Functional Cardiomyocytes Derived From Human Induced Pluripotent Stem Cells

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Abstract—Human induced pluripotent stem (iPS) cells hold great promise for cardiovascular research and therapeutic applications, but the ability of human iPS cells to differentiate into functional cardiomyocytes has not yet been demonstrated. The aim of this study was to characterize the cardiac differentiation potential of human iPS cells generated using OCT4, SOX2, NANOG, and LIN28 transgenes compared to human embryonic stem (ES) cells. The iPS and ES cells were differentiated using the embryoid body (EB) method. The time course of developing contracting EBs was comparable for the iPS and ES cell lines, although the absolute percentages of contracting EBs differed. RT-PCR analyses of iPS and ES cell–derived cardiomyocytes demonstrated similar cardiac gene expression patterns. The pluripotency genes OCT4 and NANOG were downregulated with cardiac differentiation, but the downregulation was blunted in the iPS cell lines because of residual transgene expression. Proliferation of iPS and ES cell–derived cardiomyocytes based on 5-bromodeoxyuridine labeling was similar, and immunocytochemistry of isolated cardiomyocytes revealed indistinguishable sarcomeric organizations. Electrophysiology studies indicated that iPS cells have a capacity like ES cells for differentiation into nodal-, atrial-, and ventricular-like phenotypes based on action potential characteristics. Both iPS and ES cell–derived cardiomyocytes exhibited responsiveness to β-adrenergic stimulation manifest by an increase in spontaneous rate and a decrease in action potential duration. We conclude that human iPS cells can differentiate into functional cardiomyocytes, and thus iPS cells are a viable option as an autologous cell source for cardiac repair and a powerful tool for cardiovascular research. (Circ Res. 2009;104:e30-e41.)

Key Words: induced pluripotent stem cells ■ embryonic stem cells ■ cardiomyocyte ■ action potential ■ differentiation

The generation of induced pluripotent stem (iPS) cells from mouse somatic cells using a combination of 4 retrovirally transduced transcription factors (OCT3/4, Sox2, Klf4, and c-Myc) has opened remarkable new avenues for basic research and regenerative medicine applications.1 The promise of applying this technology to human cells was rapidly realized with the successful generation of human iPS cells from human fibroblasts using either the same combination of transcription factors or an independently determined combination of lentivirally transduced genes (OCT4, SOX2, NANOG, and LIN28).2,3 This approach has also recently been applied to generate disease-specific iPS cell lines from patients with a variety of diseases.4,5

Like mouse ES cells, mouse iPS cells have most rigorously been proven to be pluripotent by generation of chimeric animals following blastocyst injection with subsequent germline transmission.6,7 In comparison, ethical constraints preclude human embryo experiments for human iPS cells, and so formation of teratomas following transplantation of human iPS cells to immunocompromised mice has provided evidence for pluripotency based on finding derivatives of the 3 embryonic germ layers.7,8 In addition, in vitro differentiation studies are critically important for demonstrating pluripotency of human iPS cells and for characterizing the properties of the committed cell types that form. In vitro differentiation studies of various human iPS cell lines have identified derivatives of the 3 primary germ layers,5,8 but detailed characterization of the ability of human iPS cells to form specific cell lineages with functional characterization of the resulting cells are generally lacking. Critical issues such as viral integration, the combination of reprogramming genes, and residual transgene expression could fundamentally impact the differentiation potential of each iPS cell line.
Cardiac differentiation of human ES cells has been well described using embryoid body (EB) formation, or more recently, using directed differentiation approaches.9–13 Detailed molecular and functional characterization of the resulting ES cell–derived cardiomyocytes revealed multiple cell types including nodal, atrial, and ventricular cardiomyocytes typically found in the human heart.12,14,15 Given the promise of human iPSCs to supply large quantities of patient-specific cells for cardiac repair without the risk of immune rejection, it is essential to evaluate for the ability of human iPSCs to undergo cardiogenesis. Furthermore, use of iPSC cell–derived cardiomyocytes as in vitro models for cardiac disease or other research applications will require careful characterization of the properties of the cardiomyocytes. The purpose of this study was to provide a detailed evaluation of the cardiac differentiation potential of recently described human iPSC cell lines induced by OCT4, SOX2, NANOG, and LIN28 in comparison with well-studied human ES cell lines, H1 and H9.3,16

