Research Article

Electrical Stimulation Promotes Cardiac Differentiation of Human Induced Pluripotent Stem Cells

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Received 29 June 2015; Revised 12 August 2015; Accepted 12 August 2015

Academic Editor: Heinrich Sauer

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Background. Human induced pluripotent stem cells (iPSCs) are an attractive source of cardiomyocytes for cardiac repair and regeneration. In this study, we aim to determine whether acute electrical stimulation of human iPSCs can promote their differentiation to cardiomyocytes. Methods. Human iPSCs were differentiated to cardiac cells by forming embryoid bodies (EBs) for 5 days. EBs were then subjected to brief electrical stimulation and plated down for 14 days. Results. In iPS(Foreskin)-2 cell line, brief electrical stimulation at 65 mV/mm or 200 mV/mm for 5 min significantly increased the percentage of beating EBs present by day 14 after plating. Acute electrical stimulation also significantly increased the cardiac gene expression of ACTC1, TNNT2, MYH7, and MYL7. However, the cardiogenic effect of electrical stimulation was not reproducible in another iPSC cell line, CERA007c6. Beating EBs from control and electrically stimulated groups expressed various cardiac-specific transcription factors and contractile muscle markers. Beating EBs were also shown to cycle calcium and were responsive to the chronotropic agents, isoproterenol and carbamylcholine, in a concentration-dependent manner. Conclusions. Our results demonstrate that brief electrical stimulation can promote cardiac differentiation of human iPS cells. The cardiogenic effect of brief electrical stimulation is dependent on the cell line used.

1. Background

After myocardial infarction, the heart fails to regenerate sufficiently to reestablish the normal cardiac function. The ability of stem cells to differentiate into cardiomyocytes for transplantation is a promising approach to repair and regenerate infarcted myocardium [1]. Specifically, human pluripotent stem cells such as embryonic stem cells (ESCs) and iPSCs are the most promising candidate cell types because of their ability to proliferate indefinitely in the undifferentiated state in vitro and to differentiate into bona fide contracting cardiomyocytes. However, application of ESCs and their derivatives have some limitations with anticipated immune rejection after transplantation. iPSCs, like ESCs, show pluripotency and self-renewal capabilities [2]. Patient-specific iPSCs, unlike ESC lines, offer an autologous source of stem cells and derivatives for personalized medicine, thus circumventing issues regarding immunological rejection [3, 4].
Spontaneous differentiation of human iPSCs into cardiomyocytes was first reported using the EB formation method [2, 5]. However, this method has a relatively low cardiac differentiation efficiency; a heterogeneous cell population is generated and it requires animal serum which limits their use in clinical application. Endogenous electric fields are present in the developing heart and are involved in cardiac development [6, 7]. Exogenous electrical stimulation has been applied to various cell types to promote cardiac differentiation including human fibroblasts [8], human mesenchymal stem cells [9, 10], human cardiac progenitor cells (c-kit/Sca-1) [11], mouse adipose-derived stem cells [12], and ESCs (both human and mouse) [13, 14]. Using electric fields that were within the endogenous physiological range (tens to hundreds of mV/mm) [7], it was found that electrical stimulation can direct the differentiation of the abovementioned cell types into immature cardiomyocyte-like cells. Here, we studied the effect of brief electrical stimulation on cardiac differentiation of human iPSCs.

