ORIGINAL ARTICLE

Controlled Release of Small Molecules for Cardiac Differentiation of Pluripotent Stem Cells

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Induced pluripotent stem cells (iPSCs) have been shown to differentiate to functional cardiomyocytes (CM) with high efficiency through temporally controlled inhibition of the GSK3/Wnt signaling pathways. In this study, we investigated the ability of temporally controlled release of GSK3/Wnt small-molecule inhibitors to drive cardiac differentiation of iPSC without manual intervention. Porous silica particles were loaded with GSK3 inhibitor CHIR99021 or Wnt inhibitor IWP2, and the particles containing IWP2 were coated with 5 wt% poly(lactic-co-glycolic acid) 50:50 to delay release by ~72 h. iPSCs reprogrammed through mRNA transfection were cultured with these particles up to 30 days. High-performance liquid chromatography suggests a burst release of CHIR99021 within the first 24 h and a delayed release of IWP2 after 72 h. Annexin V/propidium iodide staining did not show a significant effect on apoptosis or necrosis rates. Cultured cells upregulated both early (*Nkx 2.5*, *Isl-1*) and late (*cTnT*, *MHC*, *Cx43*) cardiac markers, assayed with a quantitative real-time polymerase chain reaction, and began spontaneous contraction at 3.0 ± 0.6 Hz at 15–21 days after the start of differentiation. CM had clear sarcomeric striations when stained for β -myosin heavy chain, and showed expression and punctate membrane localization of gap junction protein Connexin43. Calcium and voltage-sensitive imaging showed both action potential and calcium transients typical of immature CM. This study showed that the cardiac differentiation of pluripotent stem cells can be directed by porous silica vectors with temporally controlled release of small-molecule inhibitors. These results suggest methods for automating and eliminating variability in manual maintenance of inhibitor concentrations in the differentiation of pluripotent stem cells to CM.

Keywords: cardiac differentiation, iPS cells, controlled release

Introduction

CURRENT CARDIAC TISSUE engineering strategies are limited by a functional cell source.¹ Cardiomyocytes (CM) are terminally differentiated cells that do not proliferate.² Therefore, damaged cardiac tissue due to disease or injury cannot repair itself, leading to arrhythmias and heart failure.³

Induced pluripotent stem cells (iPSC) represent a renewable and scalable cell source for cardiac tissue engineering applications.⁴ Small-molecule inhibition of the GSK3/Wnt inhibitory pathways in iPSC resulted in cardiac differentiation efficiencies of up to 98%.⁵

Current procedures for differentiating iPSC into CM involve manual media changes and inhibitor addition. A

method of limiting handling interactions would be ideal for clinical translation of iPSC to cardiac cells. Our ultimate goal is to create a self-differentiating material that requires minimal handling interactions. In this study, we combined iPSC with particles that regulated the release of inhibitors of the GSK3 and Wnt pathways.

Porous silica particles (pSi) were tailored to release small-molecule inhibitors at desired timepoints. Drugs loaded into pSi alone without any coatings are released in a burst release, with all the drugs dispersed within 24 h.⁶ pSi have a high loading efficiency, retain drug bioactivity, and can be coated with biocompatible polymers.⁷ Polymers such as poly(lactic-co-glycolic acid) (PLGA) have been used widely in drug delivery platforms because of their tunable release

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kinetics based on co-polymer ratios and exposure of the hydrolysable chemical backbones.^{8–10} pSi have been shown to load and deliver hydrophobic small molecules for therapeutic purposes.^{11,12}

Amniotic fluid-derived stem cells (AFSC) have been widely researched for treatment of congenital heart diseases^{13–15}; however, AFSC cannot directly differentiate into functional beating CM.¹³ Our laboratory has previously reprogrammed AFSC by modified mRNA transfection and subsequently differentiated AFSC-iPSC into functional beating cardiac cells.¹⁶ Using small-molecule-loaded pSi, AFSC-iPSC could effectively differentiate into cardiac cells. The temporal exposure to the GSK3/Wnt inhibitors was shown to be critical for efficient cardiac differentiation of iPSCs,⁴ with GSK3 inhibition at day 0 and Wnt inhibition at day 3 of differentiation. Therefore, our pSi differentiation platform had two formulations of particles aiming at mimicking this inhibitory cascade, with the GSK3 inhibitor loaded within pSi particles alone and the Wnt inhibitor loaded into pSi and further coated with PLGA to delay release kinetics. Differentiation was evaluated *in vitro* for cardiac differentiation. Cells were cultured in transwells to maintain consistent inhibitor exposure released from particles as well as to maintain cellular waste/nutrient exchange. Differences between cardiac cells derived from direct exposure to GSK3/Wnt inhibitors and those exposed to particles containing the same inhibitors were evaluated through differentiation efficiency, phenotypic/genotypic analysis, and electrophysiology.