Materials and Methods

Human iPSC and ES Cell Culture

Human iPSC cell lines reprogrammed by the lentiviral-mediated transduction of 4 transcription factors (OCT4, SOX2, NANOG, and LIN28) were previously described.3 In the present study, we used a subset of those iPSC clones of fetal origin including IMR90 clone 1 (IMR90 C1), IMR90 clone 4 (IMR90 C4), and of newborn origin including Foreskin clone 1 (Foreskin C1) and Foreskin clone 2 (Foreskin C2). In addition, we used 2 human ES cell lines, H1 and H9.16 The iPSC and ES cells were maintained on irradiated mouse embryonic fibroblasts (MEFs) at a density of 19,500 cells/cm² in 6-well culture plates (Nunc) as previously described.3,12 In brief, both iPSC and ES cells were maintained in DMEM/F12 culture medium supplemented with 20% KnockOut serum replacer, 0.1 mmol/L nonessential amino acids, 1 mmol/L L-glutamine (all from Invitrogen), and 0.1 mmol/L β-mercaptoethanol (Sigma). In addition, the medium was supplemented with 100 ng/mL zebrafish basic fibroblast growth factor (purified from a bacterial expression system by us) for iPSC cells, and with 4 ng/mL human recombinant basic fibroblast growth factor (Invitrogen) for hES cells.

EB Formation and Cardiac Differentiation

Before EB formation, the iPSCs and ES cells were passaged onto a lower density of MEFs (~13,000 cells/cm²), and expanded for 3 to 4 days. Colonies were detached from 6-well culture plates by incubating with 1 mg/mL dispase (Gibco) solution at 37°C for 8 to 15 minutes and placed in ultralow attachment plates (Corning, catalog no. 3471) in suspension culture for 4 days. Differentiation medium (EB20), consisting of 80% DMEM/F12, 0.1 mmol/L nonessential amino acids, 1 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, and 20% FBS that was pretested for cardiac differentiation (Gibco, catalog no. 16000-044, lot no. 291526), was used to initiate cardiac differentiation. During suspension culture, the medium was changed at day 1 followed by culture for another 3 days without medium change. EBs were then plated on 0.1% gelatin-coated 6-well culture plates (Nunc) at the density of 50 to 100 EBs per well and cultured in EB20 medium (changed daily) for a total of 10 days from EB formation. After 10 days of differentiation, the FBS concentration was reduced to 2% (EB2 medium). The number of contracting EBs and contraction rates were measured at day 10, 20, 30, and 60 from EB formation using a microscope with a heated stage (37°C).

RT-PCR and Quantitative RT-PCR

Total RNA was isolated using TRizol Reagent (Invitrogen) from 1 well of a 6-well plate of undifferentiated iPSC cells and ES cells and from 30 to 40 contracting areas microdissected from day 60 iPSC or ES cell EBs. Total RNA from human heart tissue (obtained from donor hearts rejected for transplant because of technical reasons, following a protocol approved by the University of Wisconsin Institutional Review Board) was isolated from 1 g of left ventricular tissue using RNAzol B solution (Tel-Test, Friendswood, Tex).17 Possible genomic DNA contamination was removed by DNase I (Invitrogen) treatment for 15 minutes at room temperature. Total RNA (500 ng) was used for oligo(dT) primer–directed reverse transcription using SuperScript III First-Strand Synthesis System (Invitrogen). RT-PCR was carried out using Platinum Taq DNA Polymerase (Invitrogen). Genes of interest in RT-PCR and quantitative (Q)-PCR are listed in Table 1. PCR conditions included denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute for 35 cycles, with 72°C extension for 7 minutes at the end. ACTB (β-actin) was used as an endogenous control in RT-PCR. Quantitative RT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in triplicate for each sample and each gene. One microliter of 1:5 dilution of cDNA from reverse-transcription reaction was added as template for each RT-PCR or Q-PCR. For Q-PCR, the cDNA from undifferentiated H1 ES cells was used as a relative standard for the measurement of total and endogenous expression of OCT4 and NANOG. The expression of genes of interest was normalized to that of GAPDH in Q-PCR.

