Derivation and High Engraftment of Patient-Specific Cardiomyocyte-Sheet Using Induced Pluripotent Stem Cells Generated From Adult Cardiac Fibroblast

Zhang et al: hiPSC-CM From Cardiac Fibroblast

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Abstract

Background—Induced pluripotent stem cells (iPSCs) can be differentiated into potentially unlimited lineages of cell types for use in autologous cell therapy. However, the efficiency of the differentiation procedure and subsequent function of the iPSC-derived cells may be influenced by epigenetic factors that the iPSCs retain from their tissues of origin; thus, iPSC-derived cells may be more effective for treatment of myocardial injury if the iPSCs were engineered from cardiac-lineage cells, rather than dermal fibroblasts.

Methods and Results—We show that human cardiac iPSCs (hciPSCs) can be generated from cardiac fibroblasts and subsequently differentiated into exceptionally pure (>92%) sheets of cardiomyocytes (CMs). The hciPSCs passed through all the normal stages of differentiation before assuming a CM identity. When using the fibrin gel enhanced delivery of hciPSC-CM sheets at the site of injury in infarcted mouse hearts, the engraftment rate was 31.91%±5.75% at Day 28 post transplantation. The hciPSC-CM in the sheet also appeared to develop a more mature, structurally aligned phenotype 28 days after transplantation and was associated with significant improvements in cardiac function, vascularity, and reduction in apoptosis.

Conclusions—These data strongly support the potential of hciPSC-CM sheet transplantation for the treatment of heart with acute myocardial infarction.

Key Words: heart, stem cell, myocardium, infarction
More than 20 million people worldwide and more than 5.8 million people in the United States have been diagnosed with heart failure \(^1\), and the only treatment that is currently available for patients in the final stages of the disease is heart transplantation. However, comorbid conditions preclude many patients from undergoing transplantation surgery, and among suitable patients, the procedure is limited by high costs and an insufficient supply of donated organs. Thus, investigators continue to search for methods to improve the limited endogenous repair capacity of the heart, and stem cells are among the most promising therapeutic agents tested, because they have an unlimited capacity for self-renewal and can differentiate into numerous cell lineages. Mesenchymal stem cells \(^2\), cardiac progenitor cells \(^3\), and cardiosphere-derived cells \(^4\) have been associated with improvements in cardiac function after transplantation into the hearts of patients with myocardial infarction (MI), and promising results have been obtained in preclinical investigations with cells that have been derived from human embryonic stem cells (hESCs) \(^5\). However, human induced pluripotent stem cells (hiPSCs) may be a more appropriate source of cells for future investigations of regenerative therapy, because they can be created from the somatic cells of each individual patient and, consequently, are less likely to cause an adverse immune/inflammatory reaction, do not require a lifetime of immunosuppressive therapy, and avoid the ethical concerns associated with the use of human embryos \(^6, 7\).

The exceptionally high differentiation potential of ESCs and iPSCs could lead to tumor formation if the cells were transplanted directly into somatic tissue \(^8\); thus, iPSCs are typically induced to differentiate into a specific cell type before transplantation. In principle, iPSCs can be used to generate cells of any lineage; however, the efficiency of the differentiation procedure and subsequent function of the iPSC-derived cells may be influenced by epigenetic factors that the
iPSCs retain from their tissues of origin\textsuperscript{9,10}. If so, iPSC-derived cells may be more effective for treatment of myocardial injury if the iPSCs were created from cardiac-lineage cells, rather than (for example) dermal fibroblasts, which were the first cells used to generate iPSCs and continue to be the most common source of iPSCs for deriving cardiomyocytes (CMs). iPSCs have also been created from keratinocytes\textsuperscript{11}, neuroprogenitor cells\textsuperscript{12}, hemapoietic progenitor cells\textsuperscript{13}, endothelial cells\textsuperscript{14}, adipose stem cells\textsuperscript{15}, peripheral blood cells\textsuperscript{16}, hair follicle cells\textsuperscript{17}, and cells obtained from the urine\textsuperscript{18}. The experiments described in this report are the first to use iPSCs that have been engineered from cardiac fibroblasts. The fibroblasts were obtained from the hearts of patients with ischemia cardiomyopathy who were undergoing open-chest surgery, and genetically reprogrammed to generate human cardiac induced-pluripotent stem cells (hciPSCs). Then, the hciPSCs were differentiated into sheets of hciPSC-derived CMs (hciPSC-CMs) that were subsequently evaluated in immunodeficient mice with surgically induced MI. Notably, the hciPSCs generated for this report, unlike those used in a previous, disease-modeling study\textsuperscript{19}, were genetically normal, because they were created from the cells of a patient whose heart failure could not be attributed to a genetic cause.

\textbf{Methods}

\textbf{Isolation and characterization of cardiac fibroblasts}

The University of Minnesota Human Subjects Research Institutional Review Board (IRB) approved all protocols in this study. Human cardiac tissue was collected from the left atrial appendage of 3 patients (2 female, 1 male) who underwent open chest surgery; then, the tissue was cut into small pieces, suspended in HBSS buffer supplemented with 600 U/mL collagenase II and 60 U/mL DNaseI, and dissociated with a GentleMACS dissociator (Miltenyi Biotec).
Single cells were collected as directed by the manufacturer’s protocol and then cultured in DMEM supplemented with 10% FBS and 1x penicillin/streptomycin (Invitrogen). The culture medium was changed every three days for 2 weeks, and the purity of the cell preparation was evaluated by staining the cells for vimentin expression (R&D system, Table).

