Supplementary Information

Silicon nanowire-induced maturation of cardiomyocytes derived from human induced pluripotent stem cells

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Experimental Details

Electrically conductive silicon nanowire fabrication and harvesting. Single-crystalline SiNWs were synthesized using the nanocluster-catalyzed vapor-liquid-solid method described previously in a quartz tube connected to a gas manifold and vacuum pump and heated by a temperature controlled tube furnace.[1] Monodisperse gold nanoparticles (100 nm, Ted Pella) were dispersed on SiO_2/silicon substrates, which were placed within the central region of the quartz tube reactor. The SiNWs were synthesized at 470-485 ºC using silane (SiH_4) as the silicon reactant source, H_2 as the carrier gas, and phosphine (PH_3, 1000 ppm in H_2) as the n-type dopants. In a typical synthesis of uniform n-type, 100 nm SiNWs, the flow rates of SiH_4, PH_3 and H_2 were 1-2, 2-4 and 60 standard cubic centimetres per minute, respectively, and the total pressure 40 torr. The nanowires were collected from the oxidized silicon substrates by sonication in isopropanol for 1 min followed by centrifugation to obtain SiNWs with an average dimension of 100 nm diameter and 10 μm length. The electrical conductivity of the SiNWs were measured by using four-probe transport measurement.

Cell harvest and culture. Rat-neonatal cardiac cells were isolated from 2-day-old Sprague-Dawley rats by using the neonatal isolation kit (Worthington Biochemical Corporation, Lakewood, NJ). Rat-neonatal cardiac cells and spheroids were cultured in Dulbecco's Modified Eagle Medium (DMEM, 4500 mg/L glucose) (Thermo Scientific, Pittsburgh, PA) containing 10% heat inactivated fetal bovine serum (HI FBS) (Life Technologies, Carlsbad, CA), 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA), and 1% non-essential amino acids (Life Technologies, Carlsbad, CA). hiPSC-derived cardiomyocytes (iCell Cardiomyocytes, Cellular Dynamics International, Madison, WI, USA) were cultured according to the manufacturer's protocol. Briefly, hiPSC-derived cardiomyocytes were plated on 0.1% gelatin coated 6-well plates in iCell Cardiomyocyte Plating Medium (Cellular Dynamics International) at a density of about 3×10^5 to 4.0×10^5 cells/well and incubated at 37 °C in 5% CO_2 for 4 days. Two days after plating, the plating medium was removed and replaced with 4 mL of iCell Cardiomyocytes Maintenance Medium (Cellular Dynamics International). After 4 days of monolayer pre-culture, cells were detached using trypLE Express (Gibco Life Technologies, Grand Island, NY) and prepared for spheroid fabrication.

Spheroid fabrication and electrical stimulation. The agarose hydrogel molds were prepared using commercial master micro-molds from Microtissues, Inc (Providence, RI) as negative replicates to create non-adhesive agarose hydrogels molds containing 35 concave recesses with hemispheric bottoms (800 μm diameter, 800 μm deep) to facilitate the formation of tissue cell spheroids. 330 μL 1% sterile agarose solution was pipetted into the master micro-molds and was then carefully detached after gelation from the master mold and transferred into one well of a 24-well tissue culture plate. The schematic presentation of cell spheroids fabrication is shown in the Fig. 2C. A suspension of rat-neonatal cardiac cells and e-SiNWs in media was prepared at a 1:1 ratio (number of cells/number of SiNWs) with a concentration of 5.0×10^6 cells/mL. Similarly, hiPSC-derived cardiomyocytes were mixed with e-SiNWs in the Maintenance media at a 1:1 ratio (number of cells/number of SiNWs) with a concentration of 3.0×10^6 cells/mL. Approximately 75 μl of the cell/e-SiNW suspension (rat-neonatal cardiac cells, 5.0×10^6 cells/mL; hiPSC-CMs, 3.0×10^6 cells/mL) was pipetted into each agarose mold. After the cells had settled down into the recesses of the mold (10 min), additional media was added (5 mL) and

exchanged every 2 days for the length of the experiment. After 4 days of spheroid culture, an electrical stimulation treatment (C-Pace unit, Ion Optix, Milton, MA 02186) was started for designated groups for 7 days (15 V, 1 Hz, 2 ms). For the long term culture experiment of hiPSC cardiac spheroids, the electrical stimulation treatment was performed for 21 days for the designated groups after the initial 4 days of spheroid culture.