2. Methods

2.1. Human iPSCs Generation. Human tissue sample collection was approved by the Human Research Ethics Committee of the Royal Victorian Eye and Ear Hospital (11/1031H). Following informed consent, CERA007c6 iPSCs were generated using skin fibroblasts from a 36-year-old healthy male by the episomal method as described previously [15, 16]. Briefly, passage 4 fibroblasts were nucleofected with episomal vectors expressing OCT4, SOX2, KLF4, L-MYC, LIN28, and shRNA against p53. The nucleofected fibroblasts were then replated onto mitotically inactivated mouse embryonic fibroblasts on day 7 and allowed to grow until day 34. Reprogrammed colonies resembling human embryonic stem cell morphology were manually dissected to establish clonal cell line for expansion. The established iPSC line was cultured on mitotically inactivated mouse embryonic fibroblasts feeders in the presence of DMEM/F-12 medium containing 1x GlutaMAX, 20% knockout serum replacement, 10 ng/mL basic fibroblast growth factor, 0.1 mM nonessential amino acids, 100 μM β-mercaptoethanol, and 0.5x penicillin/streptomycin (both from Invitrogen, CA, USA). The iPS(Foreskin)-2 cell line generated from newborn fibroblasts by lentiviral transfection was obtained from Professor J. Thomson (University of Wisconsin) [4] and was used for all experiments unless otherwise indicated.

2.2. Cardiac Differentiation of Human iPSCs. Human iPSCs were maintained as previously described [17]. Cardiac differentiation of human iPSCs was induced through formation of EBs. EBs were formed by mechanically dissecting undifferentiated human iPSC colonies into approximately 0.2 mm² pieces, transferred to low attachment plates, and cultured in suspension in serum-containing media (DMEM/F-12 GlutaMAX medium, 20% fetal bovine serum (Sigma-Aldrich, MO, USA), 0.1 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, and 50 μ/mL penicillin/streptomycin, all from Invitrogen, unless otherwise stated) where they aggregated to form EBs over 5 days. On day 5 (day 0 after plating), EBs were transferred onto tissue culture plates precoated with 0.1% gelatin and 10 μg/mL fibronectin (both from Sigma-Aldrich) and cultured in serum-free media containing RPMI medium (Invitrogen) supplemented with 284 nM ascorbic acid, 68.7 nM transferrin, 38.7 nM sodium selenite, and 0.0075% bovine serum albumin (all from Sigma-Aldrich). The percentage of contractile EBs was determined as the number of EBs that showed spontaneous contraction divided by the total number of EBs plated.

2.3. Electrical Stimulation. At day 5 of EB formation, EBs were subjected to electrical stimulation using a custom made electrical stimulator. The electrical stimulator consisted of 16 gold electrodes coated with platinum that fitted into an 8-well chamber slide (BD Falcon, MA, USA) (Figure 2(a)). Each well contained an anode and cathode with an interelectrode distance of 10 mm. The electrodes were connected to an electrical stimulator that generated a charge-balanced biphasic current pulses (Figure 2(b)). The EBs were then electrically stimulated in suspension (Figure 2(a)) for different durations (1.5, 5, 10, or 15 min). The electric fields studied were 65 and 200 mV/mm at 1 Hz frequency and 1 ms pulse width. Control EBs were subjected to the same procedure but without electrical stimulation. After electrical stimulation, EBs were immediately transferred onto gelatin and fibronectin coated culture plates and cultured in serum-free media.

2.4. Quantitative Polymerase Chain Reaction (RT-qPCR). RNA was extracted from undifferentiated iPSC and beating EBs at day 14 after plating using TRI Reagent (Invitrogen) followed by RNA precipitation with chloroform and isopropanol (both from Sigma-Aldrich). In a separate experiment, RNA was extracted from a pool of 5 randomly selected EBs at days 0 (before electrical stimulation), 1, 3, and 7 after plating (without selecting beating EBs). cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). A qRT-PCR was carried out using TaqMan Universal master mix, TaqMan gene expression assays, and the 7900HT Fast Real-Time PCR machine (all from Applied Biosystems) to assess the expression of 18s (Hs99999901_s1), GATA binding protein 4 (GATA4; Hs00171403_m1), NK2 homeobox 5 (NKX2.5; Hs00231763_m1), myocyte enhancer factor 2C (MEF2C; Hs00231499_m1), T-box 5 (TBX5; Hs00361555_m1), cardiac α-actin (ACTC1; Hs01109515_m1), cardiac troponin T (TNNT2; Hs01109515_m1), myosin light chain atria isoform 7 (MYL7; Hs01085598_g1), myosin light chain ventricular isoform 2 (MYL2; Hs00166405_m1), myosin heavy chain (MYH7; Hs00165276_m1), muscarinic cholinergic receptor-2 (CHM2; Hs01085598_g1), β-adrenergic receptor-1 (ADRB1; Hs02330487_s1), and β-adrenergic receptor-2 (ADRB2; Hs00244053_s2). All readings were performed in duplicate. The fold change was calculated by applying the comparative CT method (ΔΔCT) whereby the mRNA expression levels were normalized against the level of the housekeeping gene 18s (ΔCT) with the level of candidate genes in EBs at day 0 (Figures 3(c), 3(d), 4(b), and 4(c) or undifferentiated iPSC samples (Figures 5(b) and 5(c)) were used as the reference genes (ΔΔCT). RT-qPCR reaction of
samples with reverse transcriptase omitted (−RT) was also
conducted to check the absence of genomic DNA and the
specificity of the primer probes.