**Generation and differentiation of human cardiac induced pluripotent cells**

Cardiac fibroblasts were passaged twice and then reprogrammed with the CytoTune™-iPS Reprogramming Kit (Invitrogen) as directed by the manufacturer’s instructions. Colonies of putative hciPSCs were identified via Tra1-60 live staining and chosen for expansion. Cells from the selected colonies were cultured on Matrigel-coated dishes in mTeSR or with feeder cells in DMEM-F12 supplemented with 15% KO-SR, 8ng/mL bFGF, 1x NEAA, 1x Glutamax, 1x penicillin/streptomycin, and 1% 2-mercaptoethanol. The cells were examined for morphological similarities to embryonic stem cells and for the expression of pluripotency transcription factors; three clones from the cells of each patient were selected for subsequent analyses and differentiation.

hciPSCs were differentiated into CMs via the Matrigel sandwich method, as described previously. Briefly, 1x10^6 single hciPSCs were expanded on a Matrigel-coated dish for 3-5 days; then, differentiation was induced on day 0 by culturing the cells with 100 ng/mL Activin A (R&D systems) in RPMI basal medium plus B27 without insulin. Twenty-four hours later (i.e., on day 1 of differentiation), the cells were treated with 10 ng/mL BMP4 and 7.5 ng/mL bFGF (R&D systems) in RPMI basal medium plus B27 without insulin; four days later (i.e., on day 5 of differentiation), the cells were cultured in RPMI basal medium with normal B27, and beating
cells usually appeared on ~day 8 after differentiation was initiated. The beating cells were videotaped with a Nikon camera that had been attached to the microscope.

**Karyotyping**

Karyotype analyses were performed in the University of Minnesota’s Cytogenics Core Laboratory. Cells were cultured in the wells of a 6-well plate, treated with colcemid for 3.5 hours, and then harvested according to a standard cytogenetic protocol. Metaphase G-banding was evaluated at 400-425 band resolution in 10 cells.

**Teratoma formation**

The teratoma-formation assay was performed as described previously. Briefly, 2 million cells were subcutaneously implanted into immunodeficient mice. Eight weeks later, the teratoma was excised, sectioned, H&E-stained, and examined for the presence of all three germ layers.

**Flow cytometry, in-vitro immunohistochemistry**

For flow cytometry analyses, cells were trypsinized, resuspended as single cells, permeabilized in 0.1% Triton X-100 at 4 °C for 10 minutes, blocked with UltraV block (Fisher Scientific) at room temperature for 7 minutes, incubated with primary anti-Vimentin, anti-cTnT, and anti-cardiac alpha sarcomeric actinin (cASA) antibodies (Table) or isotype control antibodies at 4 °C overnight, washed, and stained with appropriate secondary antibodies (Table). After staining, the cells were washed, re-suspended in 2% FBS/PBS containing 5 μL of propidium iodide (10 μg/mL), and evaluated with a FACS Aria instrument (BD Biosciences).
For immunohistochemistry analyses, hciPSCs and hciPSC-CMs were fixed with 4% paraformaldehyde at room temperature for 20 minutes, permeabilized in 0.1 Triton X-100 at 4 °C for 10 minutes, and blocked with UltraV block for 7 minutes. Primary antibodies against SSEA4, Tra1-60, and Oct4 (hciPSCs), or cTnT and connexin43 (hciPSC-CMs) were added to the UltraV block buffer at a concentration of 1:100, and the cells were incubated at 4 °C overnight; then, the labeled cells were washed and incubated with FITC- and TRITC-conjugated secondary antibodies (Table) in UltraV block buffer at room temperature for 1 hour, counterstained with DAPI, washed, and visualized under a fluorescence microscope (Olympus, Japan).

Quantitative RT-PCR

Total RNA was extracted and treated with DNase1 and with an RNaeasy mini kit (Qiagen). Quantitative RT-PCR was performed on an ABI 7100 real time PCR machine. Probes for Oct4, Sox2, Nanog, Brachyury, Mesp1, Nkx2.5, Gata4, Mef2C, and Tbx5 were purchased from ABI, and GAPDH levels were used as an internal control.

Measurement of Ca²⁺ Transients in iPSC-CMs

iPSC-CMs from iPSC cell lines derived from different tissues (hUCBiPSC: from human umbilical cord blood mononuclear cell, hdiPSC: from human dermal fibroblasts, or hciPSC: from human cardiac fibroblasts) were cultured on Matrigel-coated coverslips (Fisher) for 24 hours. iPSC-CMs were then loaded with 1 μmol/L fura2 AM (Invitrogen) in Tyrode’s buffer (140 mmol/L NaCl, 10 mmol/L glucose, 10 mmol/L HEPES, 4 mmol/L KCl, 1 mmol/L MgCl₂, pH 7.45) supplemented with 1.2 mmol/L Ca²⁺ for 20 minutes at 37°C. Changes in fura2 ratio
(340/380nm) were recorded using the Fluorescence and Contractility System (IonOptix) coupled to an inverted fluorescence microscope (Motic AE31) with a 40x objective.