Immunolabeling

Undifferentiated iPSCs were plated on coverslips with MEF feeders as described for iPSC cell culture. Single cardiomyocytes were isolated from day 60 microdissected contracting areas using 0.25% trypsin-EDTA (Invitrogen) plus 2% chick serum (Sigma) for 5 to 10 minutes at 37°C. The cells were washed and plated on coverslips coated with 0.1% gelatin solution in EB20 medium for 3 days to allow attachment. Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, rinsed twice in PBS, and permeabilized in 0.2% Triton X-100 (Sigma) for 1 hour at room temperature. Samples were blocked with 5% nonfat dry milk (Bio-Rad) in 0.2% Triton X-100 solution followed by 3 washes with PBS. Primary antibodies, including monoclonal anti-Oct-4 (IgG, 1:100 dilution; Santa Cruz Biotechnology), polyclonal anti-Nanog (IgG, 1:100 dilution; Cosmo Bio Co Ltd), monoclonal anti-α-actinin (IgG1, 1:500 dilution; Sigma), monoclonal anti–cardiac troponin T (TrTrx) (IgG, 1:200 dilution; Thermo Scientific), monoclonal anti-MLC2a (IgG1, 1:400 dilution; Synaptic Systems, Germany), and polyclonal anti-MLC2v (IgG, 1:200 dilution; ProteinTech Group), were added to samples and incubated overnight at 4°C. The samples were washed in PBS with 0.2% Tween 20 twice and PBS twice. The isotype-specific secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen) and Alexa Fluor 594 goat anti-mouse IgG2a (Invitrogen) were used for IgG1 and IgG2a isotype primary antibodies. Alexa Fluor 488 or 594 goat anti-rabbit IgG (H+L) (Invitrogen) were used for polyclonal antibodies. The secondary antibodies were diluted in the same solution as the primary antibodies and incubated at room temperature for 1.5 hours in the dark on a rotator. The samples were washed with 0.2% Tween 20 twice and PBS twice. The isotype-specific secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen) and Alexa Fluor 594 goat anti-mouse IgG2a (Invitrogen) were used for IgG1 and IgG2a isotype primary antibodies. Alexa Fluor 488 or 594 goat anti-rabbit IgG (H+L) (Invitrogen) were used for polyclonal antibodies.

Analysis of Cardiomyocyte Proliferation

Single cardiomyocytes were isolated from early (day 10 to 20) and late (day 60) contracting EBs following plating on glass coverslips as described above for 2 days. Cells were pulsed for 17 hours with 10 μmol/L 5-bromodeoxyuridine (BrdUrd) (Invitrogen) in EB2 medium. Cells were fixed in 4% paraformaldehyde and washed twice with PBS solution. DNA was denatured in 2 mol/L HCl/0.1% Triton X-100 in PBS solution for 15 minutes at room temperature and washed 3 times with PBS solution. Primary antibodies for sarcomeric myosin, MF20 goat anti-mouse IgG1 (Invitrogen) and MF20 goat anti-mouse IgG2a, MF50 goat anti-rabbit IgG (H+L) (Invitrogen) were used to colabel the cells as described by guest on May 2, 2016http://circres.ahajournals.org/
above. Alexa Fluor 594 goat anti-mouse IgG2b (Invitrogen) was used to detect MF20/H11001 cells, and Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen) was used to detect BrdUrd/H11001 cells. Nuclei were stained with Hoechst as described above. The BrdUrd nuclei were counted relative to the total number of nuclei (Hoechst) in MF20/H11001 cells. At least 3 coverslips of at least 100 isolated cells were analyzed for each condition.

Electrophysiology

Single spontaneously contracting EB outgrowths were microdissected and plated on glass coverslips. Microdissected outgrowths were maintained in EB2 media for 1 to 10 days before recording. Cardiomyocyte activity was assessed from 56 to 70 days post-EB formation using sharp
microelectrodes (50 to 100 MΩ; 3 mol/L KCl) in a 37°C bath continuously perfused with Tyrode’s solution (mmol/L): 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1 MgCl2, 10 Hepes, 10 glucose (pH 7.4, NaOH). Junction potentials and capacitance were nulled and data were acquired at 10 kHz using an AxoClamp2A amplifier and pClamp 9.2 software (Molecular Devices, Sunnyvale, Calif). Electric field stimulation was performed using 2 platinum electrodes coupled to a S48 stimulator (Grass, Quincy, Mass). For analysis, data were filtered off-line using a low pass Gaussian filter with a cutoff frequency of 2 kHz.

Statistics
Data are presented as means±SEM. Statistical significance was determined by Student t test (2-tail) for 2 groups or 1-way ANOVA for multiple groups with post hoc testing using Tukey method (Microcal Origin version 7.5). P<0.05 was considered statistically significant.

Results
Cardiac Differentiation of Human iPS Cells in Embryoid Bodies
As an initial test of the ability of human iPS cells to undergo cardiac differentiation, we generated embryoid bodies (EBs) from 4 previously described iPS cell clones (IMR90 C1, IMR90 C4, Foreskin C1, Foreskin C2). A fraction of the EBs generated from each of the tested iPS cell lines formed contracting outgrowths of cells suggesting cardiac differentiation. The efficiency of forming contracting EBs after 30 days in culture varied significantly among iPS cell clones from less than 1% to 10% of the EBs having contracting regions (Figure 1A). Based on these results, we chose 2 iPS clones of different origins (IMR90 C4 and Foreskin C1), which readily underwent cardiogenesis for comparison to 2 well-characterized human ES cell lines, H1 and H9.

