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TITLE:

Generation and Expansion of Human Cardiomyocytes from Patient Peripheral Blood Mononuclear Cells

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KEYWORDS:
peripheral blood mononuclear cells, PBMCs, human induced pluripotent stem cells, iPSCs, cardiac differentiation, iPSC reprogramming, cardiomyocyte expansion.

SUMMARY:

Here, we present a protocol to robustly generate and expand human cardiomyocytes from patient peripheral blood mononuclear cells.

ABSTRACT:

Generating patient-specific cardiomyocytes from a single blood draw has attracted tremendous interest in precision medicine on cardiovascular disease. Cardiac differentiation from human induced pluripotent stem cells (iPSCs) is modulated by defined signaling pathways that are essential for embryonic heart development. Numerous cardiac differentiation methods on 2-D and 3-D platforms have been developed with various efficiencies and cardiomyocyte yield. This has puzzled investigators outside the field as the variety of these methods can be difficult to

follow. Here we present a comprehensive protocol that elaborates robust generation and expansion of patient-specific cardiomyocytes from peripheral blood mononuclear cells (PBMCs). We first describe a high-efficiency iPSC reprogramming protocol from a patient's blood sample using non-integration Sendai virus vectors. We then detail a small molecule-mediated monolayer differentiation method that can robustly produce beating cardiomyocytes from most human iPSC lines. In addition, a scalable cardiomyocyte expansion protocol is introduced using a small molecule (CHIR99021) that could rapidly expand patient-derived cardiomyocytes for industrial- and clinical-grade applications. At the end, detailed protocols for molecular identification and electrophysiological characterization of these iPSC-CMs are depicted. We expect this protocol to be pragmatic for beginners with limited knowledge on cardiovascular development and stem cell biology.

INTRODUCTION

The discovery of human induced pluripotent stem cells has revolutionized modern cardiovascular medicine[1,2]. Human iPSCs are capable of self-renewing and generating all cell types in the heart, including cardiomyocytes, endothelial cells, smooth muscle cells and cardiac fibroblasts. Patient iPSC-derived cardiomyocytes (iPSC-CMs) can serve as indefinite resources for modeling genetically inheritable cardiovascular diseases (CVDs) and testing cardiac safety for new drugs[3]. In particular, patient iPSC-CMs are well poised to investigate genetic and molecular etiologies of CVDs that are derived from defects in cardiomyocytes, such as long QT syndrome[4] and dilated cardiomyopathy (DCM)[5]. Combined with CRISPR/Cas9-mediated genome editing, patient iPSC-CMs have opened an unprecedented avenue to understand the complex genetic basis of CVDs including congenital heart defects (CHDs)[6-8]. Human iPSC-CMs have also exhibited potentials to serve as autologous cell sources for replenishing the damaged myocardium during a heart attack[9]. In recent years, it has become paramount to generate high-quality human iPSC-CMs with defined subtypes (atrial, ventricular and nodal) for cardiac regeneration and drug testing[10].

Cardiac differentiation from human iPSCs has been greatly advanced in the past decade. Differentiation methods have gone from embryoid body (EB)-based spontaneous differentiation to chemically defined and directed cardiac differentiation[11]. Key signaling molecules essential for embryonic heart development, such as Wnt, BMP, Nodal, and FGF are manipulated to enhance cardiomyocyte differentiation from human iPSCs[10,12]. Significant advances include sequential modulation of Wnt signaling (activation followed by inhibition) for robust generation of cardiomyocytes from human iPSCs[13,14]. Chemically defined cardiac differentiation recipes have been explored to facilitate large-scale production of beating cardiomyocytes[15,16], which have the potential to be upgraded to industrial and clinical level production. Moreover, robust expansion of early human iPSC-CMs is achieved by exposure to constitutive Wnt activation using a small chemical (CHIR99021)[17]. Most recently, subtype-specific cardiomyocytes are generated through manipulation of retinoic acid (RA) and Wnt signaling pathways at specific differentiation windows during cardiomyocyte lineage commitment from human iPSCs[18-22].

In this protocol, we detail a working procedure for robust generation and proliferation of human CMs originating from patient peripheral blood mononuclear cells. We present protocols for 1) reprogramming human PBMCs to iPSCs, 2) robust generation of beating cardiomyocytes from

human iPSCs, 3) rapid expansion of early iPSC-CMs, 4) molecular characterization of human iPSC-CMs, and 5) electrophysiological measurement of human iPSC-CMs at the single-cell level by patch clamp. This protocol covers the detailed experimental procedures on converting patient blood cells into beating cardiomyocytes.

PROTOCOL

The experimental protocols and informed consent for human subjects were approved by the Institutional Review Board (IRB) at Nationwide Children's Hospital.

1. Preparation of cell culture media, solutions, and reagents

1.1. Prepare PBMC media

1.1.1. Mix 20 mL of basal PBMC culture media (1x) and 0.52 mL of supplement. Add 20 μL of SCF and FLT3 each (stock concentration: 100 μg/mL), 4 μL of IL3, IL6 and EPO each (stock concentration: 100 μg/mL) and 200 μL of L-glutamine alternative (100x). Mix them thoroughly. Filter in a sterile hood using a 0.22-μm filter unit. Name this as Complete Blood Media.

1.1.2. Mix 20 mL of basal PBMC culture media (1x) and 0.52 mL of the Supplement. Add 200 μL of L-glutamine alternative (100x). Mix them thoroughly. Filter using a 0.22-μm filter unit. Name this as Supplement Blood Media.

1.2. Prepare complete E8 media

1.2.1. Mix 500 mL of E8 basal media and 10 mL of E8 supplement (thawed overnight at 4 ˚C) to make complete E8 media. Equilibrate to room temperature (RT) before use.

1.3 Prepare iPSC passaging media

1.3.1. Add 40 μL of Y-27632 Rock inhibitor (1:5,000 dilution, stock concentration: 10 mM) to 200 mL of complete E8 media. Mix it thoroughly. Equilibrate to RT before use.

1.4. Prepare cardiomyocyte differentiation media

1.4.1. Media I: Mix 500 mL of RPMI1640 with 10 mL of B27 minus insulin supplement (50x).

1.4.2. Media II: Add an appropriate volume of CHIR99021 (GSK3 inhibitor) stock to Media I (final concentration of 6 μM). Mix thoroughly.

1.4.3. Media III: Add an appropriate volume of IWR-1 (Wnt inhibitor) stock to Media I (to a final concentration of 5 μM). Mix thoroughly.

