Generation of Functional Murine Cardiac Myocytes From Induced Pluripotent Stem Cells

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Background—The recent breakthrough in the generation of induced pluripotent stem (iPS) cells, which are almost indistinguishable from embryonic stem (ES) cells, facilitates the generation of murine disease– and human patient–specific stem cell lines. The aim of this study was to characterize the cardiac differentiation potential of a murine iPS cell clone in comparison to a well-established murine ES cell line.

Methods and Results—With the use of a standard embryoid body–based differentiation protocol for ES cells, iPS cells as well as ES cells were differentiated for 24 days. Although the analyzed iPS cell clone showed a delayed and less efficient formation of beating embryoid bodies compared with the ES cell line, the differentiation resulted in an average of 55% of spontaneously contracting iPS cell embryoid bodies. Analyses on molecular, structural, and functional levels demonstrated that iPS cell–derived cardiomyocytes show typical features of ES cell–derived cardiomyocytes. Reverse transcription polymerase chain reaction analyses demonstrated expression of marker genes typical for mesoderm, cardiac mesoderm, and cardiomyocytes including Brachyury, mesoderm posterior factor 1 (Mesp1), friend of GATA2 (FOG-2), GATA-binding protein 4 (GATA4), NK2 transcription factor related, locus 5 (Nkx2.5), T-box 5 (Tbx5), T-box 20 (Tbx20), atrial natriuretic factor (ANF), myosin light chain 2 atrial transcripts (MLC2a), myosin light chain 2 ventricular transcripts (MLC2v), α-myosin heavy chain (α-MHC), and cardiac troponin T in differentiation cultures of iPS cells. Immunocytology confirmed expression of cardiomyocyte-typical proteins including sarcomeric α-actinin, titin, cardiac troponin T, MLC2v, and connexin 43. iPS cell cardiomyocytes displayed spontaneous rhythmic intracellular Ca²⁺ fluctuations with amplitudes of Ca²⁺ transients comparable to ES cell cardiomyocytes. Simultaneous Ca²⁺ release within clusters of iPS cell–derived cardiomyocytes indicated functional coupling of the cells. Electrophysiological studies with multielectrode arrays demonstrated functionality and presence of the β-adrenergic and muscarinic signaling cascade in these cells.

Conclusions—iPS cells differentiate into functional cardiomyocytes. In contrast to ES cells, iPS cells allow derivation of autologous functional cardiomyocytes for cellular cardiomyoplasty and myocardial tissue engineering. (Circulation. 2008;118:507-517.)

Key Words: myocytes ■ stem cells ■ differentiation

Therapeutic cell transplantation and replacement of injured tissue by in vitro engineered bioartificial substitutes may substantially improve current therapeutic procedures. For the majority of tissue types, including the heart, the lack of suitable cell sources represents one of the major hurdles to be overcome before clinical application of novel regenerative therapies. For instance, engineering of bioartificial cardiac muscle is hampered by the fact that adult cardiomyocytes have almost no potential for proliferation. Although a variety of studies reported cardiac differentiation of extracardiac and cardiac adult stem and progenitor cells,1,2 it is currently unclear whether those cell types can be expanded sufficiently or give rise to functional cardiomyocytes.3,4

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In 2006, Takahashi and Yamanaka5 described the reprogramming of somatic cells to a pluripotent state based on retroviral overexpression of the 4 transcription factors oc-
tamer binding transcription factor 3/4 (Oct 3/4), Sry-related HMG-box transcription factor (Sox2), cellular myelocytoma- tosis oncogene (c-Myc), and Kruppel-like factor 4 (Klf4) in embryonic or adult mouse fibroblasts. In contrast to this initial groundbreaking study, further reports that used optimized selection approaches and, more importantly, prolonged periods of reprogramming provided the ultimate proof of pluriptoty by generation of viable chimeric offspring after injection of induced pluripotent stem (iPS) cells into blastocysts.6–8