2.5. Immunocytochemistry. The primary antibodies used to
confirm pluripotency of CERA007c6 iPSCs were OCT4
(5 μg/mL, Santa Cruz Biotechnology, TX, USA) and TRA1-
60 (5 μg/mL, Millipore), followed by the appropriate Alexa
Fluor-488 secondary antibodies. For in vitro differentiation,
CERA007c6 iPSCs were allowed to form EBs for 12
days and subsequently plated down on gelatinized plates
to further differentiate for 15 days. Immunocytochemistry
is performed using the primary antibodies against alpha-
 fetoprotein (10 μg/mL, AFP, Millipore), alpha smooth muscle
actin (10 μg/mL, SMA, R&D Systems, MN, USA), and
Nestin (10 μg/mL, Abcam), followed by the Alexa Fluor-488
secondary antibody (10 μg/mL; Invitrogen).

For quantitative assessment of cardiomyocyte differentiation,
beating colonies at 14 days after plating were trypsinized
into single cell suspension with 0.25% trypsin-EDTA and
spun onto coated glass slides (4 min at 900 rpm; Shandon
Cytospin 4, Thermo Fisher Scientific, MA, USA). Cells were
fixed and permeabilized with 100% ice-cold methanol for
10 min at 4°C. Cells were then incubated with a serum-free
blocking solution (Thermo Fisher Scientific) for 10 min prior
to incubation with cardiac troponin T antibody (2 μg/mL,
mouse monoclonal IgG; Abcam, MA, USA) at 4°C overnight
followed by a secondary antibody, Alexa Fluor-488 goat anti-
mouse IgG for 60 min at room temperature.

For qualitative analysis, beating colonies at 14-15 days
after plating were treated with 10 μM of Y-27632 (Millipore,
MA, USA), a Rho-associated kinase (ROCK) inhibitor, for
1-2 hours at 37°C to minimise apoptosis [15]. Cells were then
washed with phosphate buffer saline (PBS) and dissociated
with TrypLE Select (Invitrogen) at 37°C for 5 min. Serum-
containing media were then added to neutralize the enzy-
matic reaction of TrypLE. Mixture of single cells and clumps
was centrifuged at 400 g for 5 min, resuspended in serum-
containing media supplemented with 10 μM of Y-27632,
and seeded onto gelatin and fibronectin coated 8-well chamber
slides. After 48 h of incubation at 37°C, the media were
removed and the cells were fixed with 4% paraformaldehyde
for 30 min at room temperature followed by Triton X-100
(0.1% in PBS) for 10 min. Cells were then incubated with
a serum-free blocking solution for 10 min prior to incubation
with antibodies against cardiac troponin T (4 μg/mL, mouse
monoclonal IgG, Abcam), sarcomeric α-actinin (25 μg/mL,
mouse monoclonal IgG, Sigma-Aldrich), myosin heavy chain
(2 μg/mL, mouse monoclonal IgG, Abcam), and connexin 43
(1.2 μg/mL, rabbit polyclonal IgG, Abcam) at 4°C overnight
followed by Alexa Fluor-488 goat anti-mouse IgG (10 μg/mL,
Invitrogen) or Cy3 fluorescence-conjugated goat anti-rabbit
IgG (7.5 μg/mL, Jackson ImmunoResearch Laboratories, PA,
USA) for 60 min at room temperature.