1.4.4. Media IV: Mix 500 mL of RPMI1640 with 10 mL of B27 supplement (50x). Mix thoroughly.

1.4.5. Media V: Mix 500 mL of RPMI1640 (no glucose) with 10 mL of B27 supplement (50x). Mix thoroughly.

1.4.6. Media VI: Add an appropriate volume of CHIR99021 stock to Media IV (to a final concentration of 2 μM). Mix thoroughly.

1.5 Prepare iPSC-CM passaging media

1.5.1. Add 10 mL of Knockout Serum Replacement (KSR) to 90 mL of Media IV (KSR final concentration: 10%). Mix well.

1.6 Prepare iPSC-CM freezing media

1.6.1. Add 1 mL of DMSO to 9 mL of KSR (final concentrations: 10% DMSO/90% KSR) and mix well.

1.7 Prepare basement membrane matrix medium-coated plates

1.7.1. Thaw basement membrane matrix medium at 4 ˚C overnight and aliquot in 1.5 mL tubes. Add 1 mL of this medium to 250 mL of DMEM/F12 media (1:250 dilution) and mix them thoroughly. Apply 2 mL of the diluted solution per well in a 6-well plate and incubate in 5% CO_2 at 37 ˚C for 30 min before use.

2. iPSC reprogramming of PBMCs

2.1. Separate PBMCs from blood samples.

2.1.1. Collect patient blood samples (~5 mL) and transfer into blood cell separation tubes (see **Table of Materials**). Mix by inverting 10x.

2.1.2. Centrifuge at 1,500 x *g* for 30 min at room temperature.

2.1.3. Take the tubes out carefully and spray with 70% ethanol. Under a biosafety hood, remove the caps without disturbing the mononuclear cells (buffy layer). PBMCs will be in a whitish layer just under the plasma layer (**Figure 1A**). Collect the whole buffy layer using a 1000 μL pipette and transfer to a 15 mL conical tube.

2.1.4. Count the cell number using an automated cell counter. Spin the tube at 300 x *g* for 25 min at RT.

2.1.5. Discard the supernatant. Wash with 10 mL of DPBS (Ca^{2+}/Mg^{2+} free).

2.1.6. Spin the tube at 300 x *g* for 15 min at RT.

2.1.7. Remove the supernatant. Resuspend cell pellets in 1 mL of the freezing media (KSR plus 10% DMSO). Adjust cell density to make 1 x 10^6 cells per vial.

2.1.8. Place PBMC cryovials in a cell freezing container and keep at -80 °C overnight. Transfer to a liquid nitrogen tank for long-time storage the next day.

2.2. iPSC reprogramming.

2.2.1. Add 3 mL of Supplement Blood Media in a 15 mL conical tube. Thaw PBMCs in 37 ˚C water bath and transfer them to a conical tube. Spin at 300 x *g* for 7 min at RT.

2.2.2. Discard the supernatant. Resuspend PBMCs with Complete Blood Media. Seed them into two wells of a 24 well tissue culture plates (no basement membrane matrix).

2.2.3. Incubate in 5% CO_2 at 37 ˚C overnight. The next day gently remove half of the old media (0.5 ml) and add 0.5 mL of fresh Complete Blood Media.

2.2.4. Change media every other day by refreshing half of the old media.

2.2.5. After a week, aggressively wash the well with 1 mL of Supplement Blood Media and transfer cells into a 15 mL centrifuge tube.

2.2.6. Count the cell number. Take 2 x 10^5 cells and centrifuge at 300 x *g* for 7 min.

2.2.7. Discard the supernatant. Resuspend cells with 300 μL of Complete Blood Media. Perform transfection by adding appropriate volume of Sendai virus reprogramming vectors according to the manufacturer's instructions. Transfer them into one well of a 24-well plate (no basement membrane matrix). Incubate in 5% CO_2 at 37 ˚C overnight.

2.2.8. The next day spin at 300 x *g* for 7 min. Remove the supernatant and resuspend in 2 mL of Complete Blood Media. Transfer into one well of a 6 well plate pre-coated with basement membrane matrix. This is Day 1 (D1).

2.2.9. Don't touch the plate the next day.

2.2.10. On D3, remove 1 mL of the old media. Add 1 mL of Supplement Blood Media.

2.2.11. Repeat step 2.2.10 on D5.

2.2.12. On D7, remove 1 mL of the old media. Add 1 mL of complete E8 media.

2.2.13. On D8, Repeat step 2.2.12.

2.2.14. On D9, remove the old media. Add 2 mL of complete E8 media. Completely reprogrammed cells are expected to attach and start forming colonies.

2.2.15. Refresh with 2 mL of complete E8 media every day.

2.2.16. Around 2 weeks after Sendai virus transduction, large iPSC colonies will appear and be ready for picking.

2.2.17. Cut iPSC colonies under a stereomicroscope in the hood and transfer individual colonies to a basement membrane-coated 24well plate pre-loaded with 0.5 mL of iPSC passaging media.

2.2.18. Refresh with 0.5 mL of complete E8 media every day until iPSC colonies grow large enough for passaging into a new basement membrane matrix-coated 6-well plate.

3. Human iPSC maintenance and passaging

3.1. When human iPSCs reach over 90% confluency, remove old media. Rinse with 3 mL of DPBS once.

3.2. Add 1 mL of 0.5 mM EDTA in DPBS solution. Incubate in 5% CO_2 at 37 ˚C for 5-8 min.

3.3. Remove EDTA by aspiration. Add 1 mL of iPSC passaging media. Manually dislodge iPSCs.

3.4. Take 600-900 μL of single cell suspension and re-plate them onto a basement membrane-coated 6-well plate (dilution: 1:6 to 1:10). Incubate in 5% CO_2 at 37 °C overnight.

3.5. Refresh with 2 mL of complete E8 media every day. The iPSC cultures usually reach confluency after 3-4 days.

4. Chemically defined cardiomyocyte differentiation

4.1. Culture human iPSCs in complete E8 media until 95% confluent (3-4 days).

4.2. Remove the old media. Add 2 mL of CM differentiation Media II (6 μM CHIR in RPMI1640 plus B27 minus insulin supplement) to each well of a 6-well plate. This is D0. Do not touch on D1.

4.3. On D2, replace with 2 mL of CM differentiation Media I (RPMI1640 plus B27 minus insulin supplement).

4.4. On D3, replace with 2 mL of CM differentiation Media III (5 μM IWR-1 in RPMI1640 plus B27 minus insulin supplement). Do not touch on D4.