Under these optimized conditions, murine iPS cells were generated, which were largely identical to embryonic stem (ES) cells in global gene expression,7 DNA methylation,6 and histone modification.8 These iPS cells were able to differentiate into cell types representing all 3 germ layers and were germline competent.7 Recently, 4 studies reported successful reprogramming of human somatic cells,9–12 even without the proto-oncogene c-Myc.9 In contrast to ES cells, iPS cells are not ethically controversial and provide a route to the produc- tion of patient-specific (autologous) stem cell lines. Whereas recent studies demonstrated that mouse iPS cells are able to form derivatives of all 3 germ layers, in vitro differentiation of murine iPS cells into functional cardiomyocytes and detailed investigations of the differentiation of such iPS cell derivatives have not been reported. It was therefore the aim of the present study to differentiate murine iPS cells into functional cardiomyocytes and to characterize these cells in direct comparison to cardiomyocytes generated from a well-characterized murine ES cell line.13

Methods

Culture of Undifferentiated Murine iPS and ES Cells

The murine iPS cell clone O9, reprogrammed by the transduction with retroviral vectors encoding the 4 transcription factors Oct 3/4, Sox2, c-Myc, and Klf4 and selected for the activation of the endogenous Oct 3/4 gene,8 and the murine ES cell line E14.1, 129/Ola with an EGFP transgene targeted to the Brachyury locus14 were routinely cultured and expanded on mitotically inactivated embryonic or adult mouse fibroblasts. In contrast to this study, iPS cells are not ethically controversial and provide a route to the production of patient-specific (autologous) stem cell lines.

Cardiac Differentiation of Murine iPS and ES Cells

Before the differentiation was initiated, iPS and ES cell colonies were passaged up to 4 times without feeder cells on 6-well culture dishes coated with 0.1% gelatin to eliminate contaminating MEFs. Subsequently, colonies were detached with 0.2% collagenase IV and dissociated into single cells with 0.025% trypsin and 0.1% chicken serum. To initiate embryoid body (EB) formation, “hanging drops” composed of 600 cells in 20 μL of differentiation medium were generated (day 0 of differentiation). The differentiation medium was based on Iscove’s modified Dulbecco’s medium (Invitrogen) and supplemented with 15% fetal calf serum, 0.2 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, and 0.1 mmol/L nonessential amino acid stock. At day 3 of differentiation, the EBs were transferred onto nonadherent 1% agarose-coated 96-well culture dishes (Nunc) with 1 EB per well and cultivated for 2 additional days. At day 5 of differentiation, ~10 EBs per well were seeded on a 0.1% gelatin-coated 6-well culture dish or ~3 EBs per well on a gelatin-coated 12-well culture dish (Nunc) for immunofluorescence analyses. From day 5 until day 24, differentiation medium was replaced every second to third day. Starting on day 6 of differentiation (1 day after plating), each EB outgrowth was examined daily for spontaneously beating areas.

Analysis of mRNA Expression by Reverse Transcription Polymerase Chain Reaction and Quantitative Real-time Polymerase Chain Reaction

Total RNA was isolated from undifferentiated cells (day 0) and from EBs collected on days 3, 5, 10, and 21 of differentiation with the use of Trizol (Invitrogen), according to the manufacturer’s instructions. Integrity of total RNA was controlled with an Agilent 2100 Bioan- alyzer (Agilent Technologies, California). Potential contaminants were digested by DNase I (Stratagene, La Jolla, Calif) for 15 minutes at 37°C followed by phenol/chloroform extraction. After precipitation, 750 ng RNA was used for oligo(dT)12-18- primed cDNA synthesis with SuperScript II, RNase H Reverse Transcriptase (Invitrogen).

One microliter of cDNA was amplified by polymerase chain reaction (PCR) with 1.25 U REDTag DNA-polymerase (Sigma-Al- drich) in REDTag ×10 reaction buffer, with the use of 0.4 μmol/L of each primer and 0.4 μmol/L dNTPs in a 25-μL reaction. PCR conditions included denaturation at 94°C for 1 minute, annealing at T, for 1 minute (for annealing temperatures of individual primer pairs, see Table I in the online-only Data Supplement), and polymerization at 72°C for 1 minute and 30 seconds; after 35 cycles, an extension step of 10 minutes at 72°C was added. Primers were designed with the use of Primer3 Software (http://primer3.sourceforge.net/). Controls without reverse transcriptase excluded false-positive results due to contamination with genomic DNA.