All preparations were counterstained with DAPI
(1 μg/mL, Invitrogen) for nuclear staining and mounted with
fluorescent mounting medium (Dako, Victoria, Australia).
Images were taken using a fluorescence microscope (BX-61,
Olympus, Tokyo, Japan). For quantification, at least 100 cells
were counted from 10 random fields.

2.6. Calcium Imaging. Intracellular free calcium (Ca²⁺)
ing was performed in beating EBs collected at day 14 after
plating using the fluorescent calcium indicator Fluo-4 AM
(Invitrogen). Cells were incubated with 2 μg/mL of Fluo-4
AM for 20 min in serum-containing media at 37°C. Gray
scale images were captured (15 frames per second) on an
inverted microscope (IX71, Olympus) with a fluorescence
source (X-Cite 120Q; Lumen Dynamics, ON, Canada) and a
digital camera (DP72; Olympus). The fluorescence signal
was visualized with a 492 nm excitation beam (excitation filter
BP492/18 with a xenon light source).

2.7. Microelectrode Array Recordings. The electrophysio-
logical properties of the cardiomyocytes derived from
iPSCs were assessed using a microelectrode array (MEA)
recording system (Multichannel Systems, Reutlingen,
Germany). Beating EBs at 14 days after plating were microdis-
sected and transferred onto MEA plates coated with 0.1% gelatin and 10 μg/mL fibronectin and cultured in serum-
containing media. The responsiveness of cells to isopro-
terol hydrochloride (1-1000 nM) and carbamylcholine (1-
1000 nM) (all from Sigma-Aldrich) was tested 4 days later
in Krebs-Ringer buffer (composition in mM: 125 NaCl, 5
KCl, 1 NaHPO₄, 1 MgSO₄, 20 HEPES, 5.5 glucose, and 2
CaCl₂; pH 7.4, at 37°C). Beating EBs from each MEA plate
were treated with all drugs in random order, and cells were
allowed to recover to their baseline contraction in fresh
Krebs-Ringer buffer between drug treatments. Extracellular
field potentials and beating rate were recorded at baseline and
at 2 min after the addition of the drugs. Data were analyzed
offline with MC Rack software, version 4.3.5 (Multichannel
Systems) for beating rate, RR interval, and extracellular field
potential duration (FPD). RR interval was defined as the
time elapsed between two consecutive beats, and FPD was
defined as the time interval between the initial deflection
of the field potential and the maximal local T wave. To avoid
the influence of beat frequency on FPD, FPD measurements
were normalized (corrected field potential duration (cFPD)) with
the Bazett correction formula: cFPD = FPD / √ (RR interval).

2.8. Statistical Analysis. Data are expressed as mean ± SEM.
Statistical analysis was performed on Graphpad Prism soft-
ware (version 6). The significance of the differences was eval-
uated using the unpaired Student t-test and one-way analysis
of variance (ANOVA), followed by Bonferroni multiple-
comparison post hoc analysis where appropriate. Values of
p < 0.05 were considered statistically significant.

3. Results

3.1. Generation of the iPSC Line CERA007c6. We generated
new iPSC lines from skin fibroblasts of a healthy donor. We
undertook nucleofection to deliver episomal vectors overex-
pressing OCT4, SOX2, KLF4, L-MYC, LIN28, and shRNA
against p53 into fibroblasts. Following reprogramming, we
isolated and expanded six iPSC clones. In the present study,
clone 6 of the generated iPSCs was used (CERA007c6). Immunocytochemistry analysis showed that CERA007c6
iPSCs are positive for pluripotent markers OCT4 and TRA1-60 (Figures 1(a) and 1(b)). CERA007c6 iPSCs are differentiated in vitro by embryoid bodies formation, and we showed the presence of cells representative of endoderm (alpha-fetoprotein, AFP), mesoderm (smooth muscle actin, SMA), and ectoderm (Nestin). (f) An IgG negative control. Scale bar = 100 μm.