We first compared the in vitro differentiation of the iPS and ES cell lines by determining the time courses for formation of spontaneously contracting EBs and their associated rates of contraction. Contractions were first observed 8 to 9 days after EB formation for all of the iPS and hES cell lines (Movies 1 through 4 in the online data supplement). As shown in Figure 1B, contracting EBs developed over a similar time course during 60 days of observation, although the overall efficiency varied from line to line. H9 ES cells showed the greatest efficiency, reaching a maximum of 22%, in contrast to the least efficient, Foreskin C1, which reached 4.2%. The human iPS cell line IMR90 C4 and ES cell line H1 displayed nearly identical efficiencies (≅10%) for the formation of contracting outgrowths. Comparison of the rates of spontaneous contractions showed an increase in rate over the period of observation with some differences observed between the lines (Figure 1C). H9 EBs exhibited the highest contraction rates, whereas Foreskin C1 was slowest at most time points. Again, IMR90 C4 and H1 EBs performed comparably. In summary, there was no observable difference in the time course for the development of contraction for iPS- and ES-derived EBs, although the efficiency of forming contracting EBs varied for both iPS and ES cell lines.

Cardiac and Pluripotency Gene Expression in Cardiomyocytes Derived From iPS Cells
To provide a more detailed comparison of the cardiomyocytes derived from iPS cells and ES cells, we examined the gene expression patterns present in microdissected contracting regions from EBs using RT-PCR. Pluripotency and cardiac muscle gene expression was analyzed in day 60 EBs when cardiogenesis reached a stable plateau. The gene expression patterns of stem cell–derived cardiomyocytes were compared to those of undifferentiated iPS and ES cells, as well as adult human left ventricular myocardium.
We examined a variety of cardiac genes: the transcription factor Nkx2.5 (NKX2–5); several myofilament protein genes including cardiac troponin T (TNNT2), α-myosin heavy chain (MYH6), α-actinin (ACTN2), myosin light chain 2 atrial isoform (MYL7), and myosin light chain 2 ventricular isoform (MYL2); atrial natriuretic factor (HPPA); and phospholamban (PLN). The cardiac genes showed no detectable expression in undifferentiated iPS or ES cells with the exception of low levels of transcript detected for TNNT2 and MYL7. In contrast, by day 60, there was robust expression of the full range of cardiac genes in cardiomyocytes derived from iPS and ES cell lines, comparable to that observed in adult human ventricular myocardium (Figure 2A). Overall, the cardiac gene expression pattern was quite similar in iPS and ES cell–derived cardiomyocytes, with a strong increase in expression following cardiac differentiation.

For pluripotency genes, we focused on the most extensively studied genes, OCT4 and NANOG. Expression of OCT4 and NANOG was high in undifferentiated iPS and ES cells (Figure 2A) and in day 60 EB contracting areas (Figure 2A). Expression of OCT4 and NANOG was high in undifferentiated iPS and ES cells (Figure 2A) and in day 60 EB contracting areas (Figure 2A). Error bars represent SEM (n=3). ***P<0.001 comparing total and endogenous gene expression using t test. E, Double immunolabeling for Oct4 and cTnT in undifferentiated Foreskin C1 iPS cells (left) and Foreskin C1 iPS cell–derived cardiomyocytes from day 60 EBs (right). F, Double immunolabeling for Nanog and cTnT in undifferentiated IMR90 C4 iPS cells (left) and IMR90 C4 iPS cell–derived cardiomyocytes from day 60 EBs (right).

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For pluripotency genes, we focused on the most extensively studied genes, OCT4 and NANOG. Expression of OCT4 and NANOG was high in undifferentiated iPS and ES cells, and the expression greatly decreased with differentiation, with the exception of the Foreskin C1 iPS line. In the Foreskin C1 line, expression of OCT4 and NANOG persisted to some extent (Figure 2A). To provide a more quantitative assessment of OCT4 and NANOG expression during differentiation, we performed quantitative RT-PCR. OCT4 gene expression was significantly downregulated in EBs from all lines compared to undifferentiated iPS or ES cells (Figure 2B; P<0.001), and the degree of downregulation was similar for H1 (114-fold), H9 (105-fold) and IMR90 C4 (105-fold), but less for Foreskin C1 (14-fold). There was also significant downregulation of NANOG expression in all lines during differentiation (Figure 2C; P<0.001), although the decrease in NANOG in differentiated cardiomyocytes was relatively less than that of OCT4 (H1: 34-fold; H9: 45-fold; IMR90 C4: 4-fold; Foreskin C1: 4-fold). These results confirmed a reduction in pluripotency gene expression for both iPS and ES cells during cardiogenesis in EBs, but the reduction is variably blunted in the iPS cell lines.

