Human-induced pluripotent stem cell-derived cardiomyocytes exhibit temporal changes in phenotype


Heart Failure Discovery Performance Unit, Metabolic and Cardiovascular Therapeutic Area, GlaxoSmithKline, King of Prussia, Pennsylvania

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Ivashchenko CY, Pipes GC, Lozinskaya IM, Lin Z, Xiaoping X, Needle S, Grygielko ET, Hu E, Toomey JR, Lepore JJ, Willette RN. Human-induced pluripotent stem cell-derived cardiomyocytes exhibit temporal changes in phenotype. Am J Physiol Heart Circ Physiol 305: H913–H922, 2013. First published July 5, 2013; doi:10.1152/ajpheart.00819.2012.—Human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) have been recently derived and are used for basic research, cardiotoxicity assessment, and phenotypic screening. However, the hiPS-CM phenotype is dependent on their derivation, age, and culture conditions, and there is disagreement as to what constitutes a functional hiPS-CM. The aim of the present study is to characterize the temporal changes in hiPS-CM phenotype by examining five determinants of cardiomyocyte function: gene expression, ion channel functionality, calcium cycling, metabolic activity, and responsiveness to cardioactive compounds. Based on both gene expression and electrophysiological properties, at day 30 of differentiation, hiPS-CMs are immature cells that, with time in culture, progressively develop a more mature phenotype without signs of dedifferentiation. This phenotype is characterized by adult-like gene expression patterns, action potentials exhibiting ventricular atrial and nodal properties, coordinated calcium cycling and beating, suggesting the formation of a functional syncytiurn. Pharmacological responses to pathological (endothelin-1), physiological (IGF-1), and autonomic (isoproterenol) stimuli similar to those characteristic of isolated adult cardiomyocytes are present in maturing hiPS-CMs. In addition, thyroid hormone treatment of hiPS-CMs attenuated the fetal gene expression in favor of a more adult-like pattern. Overall, hiPS-CMs progressively acquire functionality when maintained in culture for a prolonged period of time. The description of this evolving phenotype helps to identify optimal use of hiPS-CMs for a range of research applications.

A RELIABLE SUPPLY of homogeneous and functional human cardiomyocytes has long been an aspiration of cardiac biologists and drug discovery scientists (1, 2, 7, 16, 49, 54, 60). Human cardiomyocyte cell lines could not be established, and the severely limited availability of primary human cells led to significant reliance on animal sources of cardiomyocytes: in particular, neonatal or adult rat ventricular myocytes or transformed (cancer or embryonic) cell lines, e.g., H9C2 or HL-1. These cardiomyocyte models are valuable for basic research, but their incomplete differentiation and the important physiological and molecular differences among species complicate and limit the translation of findings to humans (69).

In vitro differentiation of human embryonic stem cells (hESCs) into mature somatic cell types provides an alternative source of human cardiomyocytes, but ethical and legal barriers to working with hESCs limit their availability (3, 41, 43, 47). The creation of human-induced pluripotent stem cells (hiPS) by reprogramming somatic cells with embryonic transcription factors (57, 58, 66, 67) has provided an alternative for in vitro differentiation experiments. The ability of hiPS cells to differentiate into cardiomyocytes has been demonstrated by a number of laboratories (57, 65, 70, 72), and hiPS-derived cardiomyocytes (hiPS-CMs) are currently commercially available.

Human cardiomyocytes may be differentiated from stem cells in a short time period (2 to 3 wk) (9, 32, 46, 61, 70, 72). This corresponds to the observation that by day 21 of human embryonic development, cardiac mesodermal precursors have migrated to form a heart tube and have differentiated into contracting cardiomyocytes (31). However, an additional 36 wk of embryonic development and postnatal hypertrophic growth (induced by hormonal changes and increased hemodynamic load) is needed to produce mature human cardiomyocytes (15, 63), a process that has been difficult to recapitulate in cell culture. Cardiomyocytes differentiated from hES or hiPS cell lines (hES-CMs or hiPS-CMs) are described as functionally immature (4, 61). With time in culture, hiPS-CMs acquire basic cardiomyocyte functional properties including signaling pathways mediating cardioactive stimuli as well as changes in metabolism, structure, and electrophysiological properties (14, 24, 50). This occurs despite the fact that stimuli for maturation are poorly modeled using the standard tissue culture techniques (11, 19, 64).