Quantitative real-time PCR was performed in triplicate with the use of a Mastercycler ep realplex (Eppendorf, Hamburg, Germany) and the Absolute QPCR SYBR Green Mix (Abgene, Epsom, Surrey, UK). PCR reaction mixtures contained cDNA template, primers, and ×1 Absolute QPCR SYBR Green Mix in a final volume of 25 μL. Real-time PCR conditions included enzyme activation at 95°C for 15 minutes, followed by 40 cycles (denaturation at 95°C for 15 seconds, annealing at T, for annealing temperatures of individual primer pairs, see Table I in the online-only Data Supplement) for 60 seconds, and extension at 72°C for 60 seconds), with a final 10°C step. Size of amplicons and absence of nonspecific products were controlled by melting curves and gel electrophoresis.

Starting quantities of target cDNAs were calculated by comparing threshold cycle (Ct) values of each sample with Ct values of the respective standard curve with the use of Mastercycler ep realplex software version 2.0 (Eppendorf). Expression levels of target genes were normalized to β-actin transcript levels. Data are given as mean±SEM of normalized gene expression levels from 3 differen- tiation experiments.

Immunocytophlogical Staining

Colonies of undifferentiated iPS and ES cells were immunostained for expression of the stage-specific embryonic antigen-1 (SSEA-1). Plated EBs were stained on day 21 of differentiation to detect characteristic cardiomyocyte proteins. Some beating areas were dissected and dissociated to obtain small clusters of contracting cells.
that were seeded again onto 0.1% gelatin-coated 12-well culture dishes. Plated EBs (day 21) and single beating clusters (day 22), respectively, were fixed with 4% paraformaldehyde for 20 minutes at 4°C and subsequently blocked with 5% donkey/goat serum and 0.25% Triton X-100 (Sigma-Aldrich) diluted in Tris-buffered saline for 20 minutes at room temperature. The following primary antibodies were used: mouse monoclonal IgM anti-SSEA-1 antibody (3.1 μg/mL; clone MC-480, Developmental Studies Hybridoma Bank, Iowa), mouse monoclonal IgG1 anti-sarcomeric α-actinin antibody (16.8 μg/mL; clone EA-53, Sigma-Aldrich), mouse monoclonal IgM anti-titin antibody (3 μg/mL; clone 9D10, Developmental Studies Hybridoma Bank), mouse monoclonal IgG, anti-cardiac troponin T (cardiac isoform) antibody (2 μg/mL; clone 13-11, Labvision, Fremont), mouse monoclonal IgG, anti-myosin light chain 2 ventricular (MLC2v) antibody (1 μg/mL; clone F109.3E1, Biocytex, Marseille, France), and rabbit polyclonal IgG anti-connexin 43 antibody (10 μg/mL; AB1728, Millipore). The cells were incubated for 1 hour at room temperature with the primary antibody diluted in PBS (without Ca²⁺ and Mg²⁺) with 1% bovine serum albumin, then rinsed 3 times for 5 minutes with PBS. Further incubation was performed with the appropriate secondary antibody for 30 minutes at room temperature: donkey anti-mouse IgG either Cy2-labeled (1:100; Jackson Immunoresearch Laboratories, West Baltimore, Md) or Cy3-labeled (1:400; Jackson Immunoresearch Laboratories), Cy3-labeled donkey anti-mouse IgM (1:400; Jackson Immunoresearch Laboratories), and goat anti-rabbit IgG either Cy2-labeled (1:100; Jackson Immunoresearch Laboratories) or Cy3-labeled (1:400; Jackson Immunoresearch Laboratories), each diluted in PBS with 1% bovine serum albumin. The cells were rinsed once more, counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich), and analyzed with an Axio Observer A1 fluorescence microscope (Zeiss, Germany).