Together, these results confirmed that CERA007c6 iPSCs are pluripotent and retain the potential to differentiate in vitro. We have also characterised two other iPSC clones from the same donor and found consistent results (data not shown), confirming the quality of the iPSCs we generated.
3.2. Brief Electrical Stimulation Promotes Cardiac Differentiation of iPS(Foreskin)-2 Cell Line. Spontaneously beating colonies of iPS(Foreskin)-2 cells were detected as early as day 2 after plating but more frequently from day 4 after plating (Figures 3(a) and 3(b)). Acute electrical stimulation of EBs at 65 mV/mm (Figure 3(a)) or 200 mV/mm (Figure 3(b)) for 5 min statistically significantly increased the percentage of beating EBs at day 14 after plating (11.9 ± 3.4% in 65 mV/mm, 11.7 ± 2.3% in 200 mV/mm versus 4.9 ± 1.4% in control, one-way ANOVA, p < 0.05, n = 10–15). Electrical stimulation for a shorter or longer duration (1.5, 10, and 15 min) did not significantly modify the percentage of beating EBs when compared to control (Figures 3(a) and 3(b)). Electrical stimulation at either 65 or 200 mV/mm for a duration of 1.5 to 15 min did not affect the viability of EBs as determined by their gross morphology and electrically stimulated EBs were attached and grew after plating (data not shown). Compared to the unstimulated control group, acute electrical stimulation significantly increased the expression of cardiac transcription factors NKK2.5 and TBX5 (Figure 3(c)) as well as the cardiac contractile muscle proteins ACTC1, TNNT2, MYH7, and MYL7 (Figure 3(d)) on day 7 after stimulation.

3.3. Brief Electrical Stimulation Did Not Promote Cardiac Differentiation of CERA007c6 Cell Line. Acute electrical stimulation of EBs generated from the other line of human iPSC line, CERA007c6, at 200 mV/mm for 5 min did not significantly increase the percentage of beating EBs at day 14 after plating (5.5 ± 1.8% versus 3.6 ± 1.5% in control, p > 0.05, n = 8, Figure 4(a)). No significant difference in gene expression of cardiac transcription factors (Figure 4(b)) or cardiac contractile muscle proteins (Figure 4(c)) was detected between groups.

3.4. Expression of Cardiac-Specific Markers in Cardiomyocytes Derived from iPS(Foreskin)-2 Cell Line. At day 14 after plating, beating EBs expressed cardiac contractile proteins such as troponin T, sarcomeric α-actinin, and myosin heavy chain. Striations of cardiac muscle were visible in the cardiomyocytes derived from iPSCs (Figure 5(a)). In some cardiomyocytes, gap junctional protein connexin 43 was expressed in a punctuate expression pattern at the cell contact surfaces (Figure 5(a), white arrow) resembling that of neonatal cardiomyocytes [18]. Compared to undifferentiated human iPSCs, the mRNA expressions of cardiac transcription factors (GATA4, NKX2.5, MEF2C, and TBX5, Figure 5(b)) and cardiac contractile muscle proteins (ACTC1, TNNT2, MYH7, MYL2, and MYL7, Figure 5(c)) were upregulated in all beating EBs (both electrically stimulated and nonelectrically stimulated control). The gene expression ratio of MYL2 (ventricular isoform)/MYL7 (atrial isoform) was higher in beating EBs of the electrically stimulated group (at 200 mV/mm for 5 min) compared to control (1.70 ± 1.12 versus 0.03 ± 0.01, p < 0.05, n = 5–7, Figure 5(d)). Electrical stimulation at 65 mV/mm showed a similar gene expression ratio of MYL2/MYL7 as did the control group (0.05 ± 0.03, n = 5, Figure 5(d)). These results suggest the emergence of more ventricular-like than atrial-like cardiomyocytes in beating EBs that were electrically stimulated at 200 mV/mm for 5 min.