The aim of the present study was to characterize the in vitro differentiation of hiPS-CMs. We demonstrate that hiPS-CMs progressively acquire important cardiomyocyte characteristics, respond appropriately to a variety of the cardiac stimuli, and may serve as informative models of human pathologies.

MATERIALS AND METHODS

Cell culture. hiPS-CMs were obtained from Cell Dynamics International (CDI, Madison, WI) and cultured according to manufacturer’s instructions. Briefly, cells were grown in gelatin-coated plates at density of 6,000 cells/well of a 96-well plate, fed every 2 days with Pen Strep-supplemented maintenance media (DMEM base, 1 mM sodium pyruvate, 10 mM galactose, 10% FBS, supplied by the CDI) and kept in a humidified incubator at 37°C and 5% CO₂. HiPS-CM are grown for 30 days by the CDI before being shipped, and so the cell age is listed as 30+ days in culture, e.g., cells cultured for a week would be called 37 days old. HiPS-CM undergo continuous selection in culture and can be maintained as pure cultures for prolonged time by virtue of having a cardiac-specific myosin heavy chain 6 (MYH6) promoter driving antibiotic resistance—MYH6-dsRED-IREs-Blasti-
cidin Resistance cassette. HiPS-CM are fluorescent under appropriate excitation conditions. Triiodothyronine was obtained from Sigma (St. Louis, MO), KB2115 was obtained from Cayman Chemicals (Ann Arbor, MI), human recombinant endothelin-1 (ET-1) was obtained from Bachem (Torrance, CA), and human recombinant IGF-1 was obtained from R&D Systems (Minneapolis, MN). For induced maturation experiments, hiPS-CM were cultured as described above for varying lengths of time and incubated for 24 h in serum free media (SFM). Iscove’s modified Dulbecco’s medium (IMDM), (Invitrogen, CA), before being stimulated with varying concentrations of T3, IGF-1 or ET-1 in SFM for 24, 48, or 72 h. Upon the conclusion of treatment, cells were collected for RNA isolation or fixed for immunofluorescence (IFC).

Quantitative real-time PCR. After transfection and/or stimulation, cells were lysed and RNA was isolated according to Qiagen RNeasy protocol. Complimentary DNA was synthesized from 1 µg of total RNA using random hexamers and MultiScribe Reverse Transcriptase (Applied Biosystems). Quantitative real-time PCR was carried out using a HT7000 cycler (Applied Biosystems). Validated primer/probe sets for natriuretic peptide A; natriuretic peptide B; MYH6; MYH7; cardiac troponin T (cTNT); potassium inwardly rectifying channel (Kir2.1); sarco(endoplasatic reticulum calcium ATPase 2 (ATP2A2/SERCA2A); hyperpolarization-activated cyclic nucleotide-gated potassium channel 2 (HCN2); connexin 43 (Cx43); calcium channel, voltage-dependent, L-type, α1C-subunit (CACNA1C/Cav1.2); sodium channel, voltage-gated, type-V, α1C-subunit (SCN5A/Nav1.5); ryanodine receptor 2 (RYR2); thyroid hormone receptor-α (THRA); and thyroid hormone receptor-β (THRB) (Applied Biosystems) were used in multiplex reactions with either primer-limited GAPDH or β2-microtubulin as housekeeper genes (Applied Biosystems). For each sample, the level of expression of the gene of interest was determined relative to the expression of the housekeepers, GAPDH or β2-microtubulin and all the data are shown as normalized relative abundance. Representative experiments are shown, and the numbers of biological replicates/experiment are listed in figure legends.