Intracellular Calcium Measurements Using Confocal Laser Microscopy

Beating areas were dissected, dissociated, and plated onto 0.1 gelatin-coated 35-mm glass-bottom dishes (Willco Wells, Netherlands). The next day, intracellular calcium ([Ca²⁺]) signals were recorded after cells were incubated with 10 μmol/L fluo-4 acetoxymethyl ester (Invitrogen) for 15 minutes on a laser scanning confocal...
microscope (Zeiss LSM 5 PASCAL, Goettingen, Germany) as
described previously.15 Cells were washed with Tyrode's solution.
Fluo-4 was excited via an argon laser (488 nm; 30 mW), and emitted
fluorescence (F) was collected through a 505-nm long-pass emission
filter. Changes in fluo-4 fluorescence (indicating fluctuation in
cytosolic Ca2+/H11001) were recorded in frame and line scan mode while the
cells were beating spontaneously. In a subset of experiments, cells
were superfused with caffeine (10 mmol/L) to measure sarcoplasmic
reticulum (SR) Ca2+/H11001 load.16 The images were acquired and analyzed
with the use of Zeiss software, and fluorescence signals were
normalized to basal cell fluorescence after fluo-4 loading (F0).
Intracellular Ca2+/H11001 was assessed by line scan mode and calibrated by
the following pseudo-ratio equation \[\frac{\text{[Ca}^{2+}]_i}{K_d} = \frac{\text{F/F}_0}{\text{K}_d/\text{[Ca}^{2+}]_{\text{rest}}} + 1 - \text{F/F}_0\] with \(K_d = 1100 \text{ nmol/L}\) and \([\text{Ca}^{2+}]_{\text{rest}} = 100 \text{ nmol/L} \).

**Multielectrode Mapping**

To characterize functional properties of iPS cell–derived cardiomyocytes, extracellular recording of field potentials (FPs) was performed
with the use of a multielectrode array (MEA) data acquisition system
(Multi Channel Systems, Reutlingen, Germany; http://www.
multichannelsystems.com).17,18 For this analysis, beating areas in
plated EBs of iPS and ES cells were mechanically removed and
plated on MEA culture plates. The MEA system allows measurement of
frequency behavior and activity of different ion channels involved
in cardiac action potential generation as well as propagation in
3-dimensional cardiac tissue in parallel and therefore serves as an
ideal system to characterize specific functional properties of iPS
cell–derived cardiomyocytes in comparison to ES cell cardiomyocytes.18 The standard substrate-integrated MEA culture dish contains
60 titanium nitride–coated gold electrodes (30 \(\mu\)m in diameter)
arranged in an 8\(\times\)8 electrode grid with an interelectrode distance of
200 \(\mu\)m, allowing simultaneous recording of FPs from all electrodes
at a sampling rate of 1 to 50 kHz with the use of the MEA amplifier
system. Standard measurements were performed at 5 kHz in serum-
free medium. (+/−)-Isoproterenol hydrochloride (ISO), used as
standard stimulator of the \(\beta\)-adrenergic signaling cascade, and
carbachol, a synthetic acetylcholine analogue, were dissolved in
serum-free medium and stored according to the manufacturer's
guidelines. During recordings, temperature was kept at 37.0°C. Data
were analyzed offline with MATLAB (The Mathworks, Natick,
Mass) to detect and characterize FPs as previously described.20,21
The frequency of contractions was calculated by measuring the
distance between respective FP minima and denoted interspike
interval.18,22

![Figure 3](image_url)
Statistical Analysis

Results are given as mean±SEM. Data were analyzed by the parametric unpaired Student t test or the nonparametric Mann–Whitney U test. Values with P<0.05 were considered statistically significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Differentiation of iPSC and ES Cells Toward Spontaneously Contracting Cardiomyocytes

Growing either on MEFs or on gelatin-coated dishes under feeder cell–free conditions, undifferentiated murine iPS cells (Figure 1A, 1C) formed colonies very similar to murine ES cells (Figure 1D, 1F). Furthermore, colonies of both cell types showed expression of SSEA-1, a typical marker for undifferentiated cells (Figure 1A, 1C). As a result, both murine iPS and ES cell–derived EBs developed spontaneously contracting areas. The percentage continued to increase until 84% to 100% of ES cells had contracting areas. Very few iPS cell–derived EBs with spontaneously contracting areas were seen on day 8 of differentiation. Before day 15 of differentiation, the percentage of iPS cell–derived EBs with beating areas remained low. After day 15, the percentage continued to increase until 55±4.2% of ES cells had contracting areas on day 24 of differentiation (Figure 2A and Movie 1 in the online-only Data Supplement). In total, 153 iPS cell–derived EBs and 78 ES cell–derived EBs from separate differentiation experiments were included in the analysis. The number of beating regions per EB varied from 1 to 9 in ES cell–derived EBs and from 1 up to 15 in iPS cell–derived EBs. Although these beating regions differed considerably in size and morphology, iPS cell–derived beating clusters were usually smaller than those in ES cell EBs. These data correlated with the mRNA expression level of the marker gene cardiac troponin T, which was analyzed by means of quantitative real-time PCR. Real-time PCR revealed a 3 to 5 times higher expression level of cardiac troponin T in differentiating ES cell cultures on days 10 and 21 compared with iPS cell derivatives (n=3; Figure 2B).