Methods

Cell source

Cells were isolated from second-trimester human amniotic fluid and reprogrammed to iPSC through mRNA transfection as previously reported.¹⁴ Pluripotency was assessed through RNA expression of pluripotency markers OCT4, nanog, and Tra-1-81 with teratoma formation in a nude mouse as previously reported.¹⁶ Reprogrammed AFSC were then passaged onto a mouse embryonic fibroblast (MTI-Globalstem, Gaithersburg, MD) feeder layer (1.85×10^4 cells/cm²) for further expansion and maintained in knockout serum replacement iPSC media (Dulbecco's Modified Eagle's media/F12, 20% knockout serum replacement, 1% non-essential amino acids, 1% Pen/Strep, 0.1% beta-mercaptoethanol, 4 ng/mL basic fibroblast growth factor; Thermo Fisher Scientific, Waltham, MA). At 60–70% confluency, colonies were passaged to new mouse embryonic fibroblast feeder layers, every 7–10 days. All studies of primary human cells were approved by the Institutional Review Boards of both Baylor College of Medicine and Rice University, and subjects gave informed consent.

pSi synthesis

A tannic acid template and the Stöber process were used to synthesis pSi as per previous studies.¹⁷ Briefly, 272 mg tannic acid (Sigma Aldrich, St. Louis, MO) was dissolved in 50 mL ethanol while continuously stirring. Then, 25 mL ammonium hydroxide (Sigma Aldrich) was added and stirred for 1 min. Tetraethyl orthosilicate (300 μ L; Sigma Aldrich) was added to the mixture dropwise and stirred continuously for 3 h. The resulting particles were centrifuged and washed in 1:1 ethanol:water for a total of six

washings. The particles were then resuspended in 15 mL of deionized water and filtered twice through 40 μ m centrifuge tube filters. Particles were centrifuged again; the water was removed and then resuspended in 1 mL isopropyl alcohol. The particles were then dried overnight in a vacuum oven at 60°C and –36 Pa until dry. Once dry, 4 mg of pSi was rehydrated in 0.5 mL inhibitor loading solutions, consisting of CHIR99021 (Tocris bioscience, Avonmouth, Bristol, United Kingdom) or IWP2 (Tocris bioscience) re-suspended in dimethyl sulfoxide at 600 μ g/mL for 20 min at 37°C with constant mixing. The particles were then washed and lyophilized for further use.

PLGA encapsulation of pSi

Particles loaded with IWP2 were further encapsulated with a PLGA coating to delay the release kinetics by using a Solid-in-Oil-in-Water (S/O/W) method. An oil solution consisting of dissolved 5 wt% 50:50 PLGA (Lactel Absorbable Polymers, Pelham, AL) in dichloromethane was homogenized with the prepared loaded pSi. The solid in oil emulsion was then added dropwise to a water solution consisting of 2.5% polyvinyl alcohol. The solution was allowed to stir continuously for 6 h, washed, and lyophilized until ready for use.

Particle characterization

Dynamic light scattering (DLS) and Z-potential of pSi were performed by using a Zetasizer ZEN3600 (Malvern, Worcestershire, United Kingdom). Moreover, samples were prepared for a scanning electron microscope (SEM) by drying overnight on a stage and sputter coating with a 5 nm thick layer of Pt/Pi. SEM images were taken by using Nova NanoSEM 230. PLGA-pSi and pSi size distribution was also measured with the software ImageJ (NIH Image). Transmission electron microscope samples were prepared by drying nanoparticles onto 300 mesh carbon-coated copper grids and then analyzed. Fluorescein isothiocyanate (FITC)-labeled pSi has been used to verify the presence of the silica inside the PLGA shell by confocal microscopy.

Inhibitor loading efficiency and release studies

All supernatants after initial loading of inhibitors were stored at –20°C. Supernatants were analyzed by reverse-phase high-performance liquid chromatography (HPLC). Inhibitor release studies were done by sampling particle suspension inhibitor release up to 6 days. Briefly, drug release solution was prepared by using 1 mL of 1 $\times$ phosphate-buffered saline (PBS) and 1 mL of 1% bovine serum albumin. The lyophilized pSi were suspended at a concentration of 3 mg/mL of drug release solution and incubated at 37°C with constant stirring. At 1, 3, 5, 12, and every 24 h thereafter, suspensions were centrifuged and 1 mL drug release solution was taken and replaced with fresh drug release solution. Agilent Zorbax Eclipse Plus C18 (100 $\times$ 4.6 mm, 3.5 μ m pore particle size; Agilent Technologies, Santa Clara, CA) column was used for analysis. Samples flowed through the column at 1.0 mL/min and with a mobile phase consisting of acetonitrile in 0.1% trifluoroacetic acid: H₂O in 0.1% trifluoroacetic acid (V/V). Samples were analyzed at 281 nm.