Immunofluorescence. After stimulation, cells were washed in Dulbecco’s phosphate-buffered saline and fixed in 4% paraformaldehyde. Fixed cells were blocked/permeabilized in 70% TBS Superblock (Fierce, Rockford, IL) in PBS supplemented with 0.05% Tween-20 and 0.10% Triton X-100 (Sigma). Primary and secondary antibody incubations were performed in 70% TBS Superblock in PBS buffer with 0.05% Tween-20. Antibodies were as follows: Mouse anti-stage-specific embryonic antigen 4, FAB1435A (R&D); rabbit anti-cTNT, ab45932, mouse anti-cTNT, clone 1C11, ab8295, mouse anti-RYR2, clone C3-33, ab2827, (Abcam; Cambridge, MA); mouse anti-sarcomeric actinin, clone EA-53; mouse anti-MYH6, clone 3–48; mouse anti-smooth muscle actin, clone 1A4, Sigma; mouse anti-ATP2A2 (clone MA3–910) Affinity Bioreagents (Golden, CO); goat anti-ATP2A2, clone N-19, Santa Cruz Biotechnology (Santa Cruz, CA); Rabbit anti-Cx43, 71-0700, Invitrogen (Carlsbad, CA); Mouse anti-Cav1.2, clone L574/6, EMMLipill (Billericia, MA). Secondary AlexaFluor-coupled antibodies were from Invitrogen. Automated image acquisition with Operetta platform and analysis using Harmony software (PerkinElmer, MA) were utilized in some IFC experiments.

Electrophysiological recording. hiPS-CM cells were trypsinized with 0.05% trypsin-EDTA to release from the synctium and placed into recording chamber. Upon cell attachment, cells were perfused with external solution, consisting of (in mM) 140 NaCl, 4 KCl, 1 MgCl2, 2 CaCl2, 10 glucose, 10 HEPES (pH 7.4 with NaOH). For dye loading, Fluo4 was added to the chamber to 5 mM final concentration for 15 min. The chamber was perfused for 5 min at a rate of 1 to 1.5 ml/min before recording. All recordings were conducted with the hiPS-CMs paced at 0.5 Hz or higher rates by means of platinum wires embedded in the walls of the chamber. For fluorometric imaging plate reader (FLIPR) platform, hiPS-CMs were grown in 384-well plates and at 48 days loaded with Fluo4 according to the manufacturer’s protocol, using 30 µl labeling medium. The FLIPR stage was set to 37°C, and the cells were imaged within 10 min of removing them from the 7% CO2 incubator. FLIPR images were collected every 0.5 s, with exposure and intensity settings adjusted for maximum signal without saturation. Fluo4 fluorescence was recorded with excitation at 485 nm and emission at 530 nm. Images were analyzed using FLIPR platform software.

Metabolic profiling. Cells were seeded in XF96 tissue culture plates (Seahorse Bioscience) at 10,000 cells/well in 100 µl of growth medium and cultured in 37°C incubator with 5% CO2. One hour before the experiment, standard CDI maintenance medium was replaced with warm Seahorse assay media (without NaHCO3, with 1× l-glutamine, 1× sodium pyruvate, and 25 mM glucose). Isoproterenol was prepared at 10× in Seahorse assay media immediately before the experiment. Basic respiratory parameter measurements were performed using oligomycin, FCCP, and rotenone as recommended by the manufacturer.

Protein analysis. D45 hiPS-CMs were incubated in SFM for 24 h before the start of treatment. HiPS-CM were challenged with IGF-1 (100 ng/ml), ET-1 (100 nM), or vehicle for length of time indicated. Cells were lysed with Bioplex cell lysis buffer containing phosphatase/protease inhibitors (Bio-Rad). Cell lysate protein content was normalized using a standard BCS assay, and evaluated for phospho-protein content using either Bioplex [phospho (p)-IGF-1R, p-Akt, p-P38, p-GSK3β, p-ERK1/2, p-MAPK, p-JNK] or Meso Scale Discovery technology platform (Gaithersburg, MD) [%p-Akt/ total (t)-Akt] formats according to manufacturer’s instructions. Representative data (n = 4 biological replicates) from one of the three experiments performed are shown.

Data analysis. Data are presented as means ± SEM of three to seven replicates performed on a minimum of three independent occasions. Statistical analyses were performed by ANOVA for multiple comparisons and unpaired two-tailed Student’s t-test for two-group comparisons. The value for P < 0.05 was considered significant.