Reverse Transcription PCR Analyses Showed Expression of Marker Genes Typical for Mesoderm, Cardiac Mesoderm, and Cardiomyocytes in Differentiating iPS Cell Cultures

To characterize the differentiation pathway of undifferentiated murine iPS cells and of murine ES cells making the transition to functional cardiomyocytes, semiquantitative reverse transcription (RT) PCR analyses were performed (Figure 3). Controls without reverse transcriptase were used to exclude false-positive results based on contamination with genomic DNA.

The choice of target genes was based on the fact that early mesendodermal progenitors give rise to mesoderm, cardiac mesoderm, and cardiomyocytes. Endodermal marker genes were included because there is evidence that endoderm supplies important molecular signals for these differentiation steps. To control for the presence of different cellular intermediates and key regulators and to identify mature cardiomyocytes, we included a series of marker genes in the study: Oct 3/4 and Nanog expression indicates the presence of mesoderm, Cardiac Mesoderm, and Cardiomyocytes in Differentiating iPS Cell Cultures

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Figure 4. Quantification of Nanog and total Oct 3/4 expression levels in iPS and ES cells on days 0, 10, and 21 of differentiation (1 day after plating), 49.7% of the

expression level on day 21 of differentiation in both iPS and ES cells. Nanog expression in ES cells decreased to almost undetectable levels in differentiating ES cells. In contrast, Nanog expression had also decreased in iPS cell derivatives but was still detectable until day 21 of differentiation. The RT-PCR results demonstrated that, even in “undifferentiated” iPS and ES cell cultures, the majority of the chosen mesodermal and endodermal markers could be detected, albeit at lower levels than during differentiation. Even though statements about differences in expression based on semiquantitative RT-PCR should be judged with caution, our data suggest upregulation of the early mesendodermal/mesodermal markers Brachyury and Mesp1 on days 3 and 5 of differentiation followed by downregulation of both markers in ES cells and of Mesp1 (but not Brachyury) in iPS cells. Increased expression of the cardiac mesodermal marker genes FOG-2 and GATA4 was first detected in differentiating ES cell cultures on day 5 and in differentiating iPS cell cultures on day 10 of differentiation. The expression pattern of Tbx5 and Tbx20 in iPS cell derivatives was very similar to that of differentiated ES cells, with the highest levels of detectable transcripts on days 5, 10, and 21 of differentiation. Although there was increased transcription of the cardiomyocyte marker MLC2v starting on day 10 of differentiation, transcripts could be detected in undifferentiated and early differentiated iPS and ES cells as well. Similarly, we found uniform expression levels of MLC2a in undifferentiated iPS as well as ES cells and throughout differentiation. ANF transcripts were first present in 10-day differentiation cultures of iPS and ES cells. Differentiated iPS cells showed expression of the endodermal marker genes Sox17, FoxA2, and AFP with an expression level and pattern comparable to those in differentiated ES cells.

**Quantitative Real-time RT-PCR Analyses Revealed Impaired Downregulation of the Pluripotency Marker Genes Nanog and Oct 3/4 in iPS Cells During Differentiation**

On the basis of the RT-PCR analyses showing prolonged expression of the pluripotency markers during differentiation, especially in iPS cells, we quantified Nanog and Oct 3/4 expression by real-time PCR. Nanog and total Oct 3/4 (endogene and transgene) transcript levels were indeed downregulated during differentiation in both differentiating iPS cell and ES cell cultures. However, iPS cell derivatives showed impaired downregulation compared with ES cell derivatives, resulting in significantly higher Nanog and total Oct 3/4 expression levels on days 10 and 21 of differentiation (Figure 4). The main proportion of the total Oct 3/4 transcript level in iPS cells was based on the expression of endogenous Oct 3/4 with a negligible expression of the Oct 3/4 transgene of <0.02% of the total expression level (compare Figure 4 and Figure II in the online-only Data Supplement).