To investigate if persistent expression of lentiviral transgenes contributes to the blunted downregulation of OCT4 and
NANOG during cardiogenesis of iPS cells, we measured the total OCT4 or NANOG gene expression using primers located in the coding region and compared it to the expression of endogenous OCT4 or NANOG using primers located in 3’ untranslated region. The difference between total and endogenous gene expression is attributable to expression of the transgene. In undifferentiated ES cells, the total and endogenous OCT4 and NANOG expression was not different as predicted given the lack of transgenes (Figure 3A and 3B). The iPS cells exhibited significantly greater total than endogenous OCT4 expression, indicating residual transgene expression most prominently in the case of the Foreskin iPS cell line (Figure 3A). For NANOG, the range of total expression varied in the undifferentiated cells, but there was no significant difference between total and endogenous expression of NANOG in either of the iPS cell lines (Figure 3B).

To determine whether transgene expression accounted for the difference in iPS and ES cell expression of pluripotency genes following differentiation, we compared total and endogenous expression of OCT4 and NANOG in contracting outgrowths isolated from each of the 4 cell lines. Total expression of OCT4 was comparable in differentiated H1, H9, and IMR90 C4 EBs, but the level of OCT4 transcript in Foreskin C1 EBs was significantly greater (P<0.001), which could largely be accounted for by persistent expression of the transgene (Figure 3C). In the case of NANOG, the relative total expression in EBs also varied from line to line (Figure 3D), and both IMR90 C4 and Foreskin C1 EBs exhibited a relatively higher total expression of NANOG than observed for EBs from H1 and H9 (P<0.001), an effect that, again, was largely attributable to transgene expression. Together, these results demonstrate that there is some persistent expression of the OCT4 and NANOG transgenes in the 2 iPS cell lines studied following differentiation, and this is especially evident for the OCT4 transgene in the Foreskin C1 iPS cell line. Nevertheless, a strong downregulation in total OCT4 and NANOG gene expression occurs during cardiogenesis of iPS cells. Although we did not evaluate LIN28 and SOX2 transgene expression, it is possible that some level of expression for these transgenes persists as well.

Given the findings of persistent OCT4 and NANOG transgene expression in differentiated iPS cell–derived cardiomyocytes, we examined whether Oct4 and Nanog protein expression could be detected using standard immunolabeling approaches. Immunolabeling for Oct4 and Nanog was examined in undifferentiated iPS cells and differentiated cells from day 60 EBs which were colabeled for cardiac troponin T (cTnT) to detect cardiomyocytes. In experiments using Foreskin C1 cells (Figure 3E), we detected nuclear-localized Oct4 immunolabeling in the undifferentiated iPS cells but not in the surrounding mouse fibroblast feeder layer cells. There was no detectable cTnT immunolabeling in the undifferentiated cells. In contrast, cells isolated from contracting outgrowths from Foreskin C1 EBs subjected to the identical immunolabeling protocol revealed that the majority of cells were cTnT-positive and showed DAPI-labeled nuclei without detectable Oct4 labeling. Nor was Oct4 labeling detected in any surrounding cTnT negative cells. Similar results were observed for Nanog and cTnT immunolabeling in the IMR90 C4 line (Figure 3F). Nanog was detected in the undifferentiated iPS cells but not detectable in IMR90 iPS cell–derived cardiomyocytes. These results show that Oct4 and Nanog protein expression is strongly downregulated during differentiation of Foreskin C1 and IMR90 C4 iPS cells despite some persistent mRNA expression. This apparent discrepancy between mRNA and protein expression could be attributable to a variety of regulatory effects such as microRNA regulation, which may be particularly important for pluripotency genes.18

**P<0.001 in t test comparisons of early and late EBs.**

**Figure 4.** Proliferation of cardiomyocytes differentiated from iPS and ES cells. A, Colabeling for sarcomeric myosin with the MF20 antibody (red) and BrdUrd (green) in cardiomyocytes isolated from contracting areas of early EBs (10 to 20 days) from H9 and IMR90 C4 following a 17-hour pulse of BrdUrd to identify dividing cells. Nuclei were stained with Hoechst (blue). Scale bars=50 μm. B, Average percentage of BrdUrd-positive nuclei in cardiomyocytes (MF20-positive) differentiated from H9 ES cells and IMR90 C4 and Foreskin C1 iPS cells from early and late (day 60) EBs. Error bars represent SEM (n=3). **P<0.01, ***P<0.001 in t test comparisons of early and late EBs.