**Murine MI model and treatment**

All experimental procedures that involved animals were approved by the Institutional Animal Care and Use Committee of the University of Minnesota, performed in accordance with the Animal Use Guidelines of the University of Minnesota, and consistent with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH publication No 85-23). Myocardial infarction (MI) was surgically induced as described previously. Briefly, twelve-week old immunodeficient NOD/SCID/γc-/ (NSG) mice (Jackson Laboratory) were anesthetized with an intraperitoneal injection of sodium pentobarbital (35 mg/kg), intubated, and ventilated with a small animal respirator (Harvard Apparatus); then, a left thoracotomy was performed to expose the heart, and the left-anterior descending coronary artery was permanently ligated with a 7.0 surgical silk suture. Fifteen minutes after ligation, the surviving mice were randomly distributed into two experimental groups.

The differentiated hciPSC-CM cells grew to form a contiguous sheet and the sheet was harvested for transplantation after they had been beating for 10 days by gently lifting them from the surface of the culture dish. Animals in the hciPSC-CM group were treated with a sheet of hciPSC-CMs (containing 0.6-1.0x10⁶ cells) that had been beating for 10 days. The sheet was suspended in fibrinogen (500 μL, 25 mg/mL) before application, and the surface of the myocardium was pricked several times with a sterile needle to induce light bleeding, which enhanced sheet adhesion by stimulating the release of endogenous thrombin and may have promoted other
interactions between the sheet and the native myocardium; exogenous thrombin (8 μL, 80 U/mL) was also applied to the myocardial surface. Animals in the MI group were only treated with fibrin gel. The chest was closed in layers and animals allowed to recover. The echocardiography was application to evaluation cardiac function 4 weeks after the MI and cell transplantation.

**Engraftment Rate**

Hearts were cut into halves from the middle of the infarct; one of the halves was frozen in OCT for cryo-sectioning and the other half was stored in 10% formalin for paraffin-embedded sectioning. Embedded tissues were cut into 6-7 μm sections, stained with the antibodies listed in the Table, and viewed with a Nikon fluorescence microscope or a Zeiss LSM 710 confocal microscope. HNA⁺ or hcTnT⁺ cells were counted in 60 sections per animal, and the total number of cells was estimated as (mean cell density)³/² X volume; then, the engraftment rate was calculated as the ratio of the total (estimated) number of HNA⁺ or hcTnT⁺ cells to the number of cells administered.

**Apoptosis**

Hearts were embedded in Tissue Tek® OCT compound for cryosectioning or treated with 10% formalin for paraffin-embedded sectioning. Embedded tissues were cut into 7 μm sections, TUNEL stained, and viewed at 40X magnification. The number of TUNEL⁺ cells and the total number of cells were counted in five fields from each section.

**Vascularization**

Sections from the left ventricle (LV) were stained with anti-CD31 antibodies (Table) and viewed
at 20X magnification as described previously. CD31+ cells were counted in 10 sections from the infarct border zone and 10 sections from the remote zone for each animal; 2 fields were evaluated in each section.

**Echocardiographic assessments of cardiac function**

Transthoracic echocardiography was performed on a Vevo770 Imaging System equipped with an RMV 707B transducer (15–45MH) (VisualSonics Inc, Canada); both conventional two-dimensional images and M-Mode images of the heart in a parasternal short axis view were acquired as described previously. Measurements were obtained via the method recommended by the American Society of Echocardiography. LV internal diameters at end-diastole (LVIDed) and end-systole (LVIDes) were determined from 8 consecutively obtained measurements, and left-ventricular ejection fractions (EF) and fractional shortening (FS) were calculated according to the equations: EF%=(LVIDed²-LVIDes²)/LVIDed² X 100%; and FS%=(LVIDed-LVIDes)/LVIDed X 100%

**Statistical Analysis**

All data are presented as mean ± the standard error of the mean (SEM). Comparisons between groups were analyzed using the Wilcoxon rank-sum test, and with Bonferroni correction when number of groups were greater than two. The Software Stata release 13 (StataCorp. 2013. College Station, TX: StataCorp LP), was used to conduct statistical analysis. A p-value (two-sided) less than 0.05 was considered statistically significant.
Results

Generation and characterization of hciPSC

Three cell lines from cardiac specimen derived cardiac fibroblasts (Figure 1A), were obtained from the hearts of each of three patients (2 female, 1 male), expanded in culture, and then reprogrammed via viral transduction. Before reprogramming, all of the isolated and expanded cells expressed the fibroblast-specific marker vimentin (Figure 1B) but the pluripotency markers Oct4, Sox2, and Nanog were undetectable. Three weeks after reprogramming was initiated, putative hciPSCs were manually selected by examining the colonies for morphological similarities to embryonic stem cells and for expression of the pluripotency marker Tra1-60 (Figure 1C). The selected cells displayed a normal human chromosomal structure (Figure 1D), stained positively for expression of the pluripotency markers Tra1-60, SSEA4, and Oct4 (Figure 1E), and expressed levels of Oct3/4, Sox2, and Nanog mRNA that were similar to the levels observed in an established line of iPSCs (PCBC16iPS) (Figure 1F). Pluripotency of the hciPSCs was confirmed via the teratoma-formation assay (Figure 1G); cells transplanted into immunodeficient mice proliferated and differentiated to form teratoma that contained cells of all three developmental germ layers (i.e., endoderm, mesoderm, and ectoderm).