The percentage of cardiac troponin T-positive cells in each beating EB from the iPS(Foreskin)-2 cell line was 13.4 ± 5.0% in control (n = 12). Acute electrical stimulation at 65 or 200 mV/mm for 5 min did not statistically increase the percentage of cardiac troponin T-positive cells (11.1 ± 5.9% and 19.4 ± 5.7%, resp., p > 0.05 versus control, one-way ANOVA, n = 6–12) (Figure 5(e)). In iPSC line CERA007c6, the percentage of cardiac troponin T-positive cells in each beating EB was 16.7 ± 10.0% in control (n = 5) and acute electrical stimulation at 200 mV/mm for 5 min did not significantly increase this percentage (30.5 ± 9.7%, p > 0.05 versus control, n = 6).

3.5. Electrophysiological Profile of Cardiomyocytes Derived from iPS(Foreskin)-2 Cell Line. Beating cells from electrically stimulated and control beating EBs clearly cycle calcium (Figure 6(a)). To determine the electrophysiological properties of the beating EBs, extracellular electrograms were
Figure 3: Acute electrical stimulation enhances cardiac differentiation of iPS(Foreskin)-2 cell line. (a-b) The effect of acute electrical stimulation at 65 mV/mm (a) or 200 mV/mm (b) on cardiac differentiation of human iPS(Foreskin)-2 cell line (n = 5–15 independent experiments). (c-d) Quantitative RT-PCR analysis for the expression of cardiac transcription factors (c) and cardiac contractile muscle proteins (d) in electrically stimulated and control EBs at days 0, 1, and 7 after electrical stimulation (n = 7 independent experiments). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 by one-way (a-b) or two-way (c-d) ANOVA with the Bonferroni post hoc test.
Figure 4: Acute electrical stimulation did not promote cardiac differentiation of CERA007c6 iPSCs. (a) The effect of acute electrical stimulation at 200 mV/mm for 5 min on cardiac differentiation of CERA007c6 iPSCs at day 14 after plating (n = 3 independent experiments). (b-c) Quantitative RT-PCR analysis for the expression of cardiac transcription factors (b) and cardiac contractile muscle proteins (c) in electrically stimulated and control EBs at days 0, 1, and 7 after electrical stimulation (n = 3 independent experiments). Data are expressed as mean ± SEM.
Figure 5: Expression of cardiac-specific markers in cardiomyocytes derived from iPS(Foreskin)-2 cell line. (a) Beating EBs expressed cardiac contractile proteins, cardiac troponin T, sarcomeric α-actinin, myosin heavy chain, and gap junctional protein connexin 43 (Cx43, white arrow) (scale bar = 50 μm). Quantitative RT-PCR analysis for the expression of cardiac transcription factors (b) and cardiac contractile muscle proteins (c) in electrically stimulated and control beating EBs at day 14 after plating (n = 5–7 independent experiments). (d) MYL2/MYL7 gene expression ratio. (e) Percentage of cardiac troponin T-positive cells in each beating EB at day 14 after plating (n = 5–6 independent experiments). Data are expressed as mean ± SEM. ES: electrically stimulated and undiff: undifferentiated iPSCs.
Figure 6: Continued.
recorded from the beating clusters plated on top of MEA plates (Figure 6(b)). The resting beating rate per minute (bpm) was similar between control and electrically stimulated (200 mV/mm for 5 min) groups (60.7 ± 15.4 and 65.3 ± 12.7 bpm, resp., p > 0.05, n = 3). The beating EBs from the electrically stimulated group, but not the control group, showed a concentration-dependent increase in beating frequency when treated with isoproterenol (1–1000 nM) or carbachol (1–1000 nM) (n = 3 independent experiments). (c) The percentage change in beating frequency when treated with isoproterenol (1–1000 nM) showed a concentration-dependent increase in beating frequency in beating EBs from the electrically stimulated group, but not the control group (Figure 6(c)). Compared to control group, the baseline field potential duration (corresponding to the QT interval) trended higher in the electrically stimulated group but did not reach statistical significance (0.30 ± 0.19 sec versus 0.10 ± 0.02 sec in control, p > 0.05, n = 3, Figure 6(d)). The field potential duration remained unchanged when beating EBs were treated with either isoproterenol or carbachol (Figure 6(d)). Thus beating EBs from the electrically stimulated group were more responsive to chronotropic agents but electrical stimulation did not significantly affect the field potential duration. Compared to undifferentiated human iPSCs, the mRNA expression of the muscarinic cholinoreceptor (CHM2) and β-adrenergceptors receptors (ADRB1 and ADRB2) were upregulated in all beating EBs compared with undifferentiated EBs but there was no significant difference between groups (Figure 6(e)).