RESULTS

Expression of cardiac marker genes, maturation, and metabolism. A hiPS differentiation protocol used by CDI produces cardiomyocytes after 30 days, at which point the cells are collected and frozen for distribution. Thawed hiPS-CMs maintained in culture for 1 wk (day 37) are >95% positive for IFC staining of cardiac sarcomeric proteins MYH6 and cTNT; specific antibody staining is green, and red fluorescence is MYH6 promoter dsRED-IRES-BsdR cassette used for CM enrichment (Fig. 1A). MYH6 and cTNT were appropriately localized in
individual hiPS-CMs to organized sarcomeric bands (Fig. 1B). IFC staining of calcium handling proteins, SERCA2A and RYR2, shows the beginning of organization of SERCA2 in a banded pattern (inset) whereas the RYR2 staining is punctate (inset) (Fig. 1C). The totality of IFC data demonstrates that hiPS-CMs are a population of highly enriched but immature cardiac cell types expressing cardiac markers. IFC further demonstrates the stable presence of MYH6, SERCA2, and α-sarcomeric actinin and the increased expression of subunit of voltage-gated L-type calcium channel Cav1.2 from day 37 to day 45 (data not shown). To verify that hiPS-CMs are a cardiac population of cells, we tested markers of undifferentiated hiPS cells such as stage-specific embryonic antigen 4 or markers of other differentiated lineages such as α-smooth muscle actin, neurons (β-tubulin III protein), or endothelial cells (endothelial tyrosine kinase) for which cells were negative (data not shown).

Maturation of hiPS-CMs was investigated by quantifying the expression of maturation marker genes over time in culture and by examining changes in metabolic responsiveness of the cells. Quantitative real-time PCR demonstrated progressive changes in expression of transcripts encoding atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), Cx43, Nav1.5, Cav1.2, and Kir2.1 from day 37 to day 80 of culture. Importantly, the expression of the cardiac fetal genes ANP and BNP decreased substantially after 37 days to levels observed in normal adult human myocardium and remained substantially unchanged after day 45, whereas the expression of other genes associated with a mature cardiac phenotype (Cx43, Nav1.5, and Cav1.2) continued to increase throughout the time of observation (Fig. 2A). Some of the genes measured, such as Kir2.1, continued increasing throughout the experiment and only reached levels comparable with levels of human myocardium at day 80. The ratio of the mRNA expression of two cardiac myosin isoforms MYH6 and MYH7 increased in hiPS-CMs over time (MYH6/MYH7) (Fig. 2B) and were most comparable with that observed in human myocardium after day 45.
Stimulation of hiPS-CMs with isoproterenol (100 nM) led to a greater increase in the extracellular acidification rate, an indicator of a glycolysis, at day 45 than at day 37 (Fig. 2C), and no isoproterenol effects on pH were observed (data not shown).

**Electrophysiological characterization.** Biophysical and pharmacological properties of the Na\(^+\) and Ca\(^{2+}\) channels were evaluated in hiPS-CMs. A pattern of increasing Na\(^+\) and L-type Ca\(^{2+}\) current density in hiPS-CMs was observed with increasing time in cell culture (Fig. 3A). HiPS-CM exhibited action potential heterogeneity, likely reflecting the mixed nature of the cell types (atrial, ventricular, and pacemaker) and a range of cellular maturation in the culture. Nodal cells had prominent diastolic depolarization and fired action potentials spontaneously. Atrial and ventricular cells had no spontaneous action potentials but did fire action potentials when stimulated with depolarizing current pulse at 0.5 Hz. Resting membrane potential and action potential duration and plateau in hiPS-CMs differed from those reported in human mature cardiomyocytes (data not shown).

The T-type Ca\(^{2+}\) current was examined at two time points. On day 37 this current was recorded in 8% of cells, and on day 45 it was detected in 50% of cells. To determine whether the sodium current seen in hiPS-CMs was a cardiac type, we investigated the sensitivity of the Na\(^+\) channel to 4 and 10 \(\mu\)M of tetrodotoxin (TTX), a selective sodium channel blocker. TTX (4 \(\mu\)M) blocked only 50% of the current and 10 \(\mu\)M blocked 80% of the current, consistent with the properties ascribed to the TTX-insensitive cardiac type sodium channel. Nifedipine, a selective calcium channel blocker, was used to determine if the inward current recorded in isolated hiPS-CMs was an L-type Ca\(^{2+}\) channel. Nifedipine (10 \(\mu\)M) abolished this voltage-dependent inward transients could be trained to electrical field stimulation at 0.5 Hz.