Differentiation of hciPSC into CMs

hciPSCs were induced to differentiate via the Matrigel sandwich method (Figure 2A), and the cells' progress toward a CM lineage, which normally passes through an intermediate, mesodermal stage, was monitored by evaluating the expression of pluripotent, mesodermal, and cardiac markers. Five days after induction, the expression of pluripotent genes had declined significantly (Figure 2B) while expression of early mesodermal (Brachyury) (Figure 2C), and
cardiac mesodermal (Mesp1, Nkx2.5, Gata4, Mef2C, and Tbx5) (Figures 2C & 2D) markers had significantly increased. Mesodermal marker expression declined shortly after beating cells were observed on day 8 after induction (Supplemental Video), but cardiac markers continued to be expressed (Figure 2D). On the tenth day after beating was observed (i.e., ~18 days after differentiation was induced), 92.5% of the cells expressed cTnT (Figure 2E), cardiac alpha sarcomeric actinin (cASA) and the gap-junction protein Connexin 43 (between adjacent cells) were observed (Figure 2F). The cells continued beating for at least 60 days.

Quantitation of Beating Cardiomyocyte Derived from Different hiPSC Lines

We quantified both the proportion of beating CM sheets and the double positive cell population for cTnT and cardiac alpha sarcomeric actinin for differentiated CMs that were beating on day 20 after beating was observed. The results are summarized in Table S1 and Figure S1 of the revised manuscript. The proportion of beating sheets was at least 90% for all hciPSC-CM batches tested, but fell to as low as 30% and 20% for hdiPSC-CMs hUCBiPSC-CMs, respectively (Table S1). The hciPSC-CMs expressed both proteins of cTnT and cASA at the highest level compared to hdiPSC- or UCBiPSC-CMs (Figure S1).

Ca\textsuperscript{2+} Handling in CMs Derived from Different hiPS Cell Lines

To compare the Ca\textsuperscript{2+} handling in cardiomyocytes derived from different hiPS cell lines, we measured Ca2\textsuperscript{+} transients in spontaneously contracting CMs from early passage iPSCs originated from umbilical cord blood, dermal fibroblasts, and cardiac fibroblasts, respectively. Both hUCBiPSC-CMs and hciPSC-CMs produced regular pulsatile Ca\textsuperscript{2+} transients, whereas hdiPSC-CMs produced robust yet infrequent Ca\textsuperscript{2+} transients (Figure 3A). However, hdiPSC-CMs and
hciPSC-CMs produced faster rates of Ca\(^{2+}\) rise and decline compared to hUCBiPSC-CMs (Figure 3, B-C). Similarly, both hdiPSC-CMs and hciPSC-CMs had higher peak Ca\(^{2+}\) and resting Ca\(^{2+}\) compared to hUCBiPSC-CMs, suggesting that hdiPSC-CMs and hciPSC-CMs have a higher overall Ca\(^{2+}\) capacity (Figure 3, D-E). Together, these results indicate that hciPSC-CMs have a more cardiac-like Ca\(^{2+}\) handling profile compared to CMs derived from hUCBiPSC or hdiPSCs.

**hciPSC-CM sheets in a myocardial injury model**

The efficacy of hciPSC-CMs sheet for myocardial repair was evaluated in immunodeficient mice that had undergone surgically induced MI. Animals in the hciPSC-CM group (n=11) were treated by transplanting sheets of hciPSC-CMs (Supplemental Video) over the infarcted region, and animals in the MI group (n=9) recovered without the hciPSC-CM sheet (MI, n=9); a third, age-matched group of uninjured animals (NORMAL, n=5) was included for assessments of cardiac function. Because the 9 lines of hciPSC-CMs (i.e., 3 lines for each sample of cardiac fibroblasts that had been obtained from each patient) were similar, all animals in the hciPSC-CM group were treated with sheets derived from the same hciPSC clone.

Engrafted CMs were identified by staining sections of cardiac tissue from animals in the hciPSC-CM group for expression of the human-specific cTnT isoform (Figure 4F) or human-specific nuclear antigen (HNA, Figure 4C) \(^{24}\), and apoptosis was evaluated via TUNEL staining. Ample human cTnT gene expression was observed on day 1 after transplantation (Figure 4A and 4C), and apoptotic cells were 45% more common in myocardium of the MI group than in myocardium from under the cell sheet in animals from the hciPSC-CM group (p<0.05; Figure
The transplanted cell sheet appeared to protect native myocardial cells from the apoptotic response to ischemic injury. On day 28 (Figure 4C and 4F), the number of hcTNT$^+$ and HNA$^+$ cells, respectively, indicated that 31.91±5.75% (n=3) of the cells administered remained present in the recipient heart, which is substantially higher than has been achieved in any other investigation of CM therapy for the treatment of ischemic myocardial injury. Furthermore, very few apoptotic nuclei were observed in the transplanted cell sheet (Figure 4D), and CM in the cell-sheets had become structurally organized with aligned myocytes (Figure 4A lower panel). Sections stained for expression of the endothelial-lineage marker CD31 indicated that the hciPSC-CM sheets were also well vascularized (Figure 4E), but none of the vessels expressed HNA, which suggests that they evolved through the spouting of pre-existing vessels. Vascular density was also significantly higher in sections from the border zone of the infarct in animals from the hciPSC-CM group than in the corresponding regions of hearts from MI animals (Figure 5A), and these improvements were accompanied by significantly greater measurements of left-ventricular ejection fraction (EF) and fractional shortening (FS) in the hciPSC-CM group than in MI animals on day 28 (Figure 5B).