4.5. On D5, replace with 2 mL of CM differentiation Media I.

4.6. On D7, replace with 2 mL of CM differentiation Media IV (RPMI1640 plus B27 supplement). Thereafter, refresh the media every other day.

4.7. On D11 when contracting cells are observed, replace with 2 mL of CM differentiation Media V (no glucose).

4.8. On D13, replace with 2 mL of CM differentiation Media V.

4.9. On D15, replace with 2 mL of CM differentiation Media IV.

4.10. On D17-D21, replace with 2 mL of CM differentiation Media IV every other day.

5. Passage human iPSC-CMs

5.1. Remove old media and rinse cells with 3 mL of DPBS once.

5.2. Apply 1 mL of CM dissociation solution (see **Table of Materials**) to each well of a 6-well plate. Incubate in 5% CO_2 at 37 ˚C for 5-8 min.

5.3. Mechanically dissociate iPSC-CMs into single cells by vigorous pipetting.

5.4. Transfer cells into a 15 mL conical tube. Add 2 mL of CM passaging media (10% KSR in RMPI1640 plus B27 supplement) to neutralize CM dissociation solution.

5.5. Spin at 300 x *g* for 5 min at RT.

5.6. Discard the supernatant. Resuspend cells with a desired volume of CM passaging media. Seed them into a basement membrane-coated plate/dish. Human iPSC-CMs resume beating 1-3 days after passaging.

6. Expansion of human iPSC-CMs

6.1. Rinse D10-12 beating iPSC-CMs with 3 mL of DPBS for each well of a 6-well plate once. Add 1 mL of CM dissociation solution (Step 5.2). Incubate in 5% CO_2 at 37 ˚C for 7-10 min.

6.2. Mechanically dissociate iPSC-CMs into single cells by vigorous pipetting.

6.3. Transfer cells into a 15 mL conical tube. Add 2 mL of CM passaging media to neutralize CM dissociation solution.

6.4. Spin at 300 x *g* for 5 min at RT.

6.5. Discard the supernatant. Resuspend cells with an appropriate volume of CM passaging media. Pipette up and down to make single cell suspension. Seed one million of iPSC-CMs into a basement membrane-coated 10 cm dish.

6.6. The next day remove old media. Add 10 mL of cardiomyocyte proliferation media (Media VI: 2 μM CHIR99021). Change the media every other day.

6.7. When iPSC-CMs become confluent after 7-9 days' culture, repeat the passaging step for further expansion of iPSC-CMs.

7. Immunofluorescence

7.1. Before immunofluorescence staining, seed iPSC-CMs onto Matrigel-coated coverslips that are placed in a 24 well plate (seeding density: 0.5-1 x 10^6 cells/mL). Maintain iPSC-CMs in culture for at least 4 days.

7.2. Wash cells using 1 mL of DPBS once.

7.3. Add 0.5 mL of 4% paraformaldehyde (PFA) and incubate for 15 min at RT.

7.4. Wash cells using 1 mL of DPBS. Repeat once.

7.5. Add 0.5 mL of 0.1% Triton X-100 and incubate for 20 min at RT.

7.6. Wash with 1 mL of DPBS twice.

7.7. Add 0.5 mL of 0.2% BSA in DPBS (blocking solution). Incubate at RT for 1 h.

7.8. Add 200 μL of primary antibody diluted with blocking solution (dilution: 1:400-1:1000) Incubate at 4 ˚C overnight.

7.9. Wash cells using 0.5 mL of blocking solution for 3 min with shaking. Repeat twice.

7.10. Add 200 μL of secondary antibody diluted in the blocking solution. Incubate at RT for 1 h.

7.11. Rinse cells three times with 0.5 mL of DPBS, each for 3 min with shaking.

7.12. Counterstain nuclei with DAPI (1:2000 dilution) and incubate for 5 min at RT.

7.13. Rinse cells three times with 0.5 mL of DPBS.

7.14. Mount cells on the coverslips onto a microscope slide using 5 μl of mounting media. Store at 4 ˚C and protect from light.

8. Flow cytometry sample preparation

8.1. Wash human iPSC-CMs with 3 mL of DPBS once.

8.2. Add 1 mL of CM dissociation solution and incubate in 5% CO_2 at 37 ˚C for 7-10 min.

8.3. Dislodge cells using a 1,000-μL pipette. Transfer cell suspension to a round FACS tube through a strainer cap. The FACS tube is pre-filled with 1 mL of iPSC-CM passaging media (10% KSR) to neutralize the enzyme activity.

8.4. Spin at 300 x *g* for 5 min.

8.5. Remove supernatant without disturbing cell pellet. Add 250 μL of Fixation/Permeabilization solution (see Table of Materials). Incubate for 20 min at 4 ˚C.

8.6. Add 1 mL of Perm/Wash buffer. Vortex briefly and spin at 300 x *g* for 4 min.

8.7. Discard the supernatant. Add 100 μL of diluted primary antibodies (1:200-1:500) in 1x Perm/Wash buffer. Vortex briefly and incubate overnight at 4 ˚C.

8.8. Wash cells by adding 1 mL of Perm/Wash buffer. Vortex briefly and spin at 300 x *g* for 4 min.

8.9. Discard the supernatant. Add 100 μL of diluted secondary antibodies (1:500-1:1,000). Vortex briefly and incubate at RT for 1 h. Protect from light if secondary antibodies are conjugated with light-sensitive fluorescence.

8.10. Wash cells by adding 1 mL of Perm/wash buffer. Vortex briefly and spin at 300g for 4 min.

8.11. Discard the supernatant. Resuspend cells with 400 μL of FACS staining buffer (PBS/4% FBS). Store at 4 ˚C until loading to a FACS instrument.

9. Real time qPCR

9.1. Remove old media in human iPSC-CM culture. Add 500-700 μl of lysis buffer to lysate cells. Incubate for 3 min at RT. Scape cell lysate and transfer to a 1.5-ml RNase-free tube. Proceed to total RNA extraction immediately or store at -80 ˚C.

9.2. Isolate total RNA using an RNA extraction kit following the manufacturer's instruction.

9.3. Measure the RNA concentration and assess the quality of total RNA by a spectrophotometer.

9.4. Perform reverse transcription reaction using a cDNA synthesis kit. Total volume of RT reaction is 20 μL including 4 μL of reaction mix (5x), 1 μL of reverse transcriptase,1 μg of total RNA and RNase-free water.

9.5. Incubate the complete RT reaction mix in a thermal cycler using the following protocol: 25 ˚C for 5 min; 46 ˚C for 20 min; 95 ˚C for 1 min; hold at 4 ˚C.