Video and image analysis of beating spheroids. Videos of 6 spheroids from each group were recorded starting after the initial 4 days of spheroid culture using Zen 2011 software (Zeiss, Göttingen, Germany) with capture rate of 14 frames per second. Then the videos were converted to a series of TIFF format pictures by Adobe Premiere (Adobe, San Jose, CA). Threshold edge-detecting in ImageJ software (National Institutes of Health) was used on high contrast spheroid pictures and graphed to realize contraction profiles, from which other quantifiers were calculated (i.e., fractional area change and beats per minute).

Histological and immunofluorescent analysis of spheroids and cells. Freshly collected spheroids (~30-35) were placed onto a pre-labeled tissue base mold and the entire tissue block was covered with OCT. Immediately, the base mold containing spheroids was transferred into pre-cooled ethanol with dry ice to ensure that the spheroids were frozen completely. By using the cryotome, the frozen spheroids block were sectioned into 7 μm thickness layers onto glass slides for immunohistochemistry. The sections were fixed with pre-cooled acetone (-20 °C) for 10 min. The fixative was poured off and the acetone was allowed to evaporate from the sections for 20 min at room temperature. After washing (3 times at 5 min) in PBS with 0.1% Triton X-100 (PBST), 100 μl blocking buffer was added (10% goat serum in PBS) onto the sections of the slides and incubated in a humidified chamber at room temperature for 1 h. Sections were incubated with appropriately diluted primary antibody: alpha sarcomeric actinin (Abcam, Cambridge, UK), troponin I (Santa Cruz, Dallas, TX), connexin-43 (Sigma Aldrich, St. Louis, MO) and beta myosin heavy chain (Millipore, Billerica, MA) overnight at 4 °C. After washing in PBST (3 times at 5 min), tissues were incubated with coordinate secondary antibodies diluted in PBST for 1h at ambient temperature. After washing in PBST (3 times at 5 min), nuclei were counterstained with DAPI (Molecular Probes/Invitrogen, Eugene, OR) diluted in PBST for 15 min at ambient temperature. Following the final wash procedure (PBST, 3 times at 5 min), glass cover slips were added to the slides using Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA). Finally, TCS SP5 AOBS laser scanning confocal microscope (Leica Microsystems, Inc., Exton, PA) was used to get fluorescent images. Fluorescent protein expression was calculated as the fluorescence area coverage divided by the number of nuclei.

TUNEL staining for the frozen section of spheroids. In Situ Cell Death Detection Kit (Roche, Penzberg, Germany) was used to determine the viability of the cell in the frozen section of spheroids based on the protocol from website of Roche. Briefly, the frozen sections of spheroids were fixed with 4% paraformaldehyde in PBS for half hour at room temperature. Following washing in PBS for 30 minutes, samples were incubated in a permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate in PBS) for 2 minutes on ice. Then 50ul of the TUNEL reaction mixture were added to samples and incubated in 37 °C for 1 hour. After washing in PBST (3 times at 5 min), nuclei were counterstained with DAPI (Molecular Probes/Invitrogen, Eugene, OR) diluted in PBS for 15 min at ambient temperature. Following the final wash procedure (PBS, 3 times at 5 min), glass cover slips were added to the slides using Fluoro-Gel

(Electron Microscopy Sciences, Hatfield, PA). Finally, TCS SP5 AOBS laser scanning confocal microscope (Leica Microsystems, Inc., Exton, PA) was used to get fluorescent images.