The engrafted hciPSC-CM sheet was also identified by staining of human-specific cTnT antibody (hcTnT). The Abcam hcTnT antibody is specific for the human isoform of cTnT only (Figure 4Fb), while the antibody from Thermo Scientific (cTnT) is not human-specific (Figure 4F-a). Thus, the transplanted hciPSC-CMs appear red when hcTnT staining is evaluated alone (Figure 4F-b), while both the transplanted cells and the native (murine) cardiomyocytes appear green when cTnT staining is evaluated alone (Figure 4Fa). When the two images are overlaid, the hciPSC-CMs appear yellow (Figure 4F-d), while the native cardiomyocytes appear green.
Thus, the appearance of yellow regions in the overlaid images demonstrated the transplanted hciPSC-CM sheet (Figure 4F and 4Fd).

Discussion

Myocyte transplantation could be a promising therapy for patients with heart failure. The development of iPSC technology makes possible a virtually unlimited cell source for lineage-specific regenerative therapies. However, recent reports suggest that iPSCs retain some of the epigenetic characteristics of their source tissues, and that this epigenetic memory can influence both iPSC differentiation and the subsequent function of iPSC-derived cells. Thus, hciPSC-derived CMs may be more effective for myocardial recovery if the iPSCs were reprogrammed from cardiac-, rather than noncardiac-lineage cells. Here, we show that human iPSCs can be successfully generated from cardiac fibroblasts and the hciPSCs can be differentiated into CMs with >92% efficiency without any selection procedures. hciPSC-CMs have a more cardiac-like Ca\(^{2+}\) handling profile compared to CMs derived from hUCBiPSC or hdiPSCs when comparing the different hiPS cell lines (Figure 3). Using the hciPSC-CM sheet transplantation, the engraftment rate exceeds 30% at day 28 post transplantation in hearts of immunosuppressed mice with experimentally induced MI.

We have previously reported that CMs differentiation efficiency prior selection could reach up to 60-85% from hiPSC lines that derived from skin fibroblasts or cord blood mononuclear cells. In the present study, the greater than 92% hciPSC-CM differentiation efficiency likely reflects the epigenetic memory of the cardiac origin of fibroblast. The exact mechanisms of the epigenetic memory warrant future studies.
Because of the overall low engraftment rate in previous preclinical studies using CMs derived from either hESC or hiPSC and because of the high efficiency CM differentiation protocol, in the present investigation we used a method in which a CM sheet delivered the whole cell population on the surface of MI. We reasoned that the hciPSC-CMs may survive better in the host if their microenvironment of cell culture was minimally disrupted. The exceptionally high rate of CM retention was surprising, and is likely evolved from a few factors. We used a fibrin gel to position the undisturbed hciPSC-CM sheet on the surface of the injured myocardium, which has two important improvements: a) the external sheet avoids CMs facing the cyclic systolic pressure which squeezes CM out of myocardial tissue, and b) the transfer of the CM-sheet maintains cell-cell and cell-matrix interfaces, as opposed to being harvested from the cell culture environment. These two factors together likely resulted in the much higher retention rate of transplanted cells administered as a sheet vs. directly injected into the myocardium.

Maturation of CMs-hciPSC is remarkable in the \textit{in vivo} system after engraftment. Our results also indicate that although the differentiating hciPSC-CMs passed through the typical mesodermal and cardiac mesodermal stages of differentiation before assuming a CM identity, the cells remained relatively unstructured and, by extension, immature after they had been beating for many days (Figure 2F), which is consistent with previous reports that grafts containing neonatal, but not adult, CMs survive transplantation into rat hearts, and that immature CMs can continue proliferating and maturing after transplantation (Figure 4A)\textsuperscript{27,28}. Furthermore, host-derived blood vessels were prevalent throughout the transplanted CM cell sheet (Figure 4E) at week 4 after transplantation, which is an important necessity for the survival of the cell sheet. It should also be noted that unlike a previous, disease-modeling study\textsuperscript{19}, the hciPSCs used in this
report were genetically normal, because they were created from the cells of a patient whose heart failure could not be attributed to a genetic cause. By comparing CM-Sheet at day 1 vs. day 28 post transplantation (Figure 4A), it is also very interesting to note that the in vivo environment promotes myocyte maturation. The exact in vivo cues that led to this remarkable difference in CM maturation remains to be deciphered in future studies.

The LV contractile function was significantly improved in hearts that received CM-sheet transplantation (Figure 5B), which is likely secondary to a combination of the reduction of apoptosis (Figure 4B), increased vascular density (Figure 5A), decreased LV diastolic dilatation and improved wall stress \(^{29}\). Studies using the large animal model are warranted to examine the mechanisms that lead to the LV functional improvement. The hciPSC will be very useful and applicable approach for the cardiac regenerative therapy as the cardiac biopsies are easily assessable in the interventional catheter laboratory.