9.6. Dilute cDNA by 1:10 using nuclease-free water. Set up real time qPCR reaction by mixing 1 μL of cDNA template, 1 μL of primers/probe, 10 μL of qPCR master mix and 8 μL of nuclease-free water.

9.7. Run in a real-time PCR system. The cycling protocol is 50 ˚C 2 min (hold), 95 ˚C 10 min (hold), 95 ˚C 15 sec, 60 ˚C 1 min, repeat for 40 cycles.

9.8. Collect C_T values for each gene in each sample. Relative mRNA abundance is calculated by subtracting the C_T value of target gene from the C_T value of a housekeeping gene. Relative gene expression is analyzed by the $2^{-\Delta\Delta CT}$ method.

10. Whole-cell patch clamp recording

10.1. Dissociate iPSC-CMs into single cells using CM dissociation solution as previously described.

10.2. Seed cells at a low density on basement membrane matrix-coated coverslips. Culture them for 3-4 days in Media IV.

10.3. Pull pipettes (resistance 0.9-1.5 MΩ) from borosilicate glass capillaries using a horizontal microelectrode puller.

10.4. Incubate cells in Tyrode's solution (pH=7.35).

10.5. Fill pipettes with electrode solution (pH=7.3) composed of the following chemicals: 120 mM aspartic acid, 20 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES, 10 mM NaCl, 5 mM EGTA, 0.3 mM Na-GTP, 14 mM phosphocreatine, 4 mM K-ATP and 2mM creatine phosphokinase.

10.6. Place cells in the current clamp mode using a 1.5-2 diastolic threshold 5 ms current pulse at 1 Hz.

10.7. Record action potentials (APs) using a microelectrode amplifier and a software-driven acquisition board.

REPRESENTATIVE RESULTS

Human iPSC reprogramming from PBMCs

After pre-culture with Complete Blood Media for 7 days, PBMCs become large with visible nuclei and cytoplasm (**Figure 1B**), indicating that they are ready for virus transfection. After transfection with the Sendai virus reprogramming factors, PBMCs will undergo an epigenetic reprogramming process for another week. Typically, we get 30-50 iPSC colonies from the transfection of 1 x 10^5 PBMCs and the reprogramming efficiency is 0.03%-0.05%. Completely reprogrammed cells will attach and start forming colonies when they are introduced to the complete E8 media (**Figure 1C**). These early iPSC colonies are expanded for another 7 days and then mechanically cut and picked up individually. Each iPSC colony is transferred to one well of a 6-well plate to establish individual iPSC lines. After 4-5 passages, iPSC colonies will become pure with very few differentiated cells around (**Figure 1D**). At this stage, most of the cells in iPSC colonies are OCT4 and NANOG positive (**Figure 1E**), demonstrating their pluripotency. Stable iPSC lines are established by the fifth passage.

Cardiac differentiation

The cardiac differentiation protocol is depicted in **Figure 1F**. Cardiac differentiation is initiated when iPSCs are maintained for at least 10 passages. The degree of iPSC confluency is critical when CHIR99021 is applied. The cell density is more than 90% confluent but not over confluent. If iPSC colonies become too crowded, they will start spontaneous differentiation which will negatively affect the directed cardiomyocyte differentiation efficiency. Beating cardiomyocytes are usually observed after day 12 of differentiation (**Video 1**). The date in which the onset of beating occurs varies and is dependent on the iPSC lines in use. After glucose starvation and replating, iPSC-CMs show spontaneous beating (**Video 2**) and aligned sarcomere structure with intercalated cardiac troponin T (TNNT2) and α-actinin (**Figures 1G-H**). In addition, the purity of iPSC-CMs is high, with more than 93% of cells being $TNNT2^+$ as shown by FACS analysis (**Figure 1I**).

Although iPSC-CMs are relatively immature compared to adult cardiomyocytes, they show ventricular- and atrial-like action potentials measured by whole-cell patch clamp (**Figures 2A,B**). In a typical cardiac differentiation, day 30 iPSC-CMs are a mixture of ventricular-, atrial-, and nodal-like subtypes, with ventricular CMs accounting for the majority (60%, **Figure 2C**) using the abovementioned differentiation protocol (**Figure 1F**). Different differentiation protocols yield varying percentages of cardiomyocyte subtypes due to distinct signaling pathways activation during cell lineage determination[10]. Ventricular CMs are labeled with MYL2 (MLC2v, **Figure 2D**) whereas atrial iPSC-CMs are marked by NR2F2 (COUP-TFII, **Figure 2E**). These markers are highly expressed in more mature iPSC-CMs (>D30) rather than those in early stage.

Expansion of iPSC-CMs by Wnt activation

In mammals, adult cardiomyocytes do not actively divide for self-renewal. This phenomenon also takes place for human iPSC-CMs. Once mature beyond D30, cell division of iPSC-CMs is a rare event, thus limiting their ability for clinical- and industrial-level mass production. To mimic the developmental environment during embryonic cardiomyocyte proliferation, we activate the Wnt pathway by CHIR99021 to stimulate the multiplication of early iPSC-CMs. D12-14 iPSC-CMs (after purification by glucose deprivation) are seeded at a low density in the presence of 2 μM CHIR99021. Wnt activation stimulates cell division of iPSC-CMs and promotes the expression of cell cycle regulators such as Cyclin D1 (**Figure 3A**) which can push the cell cycle to advance

through the G1 phase. Interestingly, CHIR99021 enables robust proliferation of early iPSC-CMs for 2 passages compared to the controls (**Figure 3B**). However, the proliferation ability of iPSC-CMs diminishes with extensive passage (**Figure 3B**), which is consistent with the limited and well-controlled cardiac proliferation during embryonic heart development. In addition, it does not appear that CHIR99021 is able to stimulate the expansion of more mature iPSC-CMs when they reach over 30 days of differentiation and develop stable sarcomere structures.

FIGURE LEGENDS

Figure 1: Human iPSC reprogramming and cardiomyocyte differentiation. (**A**) A schematic diagram showing the PBMC layer after separation of patient blood samples. (**B**) Enlarged PBMCs are ready for transfection. (**C**) Early human iPSC colonies. (**D**) An established iPSC line at passage 5. (**E**) Human iPSCs are positive for the pluripotency markers OCT4 (green) and NANOG (red). Nuclei are counterstained by DAPI (blue). (**F**) Overview of a cardiomyocyte differentiation protocol. (**G-H**) Sarcomere structure of iPSC-CMs is revealed by immunofluorescence staining using antibodies against TNNT2 (green) and α-actinin (red). Nuclei are counterstained by DAPI (blue). (**I**) FACS analysis of iPSC-CMs using an antibody against TNNT2. Scale bars: 200 μm (**B-D**), 50 μm (**E** and **G**) and 20 μm (**H**).

