.273144 CIRCRESAHA.112.273144 [pii] (2012).
15. Ban, K. *et al.* Purification of cardiomyocytes from differentiating pluripotent stem cells using molecular beacons that target cardiomyocyte-specific mRNA. *Circulation.* **128**, 1897-1909, doi:10.1161/CIRCULATIONAHA.113.004228 CIRCULATIONAHA.113.004228 [pii] (2013).
16. Toyota, N., & Shimada, Y. Differentiation of troponin in cardiac and skeletal muscles in chicken embryos as studied by immunofluorescence microscopy. *J Cell Biol.* **91**, 497-504 (1981).
17. Saggin, L., Gorza, L., Ausoni, S., & Schiaffino, S. Cardiac troponin T in developing, regenerating and denervated rat skeletal muscle. *Development.* **110**, 547-554 (1990).
18. Kajioka, S. *et al.* Endogenous cardiac troponin T modulates Ca(2+)-mediated smooth muscle contraction. *Sci Rep.* **2**, 979, doi:10.1038/srep00979 (2012).
19. Franco, D. *et al.* Myosin light chain 2a and 2v identifies the embryonic outflow tract myocardium in the developing rodent heart. *Anat Rec.* **254**, 135-146, doi:10.1002/(SICI)1097-0185(19990101)254:1<135::AID-AR17>3.0.CO;2-S [pii] (1999).
20. Franco, D., Lamers, W. H., & Moorman, A. F. Patterns of expression in the developing myocardium: towards a morphologically integrated transcriptional model. *Cardiovasc Res.* **38**, 25-53, doi:S0008-6363(97)00321-0 [pii] (1998).
21. Poon, E. *et al.* Transcriptome-guided functional analyses reveal novel biological properties and regulatory hierarchy of human embryonic stem cell-derived ventricular cardiomyocytes crucial for maturation. *PLoS One.* **8**, e77784, doi:10.1371/journal.pone.0077784 PONE-D-13-28733 [pii] (2013).
22. Lieu, D. K. *et al.* Absence of transverse tubules contributes to non-uniform Ca(2+) wavefronts in mouse and human embryonic stem cell-derived cardiomyocytes. *Stem Cells Dev.* **18**, 1493-1500, doi:10.1089/scd.2009.0052 (2009).
23. Cao, F. *et al.* Transcriptional and functional profiling of human embryonic stem cell-derived cardiomyocytes. *PLoS One.* **3**, e3474, doi:10.1371/journal.pone.0003474 (2008).
24. Satin, J. *et al.* Calcium handling in human embryonic stem cell-derived cardiomyocytes. *Stem Cells.* **26**, 1961-1972, doi:10.1634/stemcells.2007-0591 2007-0591 [pii] (2008).
25. Itzhaki, I. *et al.* Calcium handling in human induced pluripotent stem cell derived cardiomyocytes. *PLoS One.* **6**, e18037, doi:10.1371/journal.pone.0018037 (2011).
26. Bodor, G. S., Porterfield, D., Voss, E. M., Smith, S., & Apple, F. S. Cardiac troponin-I is not expressed in fetal and healthy or diseased adult human skeletal muscle tissue. *Clin Chem.* **41**, 1710-1715 (1995).
27. Hunkeler, N. M., Kullman, J., & Murphy, A. M. Troponin I isoform expression in human heart. *Circ Res.* **69**, 1409-1414 (1991).
28. Bhavsar, P. K. *et al.* Developmental expression of troponin I isoforms in fetal human heart. *FEBS Lett.* **292**, 5-8, doi:0014-5793(91)80820-S [pii] (1991).
29. Christoffels, V. M., Keijser, A. G., Houweling, A. C., Clout, D. E., & Moorman, A. F. Patterning the embryonic heart: identification of five mouse Iroquois homeobox genes in the developing heart. *Dev Biol.* **224**, 263-274, doi:10.1006/dbio.2000.9801 S0012-1606(00)99801-4 [pii] (2000).
30. Bruneau, B. G. *et al.* Cardiac expression of the ventricle-specific homeobox gene Irx4 is modulated by Nkx2-5 and dHand. *Dev Biol.* **217**, 266-277, doi:10.1006/dbio.1999.9548 S0012-1606(99)99548-9 [pii] (2000).