Fig. 2. Time course of gene expression and metabolic responses in hiPS-CMs. A: quantitative real-time PCR (qRT-PCR) for atrial natriuretic peptide (ANP); brain natriuretic peptide (BNP); connexin 43 (Cx43); sodium channel, voltage-gated, type-V, \(\alpha_c\)-subunit (Nav1.5); calcium channel, voltage-dependent, L-type, \(\alpha_c\)-subunit (Cav1.2); and potassium inwardly rectifying channel (Kir2.1) in cells aged for 37, 45, 64, or 80 days, compared with a sample of human ventricular heart tissue (pooled sample). D, day. B: ratio of relative abundance of MYH6 to MYH7 over time; *P < 0.05 vs. day 37. C: Seahorse extracellular acidification, extracellular acidification rate (ECAR), in response to isoproterenol (Iso, 100 nM) in day 37 vs. day 45 cells (\(n = 3\) biological replicates).
or 1 Hz (Fig. 4B) as observed with Fluo4 fluorescence intensity measurements. Calcium transients responded to β-adrenergic stimulation (isoproterenol, 20 nM) with an increase in frequency; however, no effects on transient amplitude were noted, despite isoproterenol-induced increases in the L-type Ca\(^{2+}\) current (65 ± 13%) recorded on day 45 in isolated individual hiPS-CMs (Fig. 4D). Calcium transients were also abolished by the application of methacholine (25 μM), a muscarinic agonist, suggesting a component of pacemaker-stimulated contractility (Fig. 4C). Overall, the Fluo4 fluorescence data suggest that a functional syncytium develops over time.

**Signaling pathways.** The effects of known activators of pathological (ET-1) and physiological (IGF-1) hypertrophic stimuli on MAPK and phosphatidylinositol 3-kinase-Akt signaling are well defined in human cardiomyocytes and are important in the development and maintenance of hypertrophy (29, 55). These stimuli can be used to benchmark the similarity of signaling pathways in hiPS-CMs to those of isolated mature human cardiomyocytes. Treatment of day 45 hiPS-CMs with ET-1 (2 nM) for 24 h produced changes in gene expression consistent with activation of pathological pathways, e.g., induction of transcripts encoding BNP and MYH7, suggesting competent GqG protein-coupled receptor signaling. In a 24-h experiment, ET-1-induced increases in BNP and MYH7 transcript expression were not blocked by the physiological hypertrophy stimulus IGF-1 (10 ng/ml) (Fig. 5A). Interestingly, in a shorter experiment, IGF-1 (100 ng/ml) stimulation increased the ratio of p-Akt to t-Akt, whereas ET-1 stimulation (100 nM) decreased the ratio of p-Akt to t-Akt, both at 15 and 30 min of treatment. ET-1, but not IGF-1 treatment, induced JNK phosphorylation at 15 and 30 min of treatment, and both ET-1 and IGF-1 induced ERK phosphorylation. Only IGF-1 mildly and transiently increased p38 phosphorylation (Fig. 5B).

THRA and THRb are implicated in cardiac maturation and physiological hypertrophy in vivo (13, 21, 22, 25, 36, 59). We tested the effect of thyroid hormone T3, a THRA/THRb agonist, on cardiac gene expression in day 45 hiPS-CMs. T3 (100 nM) had differential effects on the expression of some of the genes tested, demonstrating the presence of functional THR signaling. Specifically, T3 increased the transcript expression of SERCA2A, MYH6, and THRb and reduced the expression of BNP, MYH7, and HCN4, a profile consistent with maturation and/or physiological adaptation (Fig. 6).