Figure 2: Cardiomyocyte subtypes in human iPSC-CMs. (**A-B**) Representative action potential durations for ventricular-like (**A**) and atrial-like (**B**) iPSC-CMs. (**C**) Representative percentages of ventricular-, atrial- and nodal-like subtypes in human iPSC-CMs. (**D-E**) D30 iPSC-CMs are stained with antibodies against ventricular cardiomyocyte marker MYL2 (**D**) and atrial marker NR2F2 (**E**). Cells are simultaneously stained with a TNNT2 antibody. Nuclei are counterstained by DAPI (blue). Scale bars: 50 μm (**D-E**).

Figure 3: Expansion of human iPSC-CMs by Wnt activation. (**A**) Percentage of Cyclin D1 positive iPSC-CMs is increased in the presence of CHIR99021. Cells are double stained with antibodies against TNNT2 (red) and Cyclin D1 (green). Nuclei are counterstained by DAPI (blue). (**B**) Cell number fold changes during the expansion of human iPSC-CMs with or without CHIR99021 in the first 3 passages. Y-axis shows the cell number fold changes. CHIR99021 stimulates the robust proliferation of early iPSC-CMs. Scale bars: 50 μm (**A**).

Video 1. Beating human iPSC-CMs at day 18 of differentiation.

Video 2. Beating human iPSC-CMs at day 25 after metabolic purification.

DISCUSSION

During iPSC reprogramming, it is critical to culture PBMCs for 1 week until they are enlarged with clear nuclei and cytoplasm. Because PBMCs do not proliferate, an appropriate cell number for viral transduction is important for successful iPSC reprogramming. Cell number of PBMCs, multiplicity of infection (MOI) and titer of virus should be considered and adjusted to reach the optimal transduction outcomes. For cardiac differentiation, initial seeding density is critical for iPSCs to reach over 90% confluent on the day when CHIR99021 is administered. On one hand, if iPSCs are less confluent at the time of cardiac differentiation, CHIR99021 will be toxic and lead to

substantial cell death. On the other hand, if iPSCs are over confluent, they will undergo spontaneous differentiation which will compromise the efficiency of directed cardiac differentiation. For the expansion of early iPSC-CMs, the timing and cardiomyocyte quality should be taken into account. Early iPSC-CMs can robustly multiply only when the purity of cardiomyocytes is high enough. Existing non-cardiomyocytes in the culture may also proliferate in response to CHIR99021 treatment, which will negatively affect the proliferation of early iPSC-CMs. In addition, it is crucial to stimulate cardiomyocyte expansion by day 20 of differentiation. Once iPSC-CMs pass over day 30, it will be difficult for them to resume robust dividing.

Human iPSCs were initially derived from dermal and lung fibroblasts via retrovirus-mediated transfection[1,2]. There are two major issues with these reprogramming methods that prevent the progress in clinical translation of patient iPSCs: 1) the retrovirus integrates into the host genome thus introducing potential genetic mutations; 2) patient-derived fibroblasts require skin biopsies which many patients may decline. In this protocol, we describe a protocol that utilizes commercial non-integration Sendai virus[23] and PBMCs to robustly derive patient iPSCs. These iPSCs are free of exogenous reprogramming vectors and can be maintained with self-renewal and pluripotency indefinitely. In addition, patient blood samples are easily collected in clinical laboratories. Our protocol is versatile and can be used for mass production of patient- and disease-specific iPSCs for large-scale repository and clinical translations[24].

Robust cardiomyocyte differentiation is achieved by sequential modulation of specific signaling pathways during cardiac differentiation from human iPSCs. Key pathways involved in cardiac specification and proliferation include Wnt, BMP, Activin, NOTCH, VEGF and retinoic acid (RA) [10,12]. Here we present an efficient cardiac differentiation protocol by sequential modulation of Wnt signaling by small chemicals: first activation by CHIR99021 and then inhibition by IWR-1[13,14]. Small chemicals are stable and give consistent differentiation outcomes compared to those using growth factors. Most iPSC-CMs generated by this protocol are ventricular-like cardiomyocytes, mixed with atrial- and nodal-like cells. Precision generation of subtype-specific cardiomyocytes is achieved through fine-tuning later differentiation steps[10,12]. For example, addition of RA immediately after IWR-1 treatment yields a high percentage of atrial-like cardiomyocytes whereas RA inhibition promotes generation of ventricular-like iPSC-CMs[18,22]. Wnt signaling activation at a later stage of differentiation promotes the induction of cardiac progenitor cells to nodal-like cardiomyocytes[19,21], which is promising for the generation of patient-specific biological pacemaker cells.

Human iPSC-CMs are immature and have limited proliferation ability[25]. During embryonic cardiac development, the maturation proceeds while the proliferation diminishes. There is a narrow window when iPSC-CMs can be stimulated for robust proliferation, which is reflective of embryonic cardiomyocyte expansion. Here we use a Wnt activator CHIR99021 to promote the proliferation of early iPSC-CMs for a limited period, which is consistent with a recent report[17]. It is speculated that the Wnt signaling pathway affects cardiomyocyte proliferation possibly through the crosstalk with multiple upstream pathways such as NOTCH and Hippo[26,27]. NOTCH signaling promotes cardiomyocyte proliferation whereas the Hippo pathway restricts cardiac growth and heart size[28-30]. It is still unknown how the interaction between NOTCH and Hippo

determines downstream Wnt activity and fine-tunes an appropriate degree of cardiac proliferation. Our protocol has provided an interesting model for cardiomyocyte proliferation to study disease mechanisms of congenital heart defects that are caused by the hypoplasia of ventricular cardiomyocytes, such as hypoplastic left heart syndrome (HLHS) and pulmonary atresia with intact ventricular septum (PA-IVS).

DISCLOSURES

The authors declare no competing financial interests.