Cellular toxicity to particles

pSi without any inhibitors loaded were added to iPSC cultures at a density of 2.5 ng/cell to assess apoptosis and necrosis. Annexin V and propidium iodide staining were used to quantify apoptosis/necrosis. Cells were lifted at 0 and 72 h, washed, and double stained with Annexin V-FITC and propidium iodide (Affymetrix ebioscience, Santa Clara, CA). The cells were then analyzed through flow cytometry (BD Acurri C6 Plus; BD biosciences, San Jose, CA). To assess the effect of transwell culture on cell survival, live/dead staining was done according to the manufacturer's protocol (Thermo Fisher). Cells were imaged by using fluorescent microscopy.

Differentiation of iPSC using dissolved GSK3/Wnt inhibitors

By adapting previously published protocols,⁵ AFSC-iPSC were differentiated into cardiac cells by temporally inhibiting the GSK3 and Wnt signaling pathways over a span of 5–7 days. On reaching ~70% confluency, undifferentiated colonies were dissociated in collagenase type 2 (Worthington Biochemical Corp., Lakewood, NJ) for 5 min, then manually dislocated from the feeder layer, dispersed into single-cell suspension, and finally plated as a monolayer of cells onto Matrigel (BD Biosciences) at 260,000 cells/cm². Cells were expanded for 3 days in mTeSR1 media (STEMCELL Technologies, Cambridge, MA). Medium was then changed to RPMI 1640 (Thermo Fisher) and the GSK3 inhibitor, CHIR99021, was added at a concentration of 6 μ M, representing differentiation day 0. After 24 h, medium was replaced with fresh RPMI 1640. At day 3, the Wnt inhibitor, IWP2, was added to RPMI/B27 without insulin at a concentration of 2.5 μ M. At day 7, insulin and ascorbic acid were added to the RPMI 1640 media. The occurrence of beating colonies was monitored through phase-contrast microscopy after day 7.

Differentiation of iPSC using pSi-released inhibitors

To test the effectiveness of inhibitor release from loaded pSi, dissociated iPSC were plated at 260,000 cells/cm² onto Matrigel-coated 12-well polyethylene terephthalate ThinCert cell culture inserts (Greiner Bio-One, Monroe, NC) with 0.4 μ m pore size (Fig. 3A). This dosing for pSi differentiation was calculated based on the two-dimensional (2D) differentiation with dissolved inhibitors. The calculated dosages for the 2D differentiation with dissolved inhibitors are ~67.5 μ g CHIR99021 and 25 μ g IWP2 per million cells, and, therefore, representative amounts of both particle formulations were used based on calculated loading efficiencies: ~1.5 mg CHIR99021-loaded pSi and 4.0 mg IWP2-loaded PLGA-pSi were suspended in 24 mL of RPMI 1640. At day 0 of differentiation, 1 mL of particle suspension was added to each seeded transwell and an additional 1 mL RPMI 1640 surrounded the insert. Every 48 h, 1 mL of fresh RPMI1640 replaced the media surrounding the insert. After day 8, insulin and ascorbic acid were added to the RPMI 1640 and 1 mL of media was added to each of the inserts and surroundings.

Flow cytometry

Cells were detached at day 12 into suspension with Accutase (ThermoFisher) and stained with a fluorescently

conjugated antibody for cardiac troponin T (BD Biosciences) at a dilution of 1:100. BD Acurri C6 Plus software (BD Biosciences) was used for all flow cytometry data collection. FlowJo software (Tree Star, Inc., Ashland, OR) was used for data analysis.

Gene expression analysis

Gene expression was quantified by using a quantitative real-time polymerase chain reaction (qRT-PCR). To assess the upregulation of early- and late-stage cardiac markers, cell samples were collected at 0, 1, 5, 10, and 20 days after the start of differentiation. At each timepoint, RNA samples were collected by using an RNA collection kit following the manufacturer's protocol (Affymetrix, Santa Clara, CA). mRNA samples were then reverse transcribed to DNA by using a cDNA kit following the manufacturer's protocol (Applied Biosystems, Foster City, CA). The resulting DNA samples were analyzed with qRT-PCR by using a proprietary assay system following the manufacturer's protocol (Affymetrix). All samples were assayed for expression of *Oct4*, *Isl1*, *Nkx2.5*, *cTnT*, *connexin 43*, *myosin heavy chain*, and *GAPDH* as a house keeping gene by using DNA primers (Thermo Fisher). The expression of each gene was first normalized to the level of *GAPDH*. Relative fold changes were determined by calculating $\Delta\Delta C_t$ compared with day (–3) or day 0. Biological triplicates for each group were assessed, and results were reported as mean $\pm$ standard deviation.