31. Bao, Z. Z., Bruneau, B. G., Seidman, J. G., Seidman, C. E., & Cepko, C. L. Regulation of chamber-specific gene expression in the developing heart by Irx4. *Science.* **283**, 1161-1164 (1999).
32. Nelson, D. O., Jin, D., Downs, K., Kamp, T. J., & Lyons, G. E. Irx4 identifies a chamber-specific cell population that contributes to ventricular myocardium development. *Dev Dyn.* **243**, 381-392, doi:10.1002/dvdy.24078 (2013).
33. Martin, A. F. Turnover of cardiac troponin subunits. Kinetic evidence for a precursor pool of troponin-I. *J Biol Chem.* **256**, 964-968 (1981).
34. Lundy, S. D., Zhu, W. Z., Regnier, M., & Laflamme, M. A. Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Stem Cells Dev.* **22**, 1991-2002, doi:10.1089/scd.2012.0490 (2013).
35. Farrell, H. M., Jr. *et al.* Nomenclature of the proteins of cows" milk--sixth revision. *Journal of dairy science.* **87**, 1641-1674, doi:10.3168/jds.S0022-0302(04)73319-6 (2004).
36. Micusan, V. V., & Borduas, A. G. Biological properties of goat immunoglobulins G. *Immunology.* **32**, 373-381 (1977).
37. Lyons, G. E. In situ analysis of the cardiac muscle gene program during embryogenesis. *Trends Cardiovasc Med.* **4**, 70-77, doi:10.1016/1050-1738(94)90012-4 1050-1738(94)90012-4 [pii] (1994).
38. Nakagawa, O., Nakagawa, M., Richardson, J. A., Olson, E. N., & Srivastava, D. HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. *Dev Biol.* **216**, 72-84, doi:10.1006/dbio.1999.9454 S0012-1606(99)99454-X [pii] (1999).

39. Xin, M. *et al.* Essential roles of the bHLH transcription factor Hrt2 in repression of atrial gene expression and maintenance of postnatal cardiac function. *Proc Natl Acad Sci U S A.* **104**, 7975-7980, doi:0702447104 [pii] 10.1073/pnas.0702447104 (2007).
40. Davis, L. M., Rodefeld, M. E., Green, K., Beyer, E. C., & Saffitz, J. E. Gap junction protein phenotypes of the human heart and conduction system. *J Cardiovasc Electrophysiol.* **6**, 813-822 (1995).
41. Minamisawa, S. *et al.* Atrial chamber-specific expression of sarcolipin is regulated during development and hypertrophic remodeling. *J Biol Chem.* **278**, 9570-9575 (2003).
42. Larsen, T. H. Atrial natriuretic factor in the heart of the human embryo. *Acta Anat (Basel).* **138**, 132-136 (1990).
43. Claycomb, W. C. Atrial-natriuretic-factor mRNA is developmentally regulated in heart ventricles and actively expressed in cultured ventricular cardiac muscle cells of rat and human. *Biochem J.* **255**, 617-620 (1988).
44. Franco, D. *et al.* Divergent expression of delayed rectifier K(+) channel subunits during mouse heart development. *Cardiovasc Res.* **52**, 65-75, doi:S0008636301003492 [pii] (2001).
45. Hoogaars, W. M. *et al.* The transcriptional repressor Tbx3 delineates the developing central conduction system of the heart. *Cardiovasc Res.* **62**, 489-499, doi:10.1016/j.cardiores.2004.01.030 S0008636304000689 [pii] (2004).
46. Hollenberg, S. M., Sternglanz, R., Cheng, P. F., & Weintraub, H. Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. *Mol Cell Biol.* **15**, 3813-3822 (1995).
47. Srivastava, D., Cserjesi, P., & Olson, E. N. A subclass of bHLH proteins required for cardiac morphogenesis. *Science.* **270**, 1995-1999 (1995).
48. Firulli, A. B., McFadden, D. G., Lin, Q., Srivastava, D., & Olson, E. N. Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. *Nat Genet.* **18**, 266-270, doi:10.1038/ng0398-266 (1998).