4. Discussion

While studies with several cell types have shown that acute electrical stimulation can promote differentiation of cells toward cardiomyocyte phenotype [8–14], we have now shown that acute electrical stimulation increased cardiac differentiation of human iPSCs. In iPSCs(Foreskin)-2 cell line, acute electrical stimulation at 200 mV/mm for 5 min modestly but significantly increased the rate of cardiac differentiation of human iPSCs by twofold (Figure 3(b)). This was accompanied by a significant increase in expression of the cardiac-specific markers ACTC1, TNNT2, MYH7, and MYL7. Compared to previous studies in ESCs, we showed a positive effect of acute electrical stimulation on cardiac differentiation of human iPSCs at a much lower electric field (others used 500 to 1000 mV/mm) although we required longer stimulation duration (others required 1 to 90 seconds) [13, 14]. We were able to achieve differentiation at lower field strength than others perhaps due to our utilization of a constant current charge-balanced stimulation approach. It is surprising that acute electrical stimulation at 200 mV/mm did not promote cardiac differentiation using another human iPSC line (CERA007c6), and others have observed that human iPSC shows line to line variations and different differentiation efficacy [19]. This could be related to the cell origin from different patient (neonatal fibroblasts versus adult fibroblasts) or the reprogramming method, which warrants further investigation.

Although acute electrical stimulation significantly increased the percentage of beating EBs, the percentage of cardiomyocytes determined by cardiac troponin T-positive cells in each beating EB was not significantly changed by acute electrical stimulation (Figure 5(e)). This variability in percentage of cardiomyocytes may be due to the variation in the size of the beating area and dilution by the variable number of noncardiomyocytes contained therein. The expression of cardiac progenitor transcription factors such as GATA4, NKX2.5, MEF2C, and TBX5 is associated with early cardiac development and cardiomyocyte specification [20].
Once committed to the cardiac lineage, cells begin to express more mature cardiac markers such as those encoded for contractile muscle proteins ACTC1, TNNT2, MYH7, MYL2, and MYL7 [21]. In the present study, the expression of these cardiac-specific transcription factors and structural genes was similar between the beating EBs from control and electrically stimulated groups, suggesting a comparable rate of cardiac specification and a similar proportion of cardiomyocytes in the beating EBs (Figures 5(b) and 5(c)).