In contrast to Oct 3/4 and Nanog, real-time PCR analyses revealed no significant differences in total c-Myc expression between iPS and ES cells (Figure II in the online-only Data Supplement).

**Immunofluorescence Analyses Revealed the Presence of iPS Cell Derivatives With Typical Cross-Striated Muscle Filaments**

Figure 5 shows immunofluorescence staining of iPS cell– and
ES cell–derived myocytes. Immunofluorescence analyses of 21-day differentiation cultures revealed cells with well-organized cross-striation in both iPS and ES cell cultures. In both iPS and ES cell differentiation cultures, cells positive for α-sarcomeric actinin, titin, cardiac troponin T, and MLC2v were detected (Figure 5). Immunostaining of single beating clusters on day 22 demonstrated the presence of the gap junction protein connexin 43 in iPS cell–derived cardiomyocytes (Figure 6A, 6C) that expressed the cardiac isoform of troponin T (Figure 6C). Similar staining was seen in ES cell derivatives (Figure 6E).

**Spontaneous Rhythmic Ca^{2+} Transients in iPS Cell–Derived Cardiomyocytes**

We assessed spontaneous intracellular Ca^{2+} fluctuations in iPS cell–derived and ES cell–derived cardiomyocytes 3 to 7 days after onset of beating using confocal laser microscopy. A typical cluster of iPS cell–derived cardiomyocytes (Figure 7A; Movie III in the online-only Data Supplement) as well as a single myocyte (Figure 7B; Movie IV in the online-only Data Supplement) is presented during low (left) diastolic [Ca^{2+}]_{i} and high (right) systolic [Ca^{2+}]_{i}. Ca^{2+} increased homogeneously throughout the cell, pointing to a finely regulated Ca^{2+} release from intracellular Ca^{2+} stores, most likely the SR. Simultaneous Ca^{2+} release within clusters indicates functional coupling of the cells. Rhythmic Ca^{2+} transients were found with the use of the line scan mode (Figure 7C). The amplitudes of Ca^{2+} transients measured in iPS cell–derived cardiomyocytes were 281±23 nmol/L (n=39) versus 298±24 nmol/L measured in ES cell–derived cardiomyocytes (n=20; statistically not significant; Figure 7E). Both are in the range of amplitudes measured in adult cardiomyocytes. In addition, caffeine-sensitive Ca^{2+} storage, characteristic of a functional SR, was found, and SR Ca^{2+} load was assessed with this method (Figure 7D), with mean values of 536±93 nmol/L (n=12) for iPS–derived cardiomyocytes versus 646±86 nmol/L measured in ES cell–derived cardiomyocytes (n=10; statistically not significant; Figure 7E).

**Electrophysiological Studies With MEAs Demonstrate Functionality and Presence of the β-Adrenergic and Muscarinic Signaling Cascade in iPS Cell–Derived Cardiomyocytes**

In the next batch of experiments, we sought to prove the electrophysiological integrity of the iPS cell–derived cardiomyocytes. Because one of the most critical determinants of normal cardiac electrophysiology is the intact response to hormones and transmitters of the central nervous system, we studied effects of 1 μmol/L ISO and 10 μmol/L carbachol on FP frequency in iPS and control cardiomyocytes. Figure 8 shows a representative experiment. After plating on the MEA dish (Figure 8A), both iPS cell– and ES cell–derived cardiomyocytes developed similar spontaneous electric activity, as indicated by the typical cardiac extracellular FP shape and amplitude (Figure 8B). Application of ISO led to a typical and comparable increase of the FP frequency (Figure 8C, middle panel) compared with basal frequency (left panel).
Subsequent application of carbachol plus ISO during the same measurement was able to block the ISO effect in both cell types (right panel), indicating intact and coupled \( \beta \)-adrenergic and muscarinic signaling cascades.