49. Calejo, A. I. *et al.* Differences in the expression pattern of HCN isoforms among mammalian tissues: sources and implications. *Mol Biol Rep.* **41**, 297-307, doi:10.1007/s11033-013-2862-2 (2013).
50. Stevens, S. M., & Pu, W. T. HCN4 charges up the first heart field. *Circ Res.* **113**, 350-351, doi:10.1161/CIRCRESAHA.113.301981 CIRCRESAHA.113.301981 [pii] (2013).
51. Spater, D. *et al.* A HCN4+ cardiomyogenic progenitor derived from the first heart field and human pluripotent stem cells. *Nat Cell Biol.* **15**, 1098-1106, doi:10.1038/ncb2824 ncb2824 [pii] (2013).
52. Davis, R. P., van den Berg, C. W., Casini, S., Braam, S. R., & Mummery, C. L. Pluripotent stem cell models of cardiac disease and their implication for drug discovery and development. *Trends Mol Med.* **17**, 475-484, doi:S1471-4914(11)00078-5 [pii] 10.1016/j.molmed.2011.05.001 (2011).
53. Mummery, C. *et al.* Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation.* **107**, 2733-2740, doi:10.1161/01.CIR.0000068356.38592.68 01.CIR.0000068356.38592.68 [pii] (2003).
54. Basu, S., Campbell, H. M., Dittel, B. N., & Ray, A. Purification of specific cell population by fluorescence activated cell sorting (FACS). *J Vis Exp.* (41), doi:10.3791/1546 1546 [pii] (2010).
55. Dubois, N. C. *et al.* SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. *Nat Biotechnol.* **29**, 1011-1018, doi:10.1038/nbt.2005 nbt.2005 [pii] (2011).
56. Samal, R. *et al.* OMICS-based exploration of the molecular phenotype of resident cardiac progenitor cells from adult murine heart. *J Proteomics.* **75**, 5304-5315, doi:10.1016/j.jprot.2012.06.010 S1874-3919(12)00435-6 [pii] (2012).
57. Van Hoof, D. *et al.* Identification of cell surface proteins for antibody-based selection of human embryonic stem cell-derived cardiomyocytes. *J Proteome Res.* **9**, 1610-1618, doi:10.1021/pr901138a (2010).
58. Gundry, R. L., Boheler, K. R., Van Eyk, J. E., & Wollscheid, B. A novel role for proteomics in the discovery of cell-surface markers on stem cells: Scratching the surface. *Proteomics Clin Appl.* **2**, 892-903, doi:10.1002/prca.200780122 (2008).
59. Gundry, R. L., Burridge, P. W., & Boheler, K. R. Pluripotent Stem Cell Heterogeneity and the Evolving Role of Proteomic Technologies in Stem Cell Biology *Proteomics.* **11**, 3947-3961 (2011).
60. Gundry, R. L. *et al.* A Cell Surfaceome Map for Immunophenotyping and Sorting Pluripotent Stem Cells. *Mol Cell Proteomics.* **11**, 303-316, doi:M112.018135 [pii] 10.1074/mcp.M112.018135 (2012).
61. Bryder, D., Rossi, D. J., & Weissman, I. L. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am J Pathol.* **169**, 338-346, doi:169/2/338 [pii] (2006).
62. Kondo, M. *et al.* Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol.* **21**, 759-806 (2003).
63. Shizuru, J. A., Negrin, R. S., & Weissman, I. L. Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system. *Annu Rev Med.* **56**, 509-538, doi:10.1146/annurev.med.54.101601.152334 (2005).
64. Weissman, I. L., & Shizuru, J. A. The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood.* **112**, 3543-3553 (2008).
65. Boheler, K. R., Bhattacharya S., Chuppa, S., Riordon, D. R., Kropp, E., Burridge, P. W., Wu, J. C., Wersto, R. P., Rao, S., Wollscheid, B., Gundry, R. L. A Human Pluripotent Stem Cell Surface N-Glycoproteome Resource Reveals New Markers, Extracellular Epitopes, and Drug Targets. *Stem Cell Reports.* **3**, 185-203 (2014).