Although the Ca\(^{2+}\) transient results clearly indicate that hciPSC-CMs have a more cardiac-like Ca\(^{2+}\) handling profile compared to CMs derived from hUCBiPSC or hdiPSCs (Figure 3), the study used passage 16-40 of hiPS cell lines that are relatively young pluripotent cell lines. Whether the iPS cell lines that are beyond ~ 100 passages, remain the epigenetic memory warrants future studies.

In conclusion, the results presented here demonstrate that cardiac fibroblasts can be reprogrammed to generate hciPSCs, and that the hciPSCs can subsequently be differentiated into CMs with a remarkable efficiency, suggestive of an epigenetic memory. hciPSC-CM maturation
was significantly advanced in vivo after engraftment as oppose to in vitro conditions.

Transplantation hciPSC-CM sheet into hearts with myocardial injury results in significant improvement in cell engraftment rate. Collectively, these observations strongly support the potential of hciPSC-CM sheet transplantation for the treatment of heart failure. Additional investigations are needed to identify the optimal methods for the controlled differentiation of iPS cells to cardiac precursor cells and cell delivery.

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Disclosures

None.
References


Table. Antibodies for flow cytometry and immunofluorescence analyses

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**Figure Legends**

**Figure 1. Generation and characterization of human cardiac induced-pluripotent stem cells (hciPSCs)**  (A) Cardiac fibroblasts were obtained from the hearts of patients who were undergoing open-chest surgery and transduced with Sendai viruses for Klf4, Oct3/4, Sox2, and c-Myc (KOSM) to generate hciPSCs. (B) Before transduction, the isolated fibroblasts were evaluated for expression of the fibroblast-specific marker vimentin (bottom panel) and of isotype-specific controls (upper panel) via flow-cytometry. NC=Negative control. CF=Cardiac fibroblast. (C-D) Three weeks after transduction, (C) putative hciPSCs were identified via live immunofluorescent staining for the pluripotency marker Tra1-60 and via morphological assessments of phase-contrast images; then, (D) karyotype analyses were performed to confirm that the chromosomal number and structure of the selected cells were normal. (E) Expression of the pluripotency markers Tra1-60, SSEA4, and Oct4 was evaluated in hciPSCs, and vimentin expression was evaluated in the surrounding feeder cells, via immunofluorescence; nuclei were counterstained with DAPI. (F) mRNA levels of the iPSC markers Oct3/4, Sox2, and Nanog were evaluated in hciPSCs, in a commercially available line of iPSCs (PCBC 16iPSC), and in un-transduced cardiac fibroblasts (CF); measurements were performed via qRT-PCR and normalized to GAPDH. Pluripotent genes were undetectable from CF. Statistics analysis shown are between ciPSC and PCBC 16iPSC only. (G) hciPSCs were subcutaneously transplanted into immunodeficient mice. Eight weeks later, the teratoma was excised, sectioned, and H&E-stained; then, pluripotency of the implanted hciPSCs was verified by identifying all three developmental germ layers: endoderm (i.e., glandular and duct cells), mesoderm (i.e., cartilage and bone cells), and ectoderm (i.e., pigmented and neuronal cells).
**Figure 2. Induced differentiation of hciPSCs into cardiomyocytes (hciPSC-CMs)**

(A) hciPSCs were differentiated into hciPSC-CMs via the Matrigel sandwich method. (B-D) mRNA levels of (B) the pluripotency genes Nanog, Oct3/4, and Sox2; (C) the early mesoderm marker Brachyury and the early cardiac-mesoderm marker Mesp1; and (D) the cardiac-cell markers Nkx2.5, Gata4, Mef2C, and Tbx5 were evaluated before differentiation was induced, after the differentiation medium was replaced on Day 5 (D-Day 5), and on Day 1, Day 10, and Day 15 after beating was observed (B-Day 1, B-Day 10, and B-Day 15, respectively); measurements were performed via qRT-PCR and normalized to GAPDH (**P<0.01). (E) On Day 10 after beating was observed, the proportion of differentiated hciPSC-CMs that expressed cardiac troponin T (cTnT) was determined via flow cytometry. (F) cASA, cTnT and the gap-junction protein Connexin 43 were visualized in cells that had been stained with fluorescent anti-cTnT (green) and fluorescent anti-Connexin 43 (red-orange, identified with arrows) antibodies.