**DISCUSSION**

Mouse and hiPS have been successfully differentiated to cardiomyocytes by a number of groups (23, 32, 45, 46, 51, 70),

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**Fig. 3.** hiPS-CMs express cardiac ion channel genes and exhibit cardiac ion channel activity. A: relationship of Na\(^+\) and Ca\(^{2+}\) current density with culture time. B: Na\(^+\) and T- and L-type Ca\(^{2+}\) currents at day 45. TTX, tetrodotoxin. C: L-type Ca\(^{2+}\) channel blocker nifedipine blocked intracellular Ca\(^{2+}\) transient, day 45. F/F\(_0\), fluorescence normalized to baseline fluorescence.
but a large-scale, reproducible culture was only recently developed. These hiPS-CMs hold promise for replacing primary cardiomyocytes obtained from animal or human cardiac samples and for addressing basic biology and toxicology questions (5, 16). However, CMs differentiated in vitro vary significantly from cells isolated from a mature human heart, with most of the differences likely due to our inability to appropriately incorporate all the relevant developmental and physiological cues (e.g., appropriate mix of signaling peptides, contractility, electrical pacing, 3-dimensional structure, etc.) into differentiation and maturation protocols. Furthermore, reports of varying phenotypes in hiPS-CMs may add further translational complications due in part to the different culture conditions, varying degrees of cellular maturity, and the genetic differences between the hiPS cell lines. Another important source of phenotypic variation in differentiated hiPS-CMs is the relative proportion of various cardiac cell types (atrial vs. ventricular vs. nodal) in the final population (definitive methods to isolate specific subpopulations are not available). Given these limitations and variations, a careful characterization of hiPS-CMs is necessary to understand how best to use these cells. Our findings demonstrate that unlike primary isolated cardiomyocytes that tend to undergo apoptosis or dedifferentiate (71) in long-term culture, hiPS-CMs develop and maintain a functional phenotype, including responsiveness to multiple physiological and pathological stimuli. Prolonged hiPS-CM culture is possible, and cells have been grown for up to 6 mo in our laboratory. Even though some cellular loss occurred, continued contractility as well as reactivity to cTNT antibody demonstrated that hiPS-CMs can maintain aspects of their cardiac identity in long-term culture.

We evaluated functionality, phenotypic development, and stability of hiPS-CMs in long-term culture using protocols established for primary human and rodent cardiomyocytes (7, 8, 12, 17, 39, 65). We characterized these cells by examining five aspects of the cardiomyocyte phenotype: gene expression, ion channel functionality (ion currents), intracellular calcium cycling (calcium transients), responsiveness to cardioactive pharmacological stimuli, and metabolic parameters. We suggest that evaluating these five aspects of cardiomyocyte function is necessary to characterize differentiated cardiomyocytes and that appropriate (mature) responses in these assays are a prerequisite for describing a cell as a differentiated cardiomyocyte. With each of these measures we observed that cultured hiPS-CMs undergo progressive functional changes and acquire a more mature phenotype over time. Furthermore, T3 treatment

Fig. 4. Calcium handling and chronotropic responses in day 45 hiPS-CMs. A: calcium transients in spontaneously contracting cells. Fluo4 fluorescence intensity. RLU, relative light units. B: pacing of CM: Fluo4 fluorescence intensity at 0.5- and 1-Hz stimulation. C: calcium transients in spontaneously contracting cells, Fluo4 fluorescence intensity: effect of isoproterenol (20 nM) and methacholine (25 µM) on rate of contraction. D: effect of isoproterenol (1 µM) on L-type Ca2+ current (n = 7 cells).
was found to affect a range of cardiomyocyte functions and may be used to promote development of a more mature phenotype.

When grown in a monolayer, hiPS-CMs contract rhythmically within 2 wk and may be paced electrically (6, 62). The coordinated spontaneous rhythmic calcium transients recorded in nearly every culture well after day 45 imply emergence of a functional syncytium, the presence of a dominant pacemaker focus, and an organized sarcoplasmic reticulum, consistent with a more developed phenotype. Our characterization suggests that calcium cycling becomes more developed over time. Specifically, increased expression of RYR2 and SERCA2 in older cells suggests progressive improvement in organization of the sarcoplasmic reticulum. In addition, the current density of L-type ion channels increases [similar to what was observed in other publications (53)] and we demonstrated that hiPS-CMs

Fig. 6. Effects of thyroid hormone receptor (THR) agonist T3 (THRA/THRB) 24 h treatment on cardiac gene expression in day 45 hiPS-CMs. qRT-PCR for BNP, MYH6, MYH7, hyperpolarization-activated cyclic nucleotide-gated potassium channel 2 (HCN4), THRB, and SERCA2 after 24 h of T3 (100 nM) treatment (n = 4 biological replicates). [*P < .05 vs. vehicle.]