Immunofluorescence

Cell cultures were fixed in 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA) at 4°C for 20 min. Fixed cells were permeated with Triton X100 (Sigma-Aldrich) for 5 min at room temperature. Next, cells were incubated with specific antibodies (Abcam, Cambridge, United Kingdom) for cardiac markers myosin heavy chain and connexin 43, then in DyLight-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Cells were imaged by using an epifluorescence microscope (DMI 6000B; Lieca Microsystems, Wetzlar, DE).

Calcium handling and membrane voltage potential

The calcium handling of spontaneously contracting cells was measured by imaging calcium-sensitive dye Indo-1 AM (Thermo Fisher) with an epifluorescence microscope (Olympus, Center Valley, PA) and a photomultiplier tube detection system and software (Ion Optix, Westwood, MA). Cells were washed with PBS at 37°C, replaced with Tyrode's buffer containing 2 μ L of 2 mM Indo-1 AM, and allowed to incubate for 30 min at room temperature. The cells were then washed three times with fresh Tyrode's buffer and analyzed by using the Ion Optix system. The measured field of view was ~50 μ m $\times$ 50 μ m in size. Detection wavelengths were recorded at 405 and 485 nm. Membrane voltage potential utilized the same procedure as calcium measurements but with voltage-sensitive dye Di-8-ANEPPS (Thermo). Detection wavelengths were recorded at 560 and 620 nm.

Peak shortening percentage analysis

Cells were recorded on spontaneous contraction from day 20 to 30 by using phase-contrast time-lapse video

microscopy (Nikon Eclipse TE300, Melville, NY). Images were taken every 100 ms with a CoolSNAP HQ2 CCD camera (Photometrics, Tuscon, AZ) and analyzed with ImageJ. Minimum representative rectangles were used to outline individual contracting cells, and the lengths of the smallest and largest rectangles were used to calculate the peak shortening percentage.

Western blot

Western blot antibodies were purchased from Abcam, Inc.; electrophoresis, transfer blots, and enhanced chemoluminescence (ECL) developing materials were purchased from Bio-Rad (Hercules, CA). Total protein lysates were collected from cells at 30 days of differentiation. Total protein concentration was quantified by a bicinchoninic acid kit (Thermo Scientific, Rockford, IL). Samples were normalized based on total protein concentration and run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels according to the manufacturer's protocols. Proteins were transferred to blotting membranes and then incubated overnight at 4°C with mouse monoclonal antibodies against MHC and cTnT (1:100 dilution in 10% milk in Tris Buffered Saline with 0.5% Tween 20 [TBST]) and mouse monoclonal antibodies against GAPDH (1:1000 dilution in 10% milk in TBST). The membranes were washed and incubated with secondary horseradish peroxidase antibodies (1:1000 in 10% milk in TBST) for 1 h at room temperature. The membranes were washed, then immersed in an ECL developing solution, and finally read by using Bio-rad ChemiDoc XRS+Statistics.

Statistical analyses were done in SigmaPlot (Systat Software, Inc., San Jose CA). Data were compared by using one-way analysis of variance followed by Tukey's test; $p < 0.05$ was considered significant. Results were presented as mean $\pm$ standard deviation, with number of samples/trials indicated in captions.

Results

pSi characterization and GSK3 and Wnt inhibitor release from pSi

The PLGA-pSi particles measured $8.24 \pm 3.25 \mu\text{m}$ in diameter, and uncoated pSi particles measured 265 nm per DLS. Zeta potentials for pSi and PLGA-pSi were -13.1 ± 0.42 and -39.6 ± 1.42 , respectively (Fig. 1F). CHIR99021 and IWP2 were loaded into silica particles by direct absorption from a 600 $\mu\text{g/mL}$ solution. HPLC data showed a burst release profile with $81.1 \pm 5.3\%$ of CHIR99021 released within the first 24 h (Fig. 1G). After the initial 24 h, CHIR99021 continued to be released through day 7 with nearly 100% cumulative release at 144 h.

When IWP2 was encapsulated within the pSi and coated with 5 wt% PLGA 50:50, the results showed a cumulative release of $27.8 \pm 7.2\%$ within the first 48 h (Fig. 1). The particles were shown to have $40.2 \pm 9.1\%$ cumulative release at the end of 72 h. After the 144 h, IWP2 was shown to have a cumulative release of $56.4 \pm 7.2\%$.

Cellular toxicity with pSi exposure

Forward scattering of annexin V-labeled cells measured by flow cytometry showed that $27.3 \pm 2.1\%$ of cells internalized uncoated pSi and $3.61 \pm 1.4\%$ of cells internalized PLGA-pSi

after 3 days in culture (Fig. 2A). Uncoated pSi and PLGA-pSi were tested for cellular toxicity through an annexin V and propidium iodide assay (Fig. 2B). After 3 days, annexin V staining of iPSC exposed to pSi loaded with both GSK3 and Wnt inhibitors ($12.5 \pm 1.9\%$) showed no significant difference compared with the cells alone ($10.1 \pm 3.3\%$), suggesting no increase in apoptosis. Propidium iodide staining also showed no significant difference between iPSC exposed to pSi loaded with both inhibitors ($3.1 \pm 1.6\%$) and cells alone ($4.1 \pm 2.5\%$), suggesting no increase in cell necrosis.