**Figure 3. Ca\(^{2+}\) Transients in iPSC-CMs.** iPSC-CMs derived from hUCBiPSC, hdiPSC, and hciPSC were cultured on Matrigel-coated coverslips for 24 hours. iPSC-CMs were then loaded with 1 μmol/L fura-2 in Tyrode’s buffer (140 mmol/L NaCl, 10 mmol/L glucose, 10 mmol/L HEPES, 4 mmol/L KCl, 1 mmol/L MgCl\(_2\), pH 7.45) supplemented with 1.2 mmol/L Ca\(^{2+}\) for 20 minutes at 37°C. To measure Ca\(^{2+}\) transients, changes in fura2 ratio (340/380nm) were recorded using the Fluorescence and Contractility System (IonOptix). Data were collected from 6 clusters of iPSCs from each group. (A) Calcium transients in hciPSC-CMs (dark grey), hdiPSC-CMs (light grey), and hUCBiPSC-CMs (black) were recorded and used to calculate (B) the rate of Ca\(^{2+}\) increase, (C) the rate of Ca\(^{2+}\) decline, (D) peak Ca\(^{2+}\) levels, and (E) resting Ca\(^{2+}\) levels.
Figure 4. Characterization of transplanted hciPSC-CM sheets  Myocardial infarction was surgically induced in mice; then, animals in the hciPSC-CM group were treated by transplanting sheets of hciPSC-CMs that had been beating for 10 days over the site of myocardial injury, and animals in the MI group recovered without the transplanted hciPSC-CM sheets. (A) Transplanted cells were identified in the hearts of hciPSC-CM animals on Day 1 and Day 28 after injury and treatment by staining cardiac sections with antibodies against the human-specific isoform of cardiac troponin T (hcTnT, green). (B) On Day 1, infarcted native tissue was harvested from MI animals (i.e., at the site of infarction) and from hciPSC-CM animals (i.e., from under the transplanted cell sheet); then, apoptotic cells were identified via the TUNEL assay (red) and CMs were visualized with antibodies against non-human specific cardiac troponin T (cTnT) (green). Apoptosis was quantified by determining the proportion of CMs that were TUNEL positive. (C-E) Cardiac tissue was harvested from the site of hciPSC-CM sheet application in the hearts hciPSC-CM animals on Day 28. Surviving transplanted cells were identified via fluorescent immunostaining for (C) human-specific nuclear antigen (HNA, red) and (D-E) hcTnT (green); (D) apoptotic cells were identified via the TUNEL assay (red); and (E) vascular structures were identified via immunofluorescent staining for expression of the endothelial-cell marker CD31 (red). (F) Sections containing portions of the transplanted cell sheet and the underlying infarcted tissues were harvested from hciPSC-CM animals on Day 28 after injury and treatment. CMs were visualized via fluorescent immunostaining for cTnT (green in panel a, the white arrowheads point the mouse cells in Panel a), and surviving transplanted cells were identified via fluorescent immunostaining for hcTnT (red in panel b). After overlaying the panel a and b, the transplanted cells were shown in yellow (in Panel d). Panel c is DAPI counter staining.
Figure 5. Assessments of cardiac function in animals treated with or without transplanted hPSC-CM sheets after MI injury (A) Sections were harvested from the border-zones (BZ) and remote zones (RZ) of hearts from animals in the MI (top row) and hPSC-CM (middle row) groups on Day 28 and from the myocardium of animals that did not undergo surgically induced MI (i.e., the Normal group; bottom panel); then, the sections were immunofluorescently stained for CD31 expression (green), and vascular density was quantified. (B) Echocardiographic assessments of left-ventricular ejection fraction (EF) and fractional shortening (FS) were performed in animals that did not undergo surgically induced MI (Normal). Statistical analyses were performed to compare between the two groups of MI and MI+CELL at Week 1 and Week 4, respectively.
Fig. 1A

Patient Cardiac Tissue → Cardiac Fibroblast → iPSC

KOSM transduction

1B

NC

CF

1C

Tra1-60-GFP

Phase-contrast

1D

Vimentin-FITC
Fig. 1E

Overlay shows the expression of Tra1-60, SSEA4, Oct4, and Vimentin in iPSCs. The mRNA levels of OCT3/4, SOX2, and Nanog are normalized and compared between different cell lines: CF (n=4), ciPSC (n=4), and PCBC 16iPSC (n=4). The p-values for OCT3/4 are p=0.31, p=0.61, and p=0.83, respectively.
Teratoma

Endoderm

Gland (200x)

Duct (100x)

Mesoderm

Cartilage (200x)

Bone (200x)

Ectoderm

Pigmented cells (200x)

Neuronal cells (200x)
Fig. 2A

Time
-4 -1 0 1 4 5 10 15

Single cell seeding in mTeSR1 with RI on Matrigel
Matrigel overlay
BMP4
bFGF
ActivinA
RPMI/B27 without insulin
RPMI/B27 with insulin

(Adapted from Zhang J. et al 2012 Circ. Res. 11,1125–1136)

2B

mRNA levels (normalized)

Before D-Day5 B-Day1 B-Day10 B-Day15

Nanog Oct3/4 Sox2
2E

Isotype control                               cTnT

2F

cTNT-FITC

Connexin43

DAPI

cTnT

DAPI

Alpha Sarcomeric Actinin

DAPI

cTNT

DAPI

Connexin43

DAPI
Fig. 3

3A

![Graph showing Fura2 Ratio (340/380) over time (sec) for hciPSC-CMs, hdiPSC-CMs, and hUCBiPSC-CMs.](image)

3B

![Bar graph showing Rate of Ca^2+ Rise (Fura2 Ratio/sec) for hUCBiPSC-CMs, hdiPSC-CMs, and hciPSC-CMs with p-values: p=0.012 for hUCBiPSC-CMs, p=0.012 for hdiPSC-CMs, and p=0.63 for hciPSC-CMs.](image)