**Discussion**

A series of recent articles\(^6\)–\(^12\),\(^15\),\(^23\)–\(^25\) based on the groundbreaking study of Takahashi and Yamanaka\(^5\) demonstrated that the reprogramming of somatic cells to a pluripotent state can be achieved by simple retroviral overexpression of specific transcription factors and that the resulting iPSCs are almost indistinguishable from ES cells.\(^6\)–\(^8\) The latest development showing production of human iPSCs, even in the absence of the proto-oncogene c-Myc\(^9\),\(^12\) and without the need for drug selection,\(^9\),\(^24\) opens up new opportunities for the establishment of patient-specific stem cell lines.

Although pluripotency has been demonstrated by the generation of teratomas and by the live birth of chimeric mice after blastocyst injection,\(^6\)–\(^8\) very limited data are available concerning the in vitro differentiation potential of iPSCs into specific cell lineages.\(^9\),\(^11\) It was therefore the aim of the present study to investigate the cardiac differentiation potential of a murine Oct 3/4-neo selected iPS cell clone (O9), to compare it with a well-established murine ES cell line (E14.1, 129/Ola, which carries an EGFP transgene targeted to the *Brachyury* locus) and to characterize the iPSC cell–derived cardiomyocytes on molecular, structural, and functional levels.

On the basis of a well-established standard differentiation protocol for ES cells, iPSC cell clone O9 showed delayed and less efficient cardiac differentiation compared with the ES cell clone, nevertheless yielding a maximum of 55% spontaneously beating EBs on day 24 of differentiation (Figure 2A). Furthermore, beating areas in iPSC cell–derived EBs were usually smaller than those in ES cell EBs. These observed differences between the 2 analyzed cell clones were in agreement with the evaluated mRNA expression level of cardiac troponin T. The 3- to 5-fold lower expression level of cardiac troponin T in differentiating iPSC cell cultures on days 10 and 21 compared with ES cell derivatives (Figure 2B) likely reflects the lower number of iPSC cell EBs with contracting areas and the predominantly smaller beating clusters.

At this point, it is unclear whether our observations reflect general differences between ES and iPSC cells. Similar differences in differentiation potential and efficiency are frequently observed among individual ES cell lines, and the observed differences between the analyzed ES and iPSC cell clones may

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**Figure 7.** Confocal laser microscopy images (fluor 4-AM) showing spontaneous Ca\(^{2+}\) fluctuations in iPS cell–derived cardiomyocytes. A, Frame scan of a typical cluster of cardiomyocytes demonstrating functional coupling of the cells with low diastolic (left) and high systolic (right) free intracellular Ca\(^{2+}\) fluctuations throughout the whole cluster. B, Frame scan of single cardiomyocyte showing homogeneous increases of intracellular Ca\(^{2+}\) from diastole (left) to systole (right). C, Representative line scan showing rhythmic spontaneous Ca\(^{2+}\) transients (top) and calculated absolute free intracellular Ca\(^{2+}\) levels (bottom). D, Representative caffeine-induced SR Ca\(^{2+}\) release, demonstrating caffeine-sensitive Ca\(^{2+}\) stores and fractional release of total SR Ca\(^{2+}\) load during spontaneous activation. E, Average intracellular Ca\(^{2+}\) transients and SR Ca\(^{2+}\) load in iPS cell– (n=39 and n=12) and ES cell–derived cardiomyocytes (n=20 and n=10).
reflect similar clonal differences. In addition, the different cardiac differentiation potential may be attributed to the fact that the transgenic ES cell line (but not the O9 clone) was preselected for both robust in vitro proliferation and efficient cardiac differentiation. This ES cell line can be differentiated to give >90% beating EBs under suitable conditions. On the other hand, delayed differentiation of iPS cell clone O9 might be in relationship to the sustained expression of the pluripotency marker genes Nanog and Oct 3/4 during differentiation. Whether in this case the significantly higher expression levels of Nanog and total Oct 3/4 during iPS differentiation compared with ES cell derivatives are based directly on the remaining low transgene expression levels is not clear. However, because the expression levels of the 2 investigated transgenes Oct 3/4 and c-Myc were negligible compared with the respective total gene (endogene and transgene) expression (Figure 4 and Figure II in the online-only Data Supplement), incomplete transgene silencing as the basic cause for the delayed and less efficient cardiac differentiation of the iPS clone O9 appears unlikely.