ACKNOWLEDGEMENTS

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Figure 1

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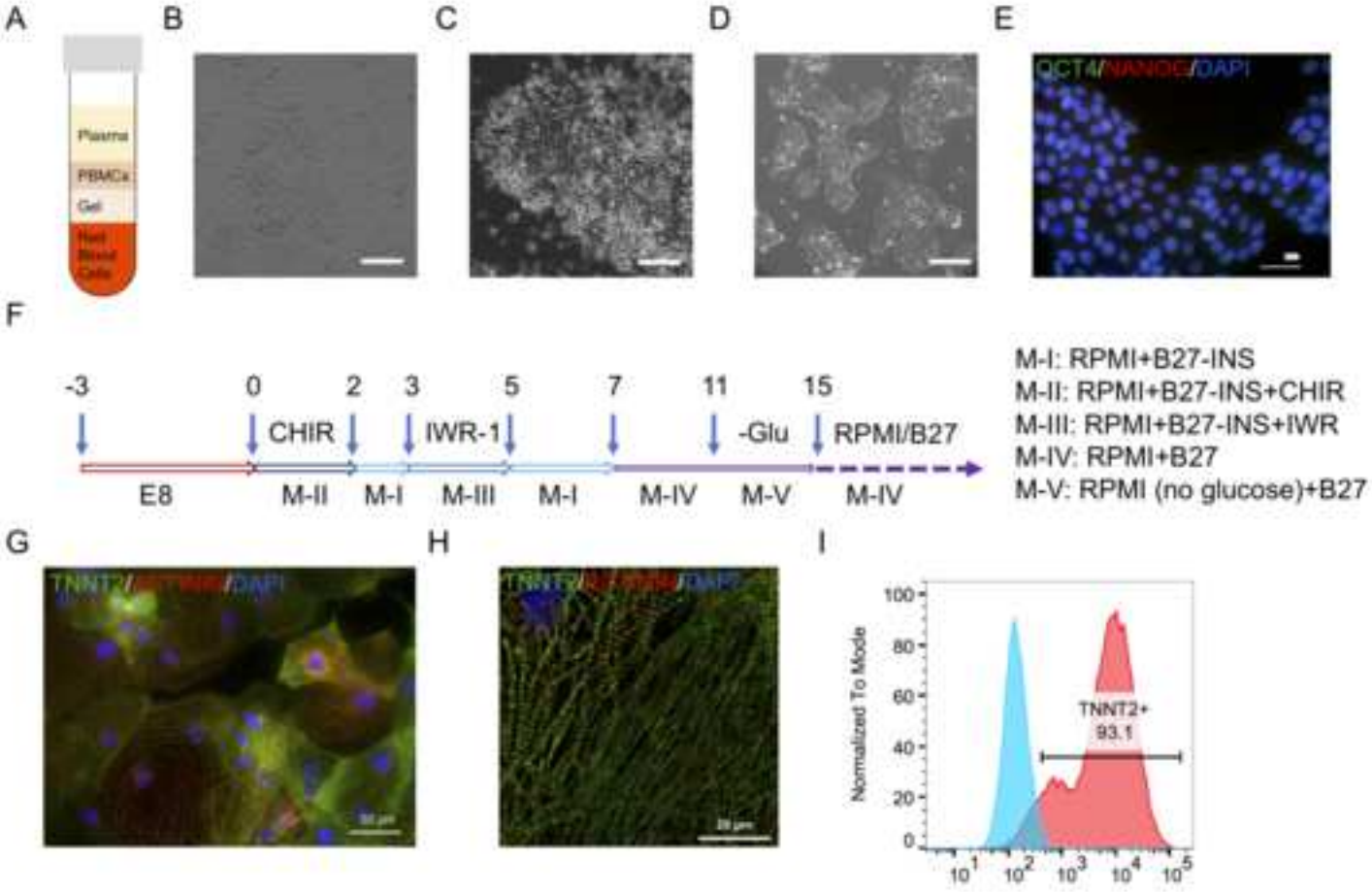