Cardiac gene expression in pSi differentiation comparable to dissolved inhibitors

Neither the 2D monolayer culture nor transwell culture showed any significant difference in viability after 3 days in culture. The morphology of differentiating cells in transwells at day 0 showed denser cell localization and less cell spreading compared with 2D monolayer differentiation. During the first 5 days of differentiation, the 2D monolayer differentiation had a phase-dark layer of fibroblastic cells below a layer of spherical phase-bright cells, whereas a phase-bright cell layer was absent in transwell culture. At timepoints greater than 10 days, both groups exhibited a clustered morphology with elongated web-like sheets. Spontaneous beating occurred in all groups at ~ 18 –25 days of differentiation (Supplementary Video S1; Supplementary Data are available online at www.liebertpub.com/tea). Localized beating colonies were observed in all groups and were not synchronously paced.

cTnT expression showed $30.3 \pm 1.9\%$ differentiation efficiency in pSi differentiated cells at day 15. Differentiation efficiency was greater in both 2D and transwell dissolved inhibitor groups with $45.6 \pm 3.5\%$ and $39.3 \pm 5.3\%$, respectively (Fig. 3B).

pSi released inhibitor differentiation expressed markers of cardiac differentiation

Cardiac differentiation was monitored through gene expression of brachyury, early-stage cardiac markers (*nkx2.5* and *isl-1*), and later-stage cardiac markers (*cTnT*, β -MHC, *Cx43*). Pluripotent stem cell marker OCT4 was also monitored throughout the differentiation to quantify remaining pluripotency in the cell population.

Brachyury expression was measured 24 h after the introduction of CHIR99021 in both conditions. The relative expression compared with day (–3) of differentiation showed that all groups had little to no *brachyury* expression at the start of differentiation, but after 24 h all groups showed significant upregulation. The 2D monolayer differentiation and transwell dissolved inhibitors had a fold change increase of 236.3 ± 6.7 , whereas the transwell with inhibitor loaded particles showed a significant fold change increase of 99.3 ± 27.1 (Fig. 4B).

When compared with day (–3), *OCT4* expression was shown to be downregulated over the course of differentiation in all groups (Fig. 4C). At day 10 and 20, *OCT4*-fold expression in all conditions was below 0.06 ± 0.01 and 0.007 ± 0.006 , respectively.

Early-stage cardiac markers, *Nkx2.5* and *Isl-1*, were upregulated throughout the differentiation compared with day 0 (Fig. 4D, E). At each of the timepoints, *Nkx2.5* expression was lower in the transwell groups compared with the 2D monolayer group. When compared with the other timepoints

FIG. 1. pSi characterization. (A) Schematic showing formulation of CHIR99021 and IWP2 loaded pSi. Gray areas represent porous silica, and blue area represents PLGA coatings. (B) Transmission electron microscope image of a single porous silica particle (scale 20 nm). (C) SEM image of pSi (scale 2 μm). (D) SEM image of PLGA-pSi (scale 10 μm). (E) Confocal image of fluorescein isothiocyanate-labeled pSi within PLGA coating (scale 10 μm). (F) Zeta potential and particle diameters. (G) Cumulative release profiles of CHIR99021 and IWP2. (H) Small-molecule loading efficiencies into pSi and PLGA-pSi ($n = 3$). pSi, porous silica particles; SEM, scanning electron microscope; PLGA, poly(lactic-co-glycolic acid). Color images available online at www.liebertpub.com/tea

within groups, day 10 was shown to have significant upregulation of *Nkx2.5* (Fig. 4D).

Cardiac troponin T was shown to be upregulated as early as day 5 in the transwell group with free inhibitors when compared with day 0 (Fig. 4F). However, significant upregulation of *cTnT* was only shown in the transwell with dissolved inhibitors group at day 20 of differentiation. β -MHC was upregulated at day 10 in the 2D monolayer group, whereas later timepoints showed β -MHC to be significantly upregulated across all groups (Fig. 4G). At day 30, the

transwell group with pSi had the highest expression of β -MHC. Gap junction protein *connexin 43* expression was not significantly different compared with day 0 at each time-point in all groups (Fig. 4H).