3C

![Bar graph showing Rate of Ca^2+ Decline (Fura2 Ratio/sec) for hUCBiPSC-CMs, hdiPSC-CMs, and hciPSC-CMs with p-values: p=0.165 for hUCBiPSC-CMs, p=0.34 for hdiPSC-CMs, and p=0.75 for hciPSC-CMs.](image)

3D

![Bar graph showing Peak Ca^2+ (Fura2 Ratio) for hUCBiPSC-CMs, hdiPSC-CMs, and hciPSC-CMs with p-values: p=0.012 for hUCBiPSC-CMs, p=0.012 for hdiPSC-CMs, and p=0.60 for hciPSC-CMs.](image)

3E

![Bar graph showing Resting Ca^2+ (Fura2 Ratio) for hUCBiPSC-CMs, hdiPSC-CMs, and hciPSC-CMs with p-values: p=0.012 for hUCBiPSC-CMs, p=0.012 for hdiPSC-CMs, and p=0.63 for hciPSC-CMs.](image)

n=4, each bar
Fig. 4A

Day 1

hcTnT

DAPI

Merged

Day 28

hcTnT

DAPI

Merged
4B

TUNEL

DAPI

cTnT

Merged

MI

MI+Cell

TUNEL/DAPI (%) p<0.001

p<0.001

TUNEL/DAPI (%)

MI (n=8) MI+Cell (n=8)
Fig. 5A

MI-BZ
CD31

MI-RZ
CD31

MI+Cell-BZ
CD31

MI+Cell-RZ
CD31

Normal
CD31

Vascular density (per mm²)

P < 0.001
P = 0.57

(n=8)
(n=4)

MI (n=8)
MI+CELL (n=8)
Normal (n=4)
Fig. 5B

- EF (%)
  - Week 1: MI (p=0.90), MI+CELL (p=0.011), Normal (p=0.90)
  - Week 4: MI (p=0.011), MI+CELL (p=0.90), Normal

- FS (%)
  - Week 1: MI (n=7), MI+CELL (n=7), Normal (n=4)
Derivation and High Engraftment of Patient-Specific Cardiomyocyte-Sheet Using Induced Pluripotent Stem Cells Generated From Adult Cardiac Fibroblast

Liying Zhang, Jing Guo, Pengyuan Zhang, Qiang Xiong, Steven C. Wu, Lily Xia, Samit Sunny Roy, Jakub Tolar, Timothy D. O'Connell, Michael Kyba, Kenneth Liao and Jianyi Zhang

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ONLINE SUPPLEMENTAL MATERIALS

Supplemental Methods:

Contractile activity and cardiac-marker expression are greater when cardiomyocytes are differentiated from hciPSCs than from hdiPSCs or hUCBiPSCs.

To determine whether the differentiation of induced-pluripotent cells (iPSCs) into cardiomyocytes may be influenced by epigenetic factors that the iPSCs retain from their tissues of origin, we evaluated the proportion of cell clusters that were beating and the proportion that expressed both cardiac troponin T (cTnT) and cardiac alpha sarcomeric actinin (cASA) (Table S1) on day 20 after beating was first observed. Assessments were performed with the hciPSCs generated for this investigation and two other established iPSC lines: one generated from human dermal fibroblasts (hdiPSCs; i.e., the PCBC16 iPSC line) and one from human umbilical cord blood cells (hUCBiPSCs). The cells were cultured and differentiated as described in the Materials and Methods, beating was monitored and recorded under a microscope, and co-expression of cTnT and cASA was quantified via flow cytometry (Figure S1). The proportion of beating clusters was at least 90% for all hciPSC-CM batches tested, but fell to as low as 30% and 20% for hdiPSC-CMs and hUCBiPSC-CMs, respectively. Batches of hciPSC-CMs also had consistently higher proportions of cTnT/cASA double-positive cells.
Supplemental Tables

**Table S1** Differentiation comparison of different iPSC cell lines

<table>
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<th>Beating Clusters (%)</th>
<th>cTnT⁺/cASA⁺ Cells (%)</th>
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hciPSC: human cardiac induced pluripotent stem cells; hdiPSC: human dermal induced pluripotent stem cells; hUCBiPSC: human umbilical cord blood induced-pluripotent stem cells. P-value: the t-test calculated P-values when we compared hciPSC with either hdiPSC or UCBiPSC.

Supplemental Figures and Figure Legends:

**Figure S1.** Expression of cTnT and cASA in cardiomyocytes differentiated from hciPSCs, hdiPSCs, and hUCBiPSCs. Twenty days after beating was first observed, cells were trypsinized, resuspended as single cells, fixed with 4% paraformaldehyde at room temperature for 20 minutes, permeabilized in 0.1% Triton X-100 at 4 °C for 20 minutes, blocked with UltraV block (Fisher Scientific) at room temperature for 7 minutes, and incubated with primary anti-cASA and anti-cTnT antibodies or isotype control antibodies at 4 °C for overnight; then, the cells were washed, stained with fluorescent secondary antibodies, washed again, re-suspended in 2% FBS/PBS containing 5 µL of propidium iodide (10 µg/mL), and analyzed with a FACS Aria instrument (BD Biosciences).
Supplemental References

Supplemental Video Legend:

Supplemental Video. The spontaneous beating of hciPSC-CM was observed on day 8 after induction.