Interestingly, RT-PCR analyses revealed that both the undifferentiated iPS cells and the ES cells expressed significant amounts of mRNA for the mesodermal markers Brachyury and Mesp1 and for cardiac markers including the MLC (Figure 3). Similar results have been reported for both human and rhesus monkey ES cells and are consistent with the reports that undifferentiated ES cell cultures often contain some colonies with a “differentiated” morphology.
Further RT-PCR analyses detected expression of the marker genes typical for mesoderm, endoderm, cardiac mesoderm, and cardiomyocytes in the differentiating iPS cells (Figure 3). Although statements about differences in expression based on RT-PCR should be judged with caution, expression levels in iPS cell derivatives appeared to be in agreement with those in the differentiated ES cells.

Immunofluorescence analyses on days 21 and 22 of differentiation showed that the iPS cells, although delayed with respect to the onset of differentiation compared with the ES cells, expressed cardiomyocyte-specific proteins and had well-organized cross-striations that were indistinguishable from those seen in ES cell–derived cardiomyocytes (Figure 5). The presence of connexin 43 was taken as evidence for the presence of gap junctions between the iPS cell–derived cardiomyocytes (Figure 6).

Physiological measurements were performed on both iPS cell–derived cardiomyocytes and ES cell–derived cardiomyocytes at the same time point after the onset of beating. Electrophysiological analyses by means of MEAs and analysis of spontaneous intracellular Ca2+ transients revealed that the iPS cell–derived cardiomyocytes and the ES cell–derived cardiomyocytes were similar with respect to differentiation. The results of β-adrenergic and muscarinic stimulation with ISO and carbachol, respectively, and spontaneous intracellular Ca2+ transient recording were similar to those obtained previously with day-3 ES cell–derived cardiomyocytes20 and cardiomyocytes derived from spermatogonial stem cells.15

In conclusion, our study shows that iPS cells are capable of generating cardiomyocytes that are functionally comparable to ES cell–derived cardiomyocytes. Although iPS cell clone O9 showed delayed and less efficient differentiation compared with the well-established ES cell line, the resultant iPS cardiomyocytes were indistinguishable from ES cell–derived cardiomyocytes in most tests. Our results establish a valuable basis for creating animal models for the development of iPS cell–based cellular cardiomyoplasty. Although there are many unresolved questions and many hurdles to overcome, the reprogramming of somatic cells into iPS cells has brought new hope for the development of novel regenerative therapies for the treatment of heart failure and for the correction of congenital cardiac malformations.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

The lack of suitable cell sources is still a major hurdle for the development of cardiac cell replacement therapies. The formation of functional cardiomyocytes from adult stem cells is still controversial. Human embryonic stem cells are ethically problematic and will not readily be accessible as an autologous cell source. The availability of induced pluripotent stem (iPS) cells now represents significant progress toward the development of patient-derived autologous cells. The aim of this study was to evaluate the general usefulness of iPS cells for myocardial restoration demonstrating the generation of functional iPS cell–derived cardiomyocytes. Analyses on molecular, structural, and functional levels documented that iPS cell–derived cardiomyocytes show typical features of embryonic stem cell–derived cardiomyocytes. Reverse transcription polymerase chain reaction and immunocytoLOGY revealed expression of typical cardiac marker genes. Spontaneous rhythmic intracellular Ca²⁺ fluctuations with amplitudes of Ca²⁺ transients comparable to embryonic stem cell derivatives were observed. Simultaneous Ca²⁺ release within clusters of iPS cell–derived cardiomyocytes indicated functional coupling of the cells, and electrophysiological studies with multielectrode arrays further demonstrated functionality and presence of the β-adrenergic and muscarinic signaling cascade. In conclusion, iPS cells allow the derivation of autologous functional cardiomyocytes for cellular cardiomyoplasty and myocardial tissue engineering.
Generation of Functional Murine Cardiac Myocytes From Induced Pluripotent Stem Cells
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