Early cardiomyocyte characteristics and arrangement

Immunofluorescence staining showed expression of late-stage cardiac markers β -MHC and connexin 43 in pSi differentiation at day 30 (Fig. 5). Western blot analysis

FIG. 2. (A) Internalization of pSi and PLGA-pSi in AFSC-iPSC after 72 h. (B) Apoptosis and necrosis of AFSC-iPSC after 72 h ($n=3$). iPSC, induced pluripotent stem cells; AFSC, amniotic fluid-derived stem cells. Color images available online at www.liebertpub.com/tea

FIG. 3. AFSC-iPSC cardiac differentiation by pSi. (A) Schematic showing transwell differentiation with pSi encapsulated inhibitors. (B) Flow cytometry results showing cardiac differentiation efficiency represented by cTnT expression at day 12 ($n=3$, $p \leq 0.05$ is significant, *significant vs. other groups). (C) Live/Dead and bright-field images of differentiating cells. Color images available online at www.liebertpub.com/tea

FIG. 4. Cardiac marker expressions during differentiation. (A) Timeline of pSi differentiation and cardiac marker analysis. (B) Brachyury expression compared with D(-3). (C) Oct 4 expression compared with D(-3). (D, E) Early markers of cardiac differentiation (Nkx2.5, Isl-1) compared with D0 (F-H). Late markers of cardiac differentiation (cTnT, β-MHC, Cx43) compared with D0 ($n=3$, $p \leq 0.05$ is significant, *significant difference from day 0, #significant difference from day -3). Color images available online at www.liebertpub.com/tea

FIG. 5. Confocal immunofluorescence of differentiated AFSC-iPSC. *Top:* Cells differentiated with pSi particles, *Bottom:* Cells differentiated in 2D with dissolved inhibitors. Actinin (red), Cx43 (green), DAPI (blue) (Scale 20 μm). 2D, two-dimensional; DAPI, 4',6-diamidino-2-phenylindole. Color images available online at www.liebertpub.com/tea

supports the translation of upregulated late-stage cardiac proteins in the pSi differentiation group comparable to the 2D differentiation group (Fig. 6B).

Electrophysiological activity and peak shortening of pSi differentiated cells

Both control and experimental groups had spontaneously beating cells at days 18–21. Calcium handling measurements showed that pSi differentiated cells spontaneously beat at a frequency of 0.31 ± 0.03 Hz (Fig. 6A). Differentiation with dissolved inhibitors resulted in cells with a beating frequency of 0.48 ± 0.05 Hz (Fig. 6A). Contractile cell peak length shortening was not significantly different between dissolved inhibitor and pSi differentiation groups at $5.0 \pm 1.2\%$ and $4.8 \pm 1.0\%$, respectively.

Discussion

In this study, we have shown that GSK3/Wnt inhibitors released from pSi can be used to differentiate iPSC into cardiac cells. Previous studies showed highly efficient cardiac differentiation by temporal exposure to dissolved GSK3/Wnt inhibitors, where direct exposure to the GSK3 inhibitor (CHIR99021) occurs during the first 24 h and subsequent exposure to the Wnt inhibitor (IWP2) occurs between days 3 and 5.^{4,18} Here, we showed that controlling the release of inhibitors by pSi encapsulation could produce spontaneously contracting cardiac cells, minimizing handling interactions.

iPSC did not exhibit cellular apoptosis or necrosis when exposed to pSi. Previous studies have shown that internalization of pSi can induce cell programmed death.^{19,20} Therefore, the preservation of iPSC cellular activity was

FIG. 6. Traces of spontaneously beating cardiac cells. (A) Calcium (Indo-1) and Voltage (Di-8-ANEPPS)-sensitive dye traces for pSi and 2D differentiation at day 30. (B) Western blot analysis of MYH7 and cTnT at day 30 ($n=3$). (C) Averaged peak length shortening percentages at day 30 ($n=3$). (D) Averaged spontaneous beating frequency using transient dye measurement data. (E) Image of beating cardiac cells within transwell (scale 20 μm , Supplementary Video S1). Color images available online at www.liebertpub.com/tea

likely due to minimal particle internalization. Both pSi formulations were internalized in iPSC but did not show a significant difference in apoptosis and necrosis compared with controls. This was likely due to the high metabolic nature of iPSC and lack of cellular waste exchange for 72 h will incite apoptosis.

Inhibitors were able to direct cardiac differentiation after release from pSi. The release profiles from pSi were not binary and, therefore, had a sustained release past day 1 and 5 for GSK3 and Wnt inhibition, respectively. Temporal regulation of these inhibitors has been previously shown to be crucial for efficient cardiac differentiation, but a sustained release produces cardiac cells with less efficiency.²¹ Our data support these findings as we showed an average pSi differentiation efficiency of 30.3%. Calcium handling and membrane potentials show contractility of pSi differentiated cells similar to that of direct inhibitor differentiation. A lower rate of spontaneous contraction in pSi differentiated cells may have resulted from sampling of differing populations of contractile cells or delayed measuring of transient intensities, allowing the cultures to decrease in temperature. Future studies showing the beating frequency at different timepoints after the start of spontaneous beating will help suggest any electrophysical development over time.