Figure 2

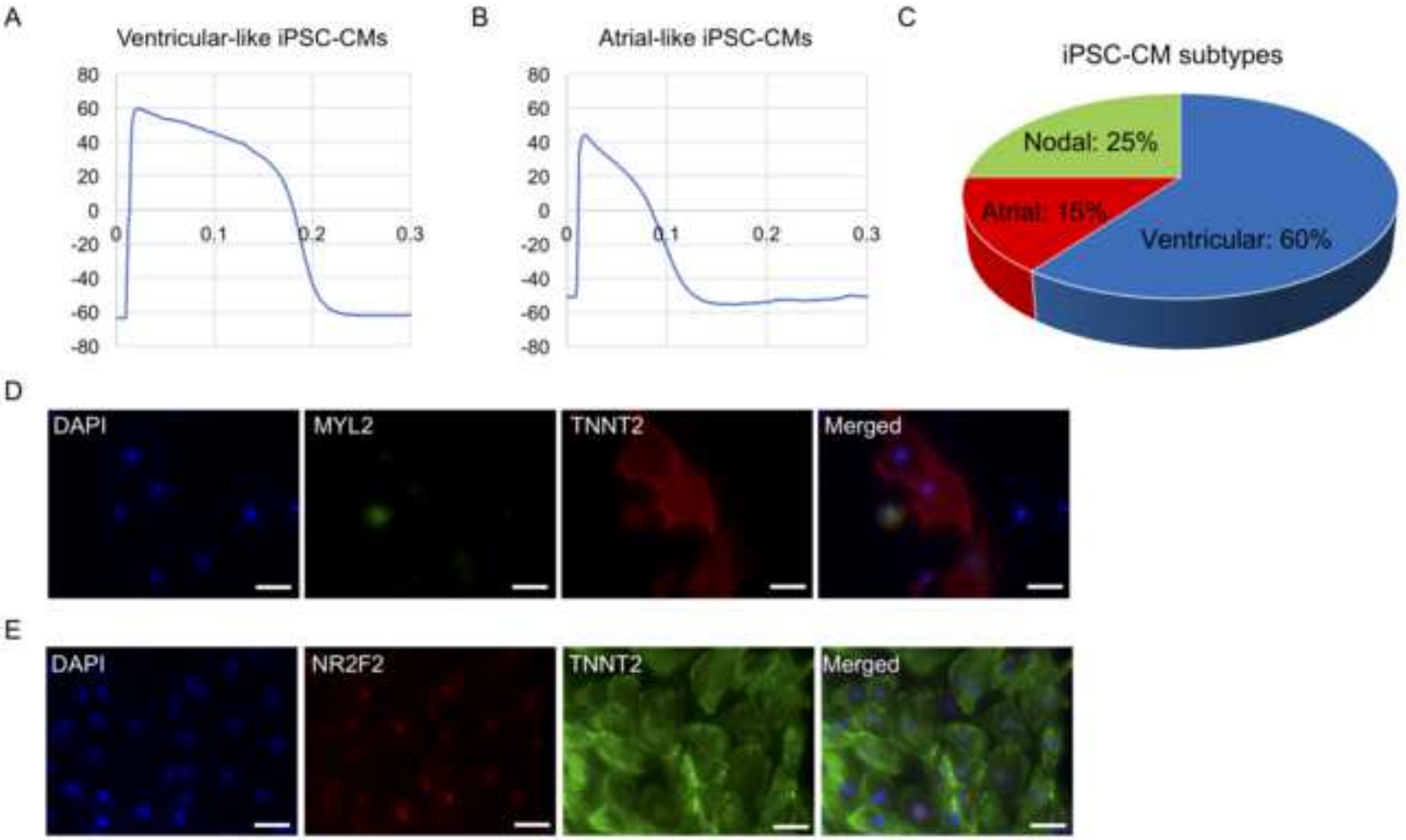
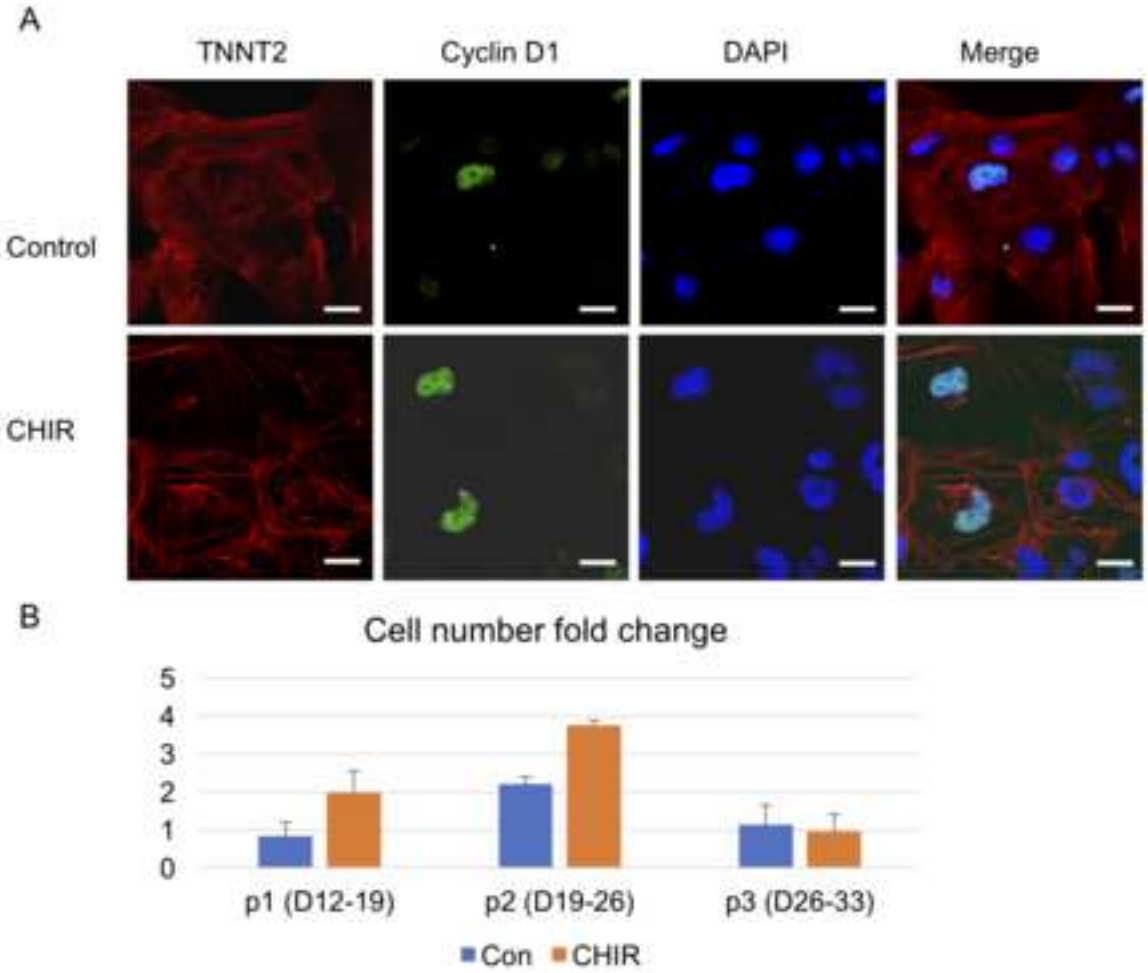


Figure 3



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Name of Material/Equipment	Company	Catalog Number
ABI 7300 Fast Real-Time PCR System	Thermo Fisher Scientific	
Axon Axopatch 200B Microelectrode Amplifier	Molecular Devices	
B27 supplement	Thermo Fisher Scientific	17504044
B27 supplement minus insulin	Thermo Fisher Scientific	A1895601
BD Cytofix/Cytoperm Fixation/Permeabilization Kit	BD Biosciences	554714
BD Vacutainer CPT tube	BD Biosciences	362753
CHIR99021	Selleck Chemicals	S2924
CytoTune-iPS 2.0 Sendai Reprogramming Kit	Thermo Fisher Scientific	A16517
Digidata 1200B	Axon Instruments	
Direct-zol RNA Miniprep kit	Zymo Research	R2050
DMEM/F12	Thermo Fisher Scientific	11330057
Essential 8 medium	Thermo Fisher Scientific	A1517001
GlutaMAX supplement	Thermo Fisher Scientific	35050061
Growth factor reduced Matrigel	Corning	356231
iScript cDNA Snythesis Kit	Bio-Rad	1708891
IWR-1-endo	Selleck Chemicals	S7086
KnockOut Serum Replacement (KSR)	Thermo Fisher Scientific	10828028
pCLAMP 7.0	Molecular Devices	
Recombinant human EPO	Thermo Fisher Scientific	PHC9631
Recombinant human FLT3	Thermo Fisher Scientific	PHC9414
Recombinant human IL3	Peprotech	200-03
Recombinant human IL6	Thermo Fisher Scientific	PHC0065
Recombinant human SCF	Peprotech	300-07
RPMI 1640 medium	Thermo Fisher Scientific	11875093
RPMI 1640 medium, no glucose	Thermo Fisher Scientific	11879020
SlowFade Gold Antifade Mountant	Thermo Fisher Scientific	S36936
StemPro-34 SFM	Thermo Fisher Scientific	10639011
TaqMan Fast Advanced Master Mix	Thermo Fisher Scientific	4444964
TrypLE Select Enzyme 10x, no phenol red	Thermo Fisher Scientific	A1217703
UltraPure 0.5 M EDTA	Thermo Fisher Scientific	15575020
Y-27632 2HCl	Selleck Chemicals	S1049

Comments/Description

Microelectrode Amplifier

Fixation/Permeabilization solution, Perm/Wash buffer
Blood cell separation tube

Sendai virus reprogramming kit
Acquisition board
RNA extraction kit

E8 media for iPSC culture
L-glutamine alternative
Matrigel
cDNA synthesis

Electrophysiology data acquisition & analysis software

Mounting media
PBMC culture media
qPCR master mix
CM dissociation solution
iPSC dissociation solution

Response to Editorial and Reviewers' Comments

Editorial Comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We have gone through the manuscript and corrected all spelling and grammar errors.