Proliferation of Cardiomyocytes Derived From iPS and ES Cells

Because the proliferative activity of cells may be impacted by overexpression of genes associated with pluripotency, we next compared cellular proliferation in iPS cell– and ES cell–derived cardiomyocytes. In normal embryonic cardiac development, the proliferative activity of cardiomyocytes declines steeply at later stages, and a similar phenomena has also been observed during in vitro differentiation of human ES cell–derived cardiomyocytes.11,19,20 In this experiment, cardiomyocytes were isolated from early (days 10 to 20) and late (day 60) contracting EBs. Immunofluorescence analysis revealed that a fraction of the MF20-positive cells contained BrdUrd-positive nuclei, indicating proliferating cardiomyocytes for both iPS and ES cell–derived populations (Figure 4A). In early EBs, there was no difference in the percentage
of BrdUrd-positive cardiomyocyte (MF20-positive) nuclei for H9 and IMR90 C4 (≈15%), but Foreskin C1 showed a significantly lower percentage of dividing cardiomyocytes (≈11%, \( P<0.05 \)) compared to the other 2 cell lines (Figure 4B). Cells from late EBs revealed significantly less proliferation compared to early EBs in all the ES and iPS cells tested (\( P<0.01 \)). H9 late EBs exhibited greater proliferation of cardiomyocytes (4%) compared to late EBs from IMR90 C4 and Foreskin C1 (≈1%, \( P<0.05 \)). These results demonstrate that iPS cell–derived cardiomyocytes like ES-cell derived cardiomyocytes show a marked reduction in proliferation during 60 days in culture, and the proliferative activity of the iPS cardiomyocytes from the cell lines studied tended to be slightly less than for cardiomyocytes formed from H9 ES cells.

iPS Cell–Derived Cardiomyocytes Exhibit Sarcomeric Organization

To evaluate the expression of myofilament proteins and the sarcomeric organization in iPS cell–derived cardiomyocytes, we performed immunolabeling with antibodies for specific myofilament proteins using enzymatically isolated cells from day 60 EBs. Cells were colabeled with the antibodies for \( \alpha \)-actinin, which is present at the Z-line of the sarcomere, and myosin light chain 2 atrial isoform (MLC2a), which is typically present at the A-band of the sarcomere. Immunofluorescence analysis revealed a clear striated pattern for \( \alpha \)-actinin labeling in cardiomyocytes from IMR90 C4 and Foreskin C1 comparable to that observed for H1 and H9 cardiomyocytes (Figure 5). Striated MLC2a labeling was also observed in cardiomyocytes from all cell lines. Overlap of \( \alpha \)-actinin and MLC2a labeling demonstrated an alternating pattern in the sarcomeres in agreement with the known localization of MLC2a to the A-band of the sarcomere, which lies between the Z-lines highlighted by the \( \alpha \)-actinin labeling.

We also performed immunolabeling for cTnT, which is a highly cardiac-specific myofilament protein. We observed comparable sarcomeric labeling of iPS and hES cell cardiomyocytes (Figure 6). Likewise, immunolabeling with an antibody to the ventricular specific protein, myosin light chain 2 ventricular isoform (MLC2v), detected presumed ventricular cardiomyocytes derived from iPS cells and ES cells (Figure 6). In summary, immunolabeling of multiple myofilament proteins indicates that a well-organized sarcomeric structure can similarly develop in iPS and ES cell–derived cardiomyocytes.

Action Potentials Reveal Multiple Types of Cardiomyocytes Derived From iPS Cells

To provide an initial assessment of the functional competence of iPS cell–derived cardiomyocytes, we performed sharp microelectrode recordings from spontaneously contracting...
EB outgrowths at 56 to 70 days post-EB formation. A total of 54 and 47 stable recordings from 23 and 20 IMR90 C4 and Foreskin C1-derived EBs, respectively, were obtained. Three major types of action potential were observed: ventricular, atrial, and nodal. Cells with ventricular-like action potentials (Figure 7A, bottom) were the most frequently encountered in both IMR90 C4– and Foreskin C1–derived EBs, and typically displayed a more negative maximum diastolic potential, a rapid action potential upstroke, and a distinct plateau phase. Atrial-like cells were distinguished from ventricular-like cells by the absence of a distinct plateau during repolarization and typically also exhibited spontaneous activity that was higher in frequency than that observed for ventricular cells (Figure 7A, middle). Finally, nodal-like cells were distinguished by maximum diastolic potentials that were less negative than those of ventricular- and atrial-like cells, smaller amplitude action potentials, a slower action potential upstroke, and a pronounced phase 4 depolarization preceding the action potential upstroke (Figure 7A, top).

Comparison of recordings from cardiomyocytes within the same EB revealed that a given action potential phenotype was predominant in each EB, as has previously been shown for human ES cell–derived EBs. Figure 7B plots action potential durations measured at 90% repolarization from the action potential peak (APD90) for EBs from which 3 or more recordings were obtained. APD90 values obtained from myocytes within the same EB clustered together much more closely than would be predicted if EBs contained myocytes of each class. The relative proportions of EBs exhibiting the nodal, atrial, and ventricular phenotypes are shown in Table 2 and compared to the proportions observed for EB outgrowths derived from the H1 and H9 cell lines. Although sample sizes are limited because of the difficulty inherent in recording, iPS and ES cells appear comparably efficient in generating cardiac myocytes of each major class.