Although plating density was the same in all groups, the differentiating cellular morphologies were different. Previous studies have shown that single iPSC can sense micro and nanoscale topographies altering their pluripotency.^{22,23} The rounded and clustered appearance of cells within the transwells was likely due to differences in substrate topography and may have promoted maintenance of pluripotency in a population of cells. After 10 days from the start of differentiation, the morphology between the two groups became more similar, with beating colonies forming in localized areas. In terms of cardiac maturation, the morphology of differentiated cardiac cells did not show a clear pattern of cellular alignment. Although immunofluorescent imaging and western blot analysis confirmed β -MHC and connexin 43 were present in differentiated cells, they ultimately lacked sarcomeric patterning and organization. Continued culture and mechanical/chemical conditioning may be required for maturation.^{24,25}

Genetic expression of cardiac differentiation markers in pSi differentiation showed comparable results to direct inhibitor differentiation. The release of CHIR99021 from uncoated pSi was sufficient in transitioning pluripotent iPSC into the mesendoderm lineage, and further Wnt inhibition by IWP2 loaded pSi upregulated late-stage cardiac markers. Peak expressions of β -MHC and connexin 43 were later in pSi differentiated cells compared with the control. Although the cumulative dosage of pSi released inhibitors was similar to that of direct addition of inhibitors, 6 μ M CHIR99021 at day 0 and 2.5 μ M IWP2 at day 3, the non-binary and sustained release may have delayed cardiac maturation. Alterations to the pSi release kinetics could be altered to further increase cardiac efficacy. The downregulation of OCT4 within all groups of differentiation is important from a translational perspective, signifying reduced risk of pluripotent cells that are able to form teratomas.²⁶

A major limitation of this study is the significantly lower differentiation efficiency compared with that of fibroblast-derived iPSC. Previous studies have shown differentiation efficiency variability based on cell line.^{27,28} This may result

from innate differences in gene expression or epigenetic traits. AFSC-derived iPSC have been previously shown to have a lower differentiation efficiency compared with fibroblast-derived iPSC lines.¹⁶ Therefore, pSi AFSC-iPSC differentiation was not expected to increase differentiation efficiency compared with the dissolved inhibitor control. A high-throughput screening of differing GSK3/Wnt inhibitors should also be investigated in future studies to possibly enhance differentiation capabilities. This could elucidate more efficient signaling inhibitors and, in turn, would require more dosage optimization if used in this pSi delivery platform. This platform used on a different cell line may produce greater differentiation efficiencies with a less heterogeneous population of resulting cells.

Although the burst release of CHIR99021 closely mimicked direct addition of the small molecule at day 0, there were still minimal concentrations over the course of cardiac differentiation. The release of IWP2, on the other hand, mimicked a more delayed and sustained release. Optimization of release parameters can also help to direct a more efficient cardiac differentiation. This could be done by altering the degradation kinetics of the particles in terms of polymer ratio and coating thickness. This would ensure the proper dosage of inhibitors per area of cell. Another limitation to this study is the diffusion of waste products. Transwell culture allowed for the isolation of iPSC and pSi, but elimination of cellular waste was limited to simple diffusion. Further development of this platform would benefit from a bioreactor ensuring sufficient exchange of nutrients.

Conclusion

This study shows that small-molecule inhibitors temporarily released from pSi can direct cardiac differentiation of iPSC. The dual release of GSK3 and Wnt inhibitors from pSi is a simple and effective way to generate spontaneously beating cardiac cells. This self-directing cardiac differentiation platform has potential in future cardiac tissue engineering applications.

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Disclosure Statement

No competing financial interests exist.

References

1. Vunjak-Novakovic, G., Tandon, N., Godier, A., *et al.* Challenges in cardiac tissue engineering. *Tissue Eng Part B Rev* **16**, 169, 2010.
2. Maltsev, V.A., Rohwedel, J., Hescheler, J., and Wobus, A.M. Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. *Mech Dev* **44**, 41, 1993.
3. Julian, D.G., Camm, A.J., Frangin, G., *et al.* Randomised trial of effect of amiodarone on mortality in patients with left-ventricular dysfunction after recent myocardial