2. Please provide an email address for each author.

Response: Here is the email address for each author.

Shiqiao Ye: shiqiao.ye@nationwidechildrens.org
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Ming-Tao Zhao: Mingtao.Zhao@nationwidechildrens.org

3. Please include a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to…"

Response: The summary is "Here, we present a protocol to robustly generate and expand human cardiomyocytes from patient peripheral blood mononuclear cells."

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have modified the text accordingly.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: StemPro-34; GlutaMax Supplement; Millipore Steriflip; Corning Matrigel; Matrigel; Eppendorf; BD vacutainer CPT tube; TrypLE Select Enzyme; BD Perm/Wash buffer; Direct-zol RNA Miniprep kit; NanoDrop; iScript cDNA Synthesis Kit; iScript; TaqMan Fast Advanced Master Mix; ABI Prism 7300; Axopatch 200B amkplifier; Axon pCLAMP 7.0 software (Axon Instruments); Digidata 1200 acquisition board etc.

Response: We have removed all these trademark symbols, registered symbols and company name in the main text. Instead, they are listed in the Table of Materials and Reagents.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: We have modified the text in the protocol section accordingly.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We have revised the protocol accordingly.

8. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

Response: We have formatted the manuscript accordingly.

9. Please include a Figure and Table legends section between representative results and discussion.

Response: We have added a Figure and Table legends section in the main text.

10. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legends.

Response: We have included scale bars for all images and defined them in the corresponding legends.

11. Please do not abbreviate journal names in the reference list.

Response: We have listed full journal names in the References.

12. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together after the Representative Results in a section called “Figure and Table Legends” in the manuscript text.

Response: We have made titles and descriptions for all figures and added “Figure Legends” in the manuscript.

13. Please sort the Materials Table alphabetically by the name of the material.

Response: We have sorted the materials alphabetically. Please see the revised materials.

Reviewer #1

1) The abbreviations in the title may be removed.

Response: We have deleted the abbreviations in the title. We have defined all abbreviations at the first use in the text.

2) There are some existing protocols to derive iPSCs from PBMCs. Please highlight the difference between your protocol and others' in the DISCUSSION.

Response: We have discussed the difference between the non-integration Sendai virus-mediated reprogramming method and other approaches in the Discussion. The advantages of our reprogramming approach are also highlighted.

"…*There are two major issues with these reprogramming methods that prevent the progress in clinical translation of patient iPSCs: 1) the retrovirus integrates into the host genome thus introducing potential genetic mutations; 2) patient-derived fibroblasts require skin biopsies which many patients may decline. In this protocol, we describe a protocol that utilizes commercial non-integration Sendai virus[23] and PBMCs to robustly derive patient iPSCs. These iPSCs are free of exogenous reprogramming vectors and can be maintained with self-renewal and pluripotency indefinitely. In addition, patient blood samples are easily collected in clinical laboratories. Our protocol is versatile and can be used for mass production of patient- and disease-specific iPSCs for large-scale repository and clinical translations[24]*"

Reviewer #2

1) Line 110. "PBMCs will be in a whitish layer...". It will be helpful to include a picture to show the different cell layers.

Response: We thank the reviewer for this suggestion. We have made an illustration in Figure 1A to indicate the PBMC layer after separation by CPT tubes.

2) Line 200. "Before immunofluorescence staining, seed iPSC-CMs onto Matrigel-coated coverslips that are placed..." It will be easier to follow if a replating cell density can be recommended.

Response: The recommended seeding density is 0.5-1 x 10^6 cells/ml. We have added this in the revised protocol.

"*Before immunofluorescence staining, seed iPSC-CMs onto Matrigel-coated coverslips that are placed in a 24-well plate (seeding density: 0.5-1 x 10^6 cells/ml). Maintain iPSC-CMs in culture for at least 4 days*."

3) Line 252. Please provide the PCR parameters.

Response: The PCR cycling protocol is 50 °C 2 min (hold), 95 °C 10 min (hold), 95 °C 15 sec, 60 °C 1min, repeat for 40 cycles. We have added this in the revised text.

"Run in a real-time PCR system. The cycling protocol is 50 ℃ 2 min (hold), 95 ℃ 10 min (hold), 95 ℃ 15 sec, 60 ℃ 1 min, repeat for 40 cycles."

4) Line 274. Please cite the figures starting from Figure 1A.

Response: We thank the reviewer for this suggestion. We have reorganized the panels in Figure 1 and cited from Figure 1A.

Reviewer #3

1) Here the authors used a non-integration Sendai virus vector to reprograming PBMCs. How many iPSC clones can they obtain? How is the reprogramming efficiency?

Response: Typically, we get 30-50 iPSC colonies from the transfection of $1x10^5$ PBMCs. The reprogramming efficiency is 0.03%-0.05%. We pick up 12 clones to establish stable iPSC lines. This information has been added to the revised text.

"Typically, we get 30-50 iPSC colonies from the transfection of $1x10^5$ PBMCs and the reprogramming efficiency is 0.03%-0.05%."

2) For PBMC isolation, how much is the minimum volume (ml) of blood that is needed? 10ml? 20ml? This would be more challenging if this much of blood is collected from newborns and children. The authors should indicate the practical volume of blood, especially when these blood samples may be shipped from long-distance medical facilities across the country.

Response: According to our experience, the minimal blood volume for iPSC reprogramming is 5 ml. This will ensure enough PBMCs are isolated and sufficient iPSC clones are obtained for establishing stable iPSC lines. We have added this in the revised text.

"*Collect patient blood samples (minimum: 5 ml) and transfer into blood cell separation tubes. Mix by inverting 10 times*."