Table 3 presents a comparison of the properties of iPS and ES cell–derived action potentials for ventricular-like cells, which were the predominant class of cardiomyocytes encountered in EBs formed from each line. Specifically, we compared spontaneous beating rate (in beats per minute), action potential durations (APDs), the maximum rates of rise during the action potential upstroke, action potential amplitudes, and maximum diastolic potentials. As observed for H9 and H1 cardiomyocytes, the action potentials of IMR90 C4 and Foreskin C1 cardiomyocytes exhibited properties that were clearly more comparable to those of human embryonic, than neonatal or adult, cardiac muscle. Measurements from IMR90 C4– and Foreskin C1–derived cells fell within range of the measurements obtained for cardiomyocytes from H9.

Table 2. Comparison of Cardiomyocyte Types in H9–, H1–, IMR90 C4–, and Foreskin C1–Derived Cardiomyocytes

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The no. of EBs examined for each cell line and the proportion of EBs exhibiting nodal-, atrial-, and ventricular-like properties are shown.
and H1 ES cell lines, and, although there were modest differences in means, these were no larger than the differences between H9 and H1 cardiomyocytes.

Cardiomyocytes typically respond to increases in heart rate with a compensatory decrease in action potential duration, and this property is likewise present in ES cell–derived cardiomyocytes. Therefore, we tested whether iPS cell–derived cardiomyocytes exhibit this typical rate adaptation in response to changing rates of electric stimulation. As shown in Figure 8A and 8B, field stimulation of microdissected Foreskin C1–derived contracting outgrowths at 1, 2, and 3 Hz resulted in incremental shortening of the observed action potential durations. Increasing the stimulus frequency from 1 to 2 and 3 Hz decreased the average APD90 and APD50 by approximately 20% and 30% (Figure 8C), respectively, with little or no change in other action potential features.

### β-Adrenergic Regulation of iPS Cell–Derived Cardiomyocytes

The above experiments suggest that iPS and ES cell–derived cardiomyocytes exhibit similar functional potential in regard to their electric activity. We next sought to determine whether β-adrenergic signaling, a canonical cardiomyocyte signaling pathway, is operational in iPS cell–derived myocytes by examining the responsiveness to isoproterenol (ISO), a non-selective β-adrenergic receptor agonist. In spontaneously active myocytes such as those derived from embryonic or neonatal hearts, as well as nodal cells in adult hearts, β-adrenergic receptor stimulation, via protein kinase A–mediated regulation of several different ion channels, results in a positive chronotropic effect that is accompanied by a shortening of APD. Figure 9A and 9B shows individual responses to ISO for ventricular-like myocytes derived from H9 (Figure 9, top) and Foreskin C1 (Figure 9, bottom) lines. Both cells displayed initial contraction frequencies slightly above 0.6 Hz during perfusion of control Tyrode’s solution and responded to perfusion of ISO (1 μmol/L), with a 2-fold or greater increase in rate. As shown in Figure 9B, the increases in rate were also accompanied by decreases in action potential duration as observed in native cardiomyocytes. Summary data are presented in Figure 9C; perfusion with ISO resulted in statistically significant increases in rate and decreases in action potential duration for IMR90 C4– and Foreskin C1–derived, as well as H9–derived, cardiomyocytes. These results suggest that β-adrenergic receptors and their associated intracellular signaling partners are present and functional in cardiomyocytes derived from iPS cells.

### Table 3. Comparison of Action Potential Properties of Ventricular-Like Cardiomyocytes Derived From H9, H1, IMR90 C4, and Foreskin C1 Cell Lines

<table>
<thead>
<tr>
<th>No. of Cells</th>
<th>Rate (bpm)</th>
<th>APD50 (ms)</th>
<th>APD90 (ms)</th>
<th>dV/dt_max (V/sec)</th>
<th>APA (mV)</th>
<th>MDP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iPS cell–derived</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMR90 C4</td>
<td>37</td>
<td>43.8±2.7</td>
<td>240.7±15.0</td>
<td>320.1±17.0</td>
<td>40.5±4.6</td>
<td>87.7±2.6</td>
</tr>
<tr>
<td>Foreskin C1</td>
<td>39</td>
<td>44.2±3.5</td>
<td>238.9±10.0</td>
<td>312.5±11.2</td>
<td>27.2±3.7</td>
<td>87.9±2.4</td>
</tr>
<tr>
<td>ES cell–derived</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9</td>
<td>45</td>
<td>44.6±2.8</td>
<td>223.9±13.4</td>
<td>312.6±15.9</td>
<td>33.5±3.7</td>
<td>83.5±2.4</td>
</tr>
<tr>
<td>H1</td>
<td>27</td>
<td>33.3±2.3</td>
<td>233.9±11.4</td>
<td>298.7±13.4</td>
<td>17.6±2.6</td>
<td>82.7±3.0</td>
</tr>
</tbody>
</table>

Data are means±SEM. dV/dt_max indicates maximum rate of rise of action potential upstroke; APA, action potential amplitude; MDP, average maximum diastolic potential for action potentials during the time period examined.
The cause of this variability is not understood.