- infarction: EMIAT. European Myocardial Infarct Amiodarone Trial Investigators. *Lancet* **349**, 667, 1997.
4. Lian, X., Hsiao, C., Wilson, G., *et al.* Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proc Natl Acad Sci U S A* **109**, E1848, 2012.
 5. Lian, X., Zhang, J., Azarin, S.M., *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ β -catenin signaling under fully defined conditions. *Nat Protoc* **8**, 162, 2013.
 6. Martinez, J.O., Chiappini, C., Ziemys, A., *et al.* Engineering multi-stage nanovectors for controlled degradation and tunable release kinetics. *Biomaterials* **34**, 8469, 2013.
 7. Minardi, S., Pandolfi, L., Taraballi, F., *et al.* PLGA-mesoporous silicon microspheres for the in vivo controlled temporospatial delivery of proteins. *ACS Appl Mater Interfaces* **7**, 16364, 2015.
 8. Hans, M.L., and Lowman, A.M. Biodegradable nanoparticles for drug delivery and targeting. *Curr Opin Solid State Mater Sci* **6**, 319, 2002.
 9. Cheng, J., Teply, B.A., Sherifi, I., *et al.* Formulation of functionalized PLGA-PEG nanoparticles for in vivo targeted drug delivery. *Biomaterials* **28**, 869, 2007.
 10. Cheng, X., Tsao, C., Saul, M., *et al.* Comparison of two nanoparticle formulations for localized delivery of platelet-derived growth factor (PDGF) from aligned collagen fibers. *Pharm Nanotechnol* **1**, 105, 2013.
 11. Li, J., Shu, Y., Hao, T., *et al.* A chitosan-glutathione based injectable hydrogel for suppression of oxidative stress damage in cardiomyocytes. *Biomaterials* **34**, 9071, 2013.
 12. Liu, D., Bimbo, L.M., Mäkilä, E., *et al.* Co-delivery of a hydrophobic small molecule and a hydrophilic peptide by porous silicon nanoparticles. *J Control Release* **170**, 268, 2013.
 13. Connell, J.P., Ruano, R., and Jacot, J.G. Amniotic fluid-derived stem cells demonstrate limited cardiac differentiation following small molecule-based modulation of Wnt signaling pathway. *Biomed Mater* **10**, 034103, 2015.
 14. Gao, Y., Connell, J.P., Wadhwa, L., Ruano, R., and Jacot, J.G. Amniotic fluid-derived stem cells demonstrated cardiogenic potential in indirect co-culture with human cardiac cells. *Ann Biomed Eng* **42**, 2490, 2014.
 15. De Coppi, P., Bartsch, G., Siddiqui, M.M., *et al.* Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* **25**, 100, 2007.
 16. Velasquez-Mao, A.J., Tsao, C.J.M., Monroe, M.N., *et al.* Differentiation of spontaneously contracting cardiomyocytes from non-virally reprogrammed human amniotic fluid stem cells. *PLoS One* **12**, e0177824, 2017.
 17. Parodi, A., Haddix, S.G., Taghipour, N., *et al.* Bromelain surface modification increases the diffusion of silica nanoparticles in the tumor extracellular matrix. *ACS Nano* **8**, 9874, 2014.
 18. Burridge, P.W., Matsa, E., Shukla, P., *et al.* Chemically defined generation of human cardiomyocytes. *Nat Methods* **11**, 855, 2014.
 19. Lee, S., Yun, H.S., and Kim, S.H. The comparative effects of mesoporous silica nanoparticles and colloidal silica on inflammation and apoptosis. *Biomaterials* **32**, 9434, 2011.
 20. Huang, X., Teng, X., Chen, D., Tang, F., and He, J. The effect of the shape of mesoporous silica nanoparticles on cellular uptake and cell function. *Biomaterials* **31**, 438, 2010.
 21. Zhang, M., Schulte, J.S., Heinick, A., *et al.* Universal cardiac induction of human pluripotent stem cells in two and three-dimensional formats: implications for in vitro maturation. *Stem Cells* **33**, 1456, 2015.
 22. Mashinchian, O., Turner, L.A., Dalby, M.J., *et al.* Regulation of stem cell fate by nanomaterial substrates. *Nanomedicine* **10**, 829, 2015.
 23. Reimer, A., Vasilevich, A., Hulshof, F., *et al.* Scalable topographies to support proliferation and Oct4 expression by human induced pluripotent stem cells. *Sci Rep* **6**, 18948, 2016.
 24. Lundy, S.D., Zhu, W.Z., Regnier, M., and Laflamme, M.A. Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Stem Cells Dev* **22**, 1991, 2013.
 25. Kamakura, T., Makiyama, T., Sasaki, K., *et al.* Ultrastructural maturation of human-induced pluripotent stem cell-derived cardiomyocytes in a long-term culture. *Circ J* **77**, 1307, 2013.
 26. Cheng, L., Sung, M.T., Cossu-Rocca, P., *et al.* OCT4: biological functions and clinical applications as a marker of germ cell neoplasia. *J Pathol* **211**, 1, 2007.
 27. Burridge, P.W., Holmström, A., and Wu, J.C. Chemically defined culture and cardiomyocyte differentiation of human pluripotent stem cells. *Curr Protoc Hum Genet* **87**, 21.3.1, 2015.
 28. Zhang, J., Wilson, G.F., Soerens, A.G., *et al.* Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ Res* **104**, e30, 2009.

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