Cardiomyocytes derived from human iPSCs showed development of calcium transients. Here we also showed that electrical stimulation increases the sensitivity of cardiomyocytes to isoproterenol and carbamylcholine when compared to the control group (Figure 6(c)). Fetal cardiomyocytes are less sensitive to isoproterenol than adult cardiomyocytes [22] suggesting the possibility that electrical stimulation may accelerate maturation of cardiomyocytes and thus explain the difference in sensitivity to β-adrenoceptor and muscarinic receptor agonists in the present study, although the expression of genes encoding these receptors was similar between the control and electrically stimulated beating EBs. The expression of β-adrenoceptor and muscarinic receptors at the protein level was not assessed, and the associated downstream signalling mechanism in the derived cardiomyocytes remains to be investigated. The lack of responsiveness to chronotropic agents of spontaneously differentiated cardiomyocytes in the control group was surprising but was in agreement with our previous finding using the same human iPSC line [17]. This could be due to the relatively “young” cardiomyocytes (day 18 after plating) used in the present study compared to others (usually >30 days) for long-term culture increases the maturity of cardiomyocytes in terms of calcium handling and the expression of ion channels, as well as cardiac structural and contractile proteins [23, 24].

The mechanism by which acute electrical stimulation promotes cardiac differentiation of human iPSCs remains unknown. Previous studies in mouse and human ESCs have implicated the involvement of reactive oxygen species and Nfκβ signalling in the cardiogenic effect of acute electrical stimulation [13, 14]. There was a positive correlation between the increased cardiac differentiation rate and generation of reactive oxygen species intracellularly when ESCs were subjected to electrical stimulation. A similar increase in beating EBs was demonstrated when ESCs were treated with low concentration of hydrogen peroxide (1–10 nM) for one hour. Furthermore, the effect of acute electrical stimulation on the cardiac differentiation of these ESCs was abolished by free radical scavengers (dehydroascorbate and pyrroldine dithiocarbamate) or the Nfκβ antagonist (N-tosyl-L-phenylalanine chloromethyl ketone) [14].

5. Conclusions

We have shown here that a single brief period of electrical stimulation can promote cardiogenic potential of human iPSCs in terms of the number of beating EBs and gene expression of cardiac transcription factors and contractile muscle proteins. However, the cardiogenic effect of acute electrical stimulation was cell line dependent. The number of cardiomyocytes in each beating cluster was not significantly affected by acute electrical stimulation. The cardiomyocytes derived from electrically stimulated human iPSCs expressed cardiac-specific markers, cycled calcium, and were responsive to adrenergic and muscarinic stimuli.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors’ Contribution

Damían Hernández performed experiments, analyzed the data, and drafted the paper. Rodney Millard built the electrical stimulator and with Robert K. Shepherd designed the well-based electrode arrays. Priyadharshini Sivakumaran was involved in the iPSC and electrophysiology assays. Duncan E. Crombie was involved in the iPSC culture and differentiation. Raymond C. B. Wong, Alex W. Hewitt, Helena Liang, and Sandy S. C. Hung provided and characterized the CERA007c6 iPSC line. Alice Pébay and Robert K. Shepherd participated in the design of different aspects of the study and discussion of results. Gregory J. Dusting and Shiang Y. Lim conceived the study, developed its design, provided funding, and extensively revised the paper. All authors read and approved the final paper. Gregory J. Dusting and Shiang Y. Lim are equally contributing authors.

Acknowledgments

These studies were supported by grants from the National Heart Foundation and National Health and Medical Research Council of Australia (NHMRC 1024817). Duncan E. Crombie is supported by an NHMRC Gustav Nossal Scholarship, Gregory J. Dusting is a Principal Research Fellow of the NHMRC, and Alice Pébay is supported by a NHMRC Career Development Award Fellow and an Australian Research Council Future Fellowship. Raymond C. B. Wong is supported by the Cranbourne Foundation Fellowship and NHMRC Project grant. Support was also provided by the J.R. and J.O. Wicking Trust and Friedreich’s Ataxia Research Alliance (research grant and 2012 Keith Michael Andrus Cardiac Research Award). The O’Brien Institute, the Centre for Eye Research Australia, and the Bionics Institute receive Operational Infrastructure Support from the Victorian State Government’s Department of Innovation, Industry and Regional Development. The authors thank James A. Thomson (University of Wisconsin) for providing the iPS(Foreskin) cell line and Owen Burns (Bionic Institute) for manufacturing the well-based electrode arrays.

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