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become more responsive to isoproterenol, all suggesting that critical ion channels and relevant signaling events continue to develop. Over time in culture the resting membrane potential becomes more polarized, approaching that described for adult primary human cardiomyocytes (20, 28, 48, 52, 68). Furthermore, hiPS-CMs cultured for a prolonged period of time (up to day 70) maintain their electrophysiological responsiveness.

Similarly, the isoproterenol-induced increase in glycolysis of hiPS-CMs changed over time in culture. Specifically, at 37 days, the metabolic effects of isoproterenol, as measured by the acidification of media, were not as robust as those observed at 45 days. The mechanism(s) underlying this maturation remain to be determined, but it is an accepted paradigm that in the course of in vivo maturation, human cardiomyocytes become less reliant on glycolysis and more reliant on β-oxidation (26, 27, 42, 44). Using extracellular acidification rates and oxygen consumption as readouts, we did not observe either a decrease in total levels of glycolysis (using extracellular acidification rate as a parameter) or an increase in β-oxidation in maturing CM. It should be appreciated that our hiPS-CMs are cultured in galactose-containing media, and this may complicate comparisons with cells cultured with other metabolic substrates.

The hiPS-CM gene expression pattern changed over time such that fetal gene expression diminished in older cells. In addition, characteristic gene expression patterns induced by both physiological (T3, IGF-1) and pathological (ET-1, isoproterenol) hypertrophic stimuli also changed over time. Specifically, ET-1 increased the expression of MYH7 and BNP and decreased the expression of MYH6 in day 45 hiPS-CMs to a greater extent than in day 30–37 cells. Interestingly, these changes reached steady-state levels sooner (around day 45) than the expression of ion channel genes. Abundance of BNP and ANP among others is higher in human fetal heart relative to adult heart, is greater at days 30–37 in hiPS-CMs, and decreases with time in culture. Since the relative induction of BNP, as well as MYH7, by pathological hypertrophic stimuli is a commonly accepted readout for in vitro hypertrophic assays, it is not surprising that the overall response to ET-1 was greater in older hiPS-CMs, when cells had lower fetal gene expression at baseline. The observed effect of ET-1 on MAPK signaling pathways is consistent with previous reports (10, 30, 40, 55), and the responsiveness to ET-1 remained constant in mature cells (day 45 to day 90), and could be observed at day 120.

THRA and THRβ are implicated in cardiac maturation and physiological hypertrophy in vivo. Expression and activity of THR receptors has been suggested as a measure of cardiomyocyte maturity and health, with THR levels and activity increasing in maturation and physiological hypertrophy, but decreasing in pathological hypertrophy in human and animal myocardium (33–35, 37, 38). T3 treatment of cardiomyocytes leads to cellular hypertrophy and to changes in expression of myosin heavy chain isoforms MYH6 and MYH7, a so-called myosin switch. Consistent with these previous observations, hiPS-CMs treated with T3 had a decreased expression of MYH7 and increased expression of MYH6, increasing the MYH6-to-MYH7 ratio. T3 also partially blocked the ET-1-induced decrease in the MYH6-to-MYH7 ratio, changes consistent with observations in neonatal or adult rat ventricular myocytes (18, 56). T3 treatment also increased expression of genes involved in calcium handling (SERCA2, RYR2) and the development of cellular action potential. Overall, changes induced by T3 are consistent with the induction of a more functionally mature phenotype, and it may be possible to design strategies for accelerating hiPS-CMs functionality by varying T3 levels in cell culture media.

The results from our molecular, pharmacological, and functional characterization suggest that hiPS-CMs are immature cardiomyocyte-like cells that progressively acquire a more functionally mature phenotype in culture. The cells also exhibit characteristic cardiomyocyte responses following challenge with putative pathological and physiological stimuli. Finally, T3 treatment of immature hiPS-CMs induces changes consistent with enhanced development. Our data help to explain how experiments at different time points may produce varying results and lead to conflicting conclusions regarding the functionality of the hiPS-CMs. The results highlight the need to characterize the phenotype of hiPS-CMs over time to use these cells appropriately. The data presented in this report add to the growing body of evidence that hiPS-CMs are important tools for exploring cardiac physiology as well as for evaluating efficacy and safety signals in drug discovery.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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