Cardiogenesis among human ES cell lines has previously been a small region of the EBs containing contracting cardiomyocytes and only with a minority of EBs developing cardiomyocytes and only a small region of the EBs containing contracting cardiomyocytes. Although EB-mediated cardiac differentiation is the classic in vitro approach, directed differentiation approaches have more recently been applied with promising results for cardiogenesis from human ES cells that likely will also be useful for iPS cardiac differentiation.\textsuperscript{9,13,25}

The time course of in vitro cardiac differentiation was not different comparing the human iPS and ES cell lines based on a similar time required to form contracting EBs. In contrast, one of the first studies to examine the time course of differentiation of mouse iPS cells to cardiomyocytes noted a substantial delay in cardiogenesis compared to mouse ES cells,\textsuperscript{26} but another study using different mouse iPS cell lines found no difference between the mouse iPS and mouse ES cell lines in cardiac differentiation.\textsuperscript{27} Given that first generation iPS cells are all subject to viral integration effects and that integration sites are highly variable,\textsuperscript{28} from a probabilistic perspective, it seems likely that some iPS cell lines will have altered cardiogenesis. However, our results and results with mouse iPS cells suggest that iPS cell lines can be readily obtained that undergo cardiogenesis in a highly similar fashion to ES cells.

Although functional cardiomyocytes have been obtained from human ES cells and iPS cells, significant challenges remain in optimizing these cells preparations for experimental and potential clinical applications. The heterogeneity of cells produced in differentiation protocols can be great even if one succeeds in isolating cardiomyocytes. For example, using a

**Figure 9.** Effect of ISO on spontaneous electric activity of iPS cell–derived cardiomyocytes. A, Time course of responses for an H9–derived (top) and a Foreskin C1–derived (bottom) cardiomyocyte before, during, and after perfusion with Tyrode's solution containing 1 \( \mu \text{mol/L} \) ISO, as indicated by measurement of the instantaneous frequencies. Bar at the top of each graph shows the duration of ISO application. B, Action potentials recorded from the cells in A before and during perfusion of ISO. C, Bar graphs of the average rate and APD90 data for H9–, IMR90 C4–, and Foreskin C1–derived cardiomyocytes before (light gray bars) and during (black bars) application of ISO. Number of cells examined for each cell type is indicated in parentheses below each graph. \(* P<0.05, ** P<0.01\) in paired \(t\) test comparisons.
mixed population of cardiomyocytes including nodal, atrial and ventricular cells in attempts at left ventricular repair raises concerns for proarhythmia effects. Likewise, a preparation including undifferentiated cells could lead to tumorigenesis. Thus, approaches to produce homogenous or well-characterized mixed cell preparations remain a great need. Techniques to isolate cardiovascular progenitor cell populations from human ES cells have now been described which help address this concern.9 Another limitation observed with the in vitro differentiation of human ES and iPS cells is that the myocytes even after 2 months of standard 2D tissue culture conditions remain embryonic in phenotype based on their size, organization, and electric properties. Techniques to promote the maturation of these cells, such as 3D culture methodology, as well application of electric and/or mechanical stimulation, will likely help address this need. The ability of human ES or iPS cell–derived cardiomyocytes to mature following transplantation has not been demonstrated, but this seems a reasonable expectation.

The technology to generate iPS cells is rapidly evolving and overcoming some of the challenges associated with the first generation iPS cells used in this study. Mouse iPS cells have now been generated using adenovirus or plasmid-mediated transfections which avoid the potential problems associated with viral integration of transgenes.29,30 Small molecules have been used to increase the efficiency of generating iPS cell lines and allowed the use of only 2 transcription factors (OCT4/SOX2 or OCT4/KLF4) to generate iPS cell lines.31,32 Given the pace of progress, it seems likely that techniques to generate human iPS cells will continue to rapidly improve; nevertheless, the present study provides evidence that with existing OCT4/ISOX2/NANOG/LIN28 human iPS cells, functional cardiomyocytes that exhibit properties highly similar to human ES cell–derived cardiomyocytes can be generated. Thus, iPS cell–derived cardiomyocytes hold significant promise for research applications studying cardiac disease models, drug development, and as an autologous source of cells for myocardial repair.

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Disclosures

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