Western blotting analysis. Following 7 days of cell culture with or without electrical stimulation, 30-35 spheroids from each rat-neonatal group were harvested from agarose molds. After centrifugation and washing by PBS once, 30 μl lysis buffer with 1% protease and phosphatase inhibitor cocktails (Pierce Biotechnology, Rockford, IL) was added into the vials containing pellet of spheroids. Thereafter, the mixture was homogenized by the FastPrep24 instrument (MP Biomedicals, Santa Ana, CA) to break down spheroids into single cells. After 30 minutes to lyse cells on ice, then tubes were centrifuged for 10 min at 10 000g at 4 °C and the supernatant was collected as protein solution. After quantifying the protein concentration by using the bicinchoninic acid methods, the protein solution was mixed with 4x LDS sample loading buffer (Pierce Biotechnology) and boiled for 5 min. Protein samples of equal amount were separated in a 4%–12% Bis Tris NuPAGE gel (Life Technologies, Carlsbad, CA). Proteins were transferred to a PVDF membrane (Life Technologies) and blocked with 5% nonfat milk for one hour, followed by incubation with the following primary antibodies: alpha sarcomeric actinin (Abcam, Cambridge, UK), connexin-43 (Sigma Aldrich, St. Louis, MO), beta myosin heavy chain (Millipore, Billerica, MA), and GAPDH (Sigma Aldrich) overnight at 4°C. Blots were then probed with horseradish peroxidase-labeled secondary antibodies (Cell Signal, Danvers, MA) and visualized by an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech (GE Healthcare), Pittsburgh, PA). The intensity of each signal was analyzed by using ImageJ software.

Spheroid spreading assay. Spheroids were seeded onto 0.1% gelatin-coated glass cover slips and incubated at 37 °C, 5% CO_2, 20% O_2. Cell culture medium was changed every other day. After 12 days culture, the spheroids spread into a monolayer structure, which was suitable for immunofluorescent staining for high resolution, single cell, sarcomere structure analysis.

Single cell cardiomyocyte analysis. The average sarcomere length was defined as spacing between α-SA striations and was measured using black and white renderings of confocal α-SA-stained cardiomyocyte images, according to previous methods.[2] Using ImageJ, fluorescence profiles along lines passing perpendicular through 3 different striated regions of at least 9 cells, containing at least 6 consecutive sarcomere structures, were measured and divided by the number of sarcomeres (space between profile peaks). Z-line width, as previously explored,[3] was measured directly on α-SA-stained cardiomyocyte images in 12 cells with 15 measurements per cell. Z-line alignment was defined to establish a sensitive method for sarcomere alignment to reflect the enhanced contraction and synchronization. Calculations were made using an ImageJ plug-in, OrientationJ, which creates an orientation distribution output.[4] The area under the curve at ±20° the peak orientation degree divided by the total area under the curve was established as the fraction Z-line alignment.

Calcium transient imaging of cardiac spheroids. Fluo-4 Direct Calcium Assay Kits (Life Technologies, Carlsbad, CA) was used to label calcium ion in the whole spheroids based on the protocol from Life Technologies. Briefly, spheroids were seeded onto 0.1% gelatin-coated glass cover slips and incubated at 37 °C, 5% CO_2, 20% O_2. Cell culture medium was changed every other day. After 4 days culture, the spheroids were rooted on the cover slips. Then cover slips

with the spheroids were put into 12 wells plates with 2 ml calcium dye solution per well and incubated at 37 °C, 5% CO_2, 20% O_2 for 1h. TCS SP5 AOBS laser scanning confocal microscope (Leica Microsystems, Inc., Exton, PA) was used to collect the videos of the calcium transient of whole spheroids with a capture rate of 14 frames per second. Finally, we used the software of LAS AF from Leica to conduct the quantification of videos collected by confocal.

qRT-PCR. Total RNA was isolated according to the kit and protocol of an RNeasy Micro Kit (Qiagen, Vinlo, Netherlands) with the addition of the QIAShredder (Qiagen) during the homogenization step for spheroids. For each group, 20-35 spheroids were used for RNA isolation. At least 25 ng of total RNA for each group was subjected to cDNA synthesis using the Bio-Rad (Hercules, USA) iScript cDNA synthesis kit. qRT-PCR step was performed using "best coverage" validated Taqman primers (Life Technologies, Carlsbad, USA) in 10 µl reactions for the following genes: CACNA1C, CACNA1G, GAPDH, GJA1, MYL2, ACTB. Data was normalized as the change in cycle threshold (Ct) to GAPDH and ACTB (dCt) and analyzed using, mRNA expression = 2^(-(dCt)).

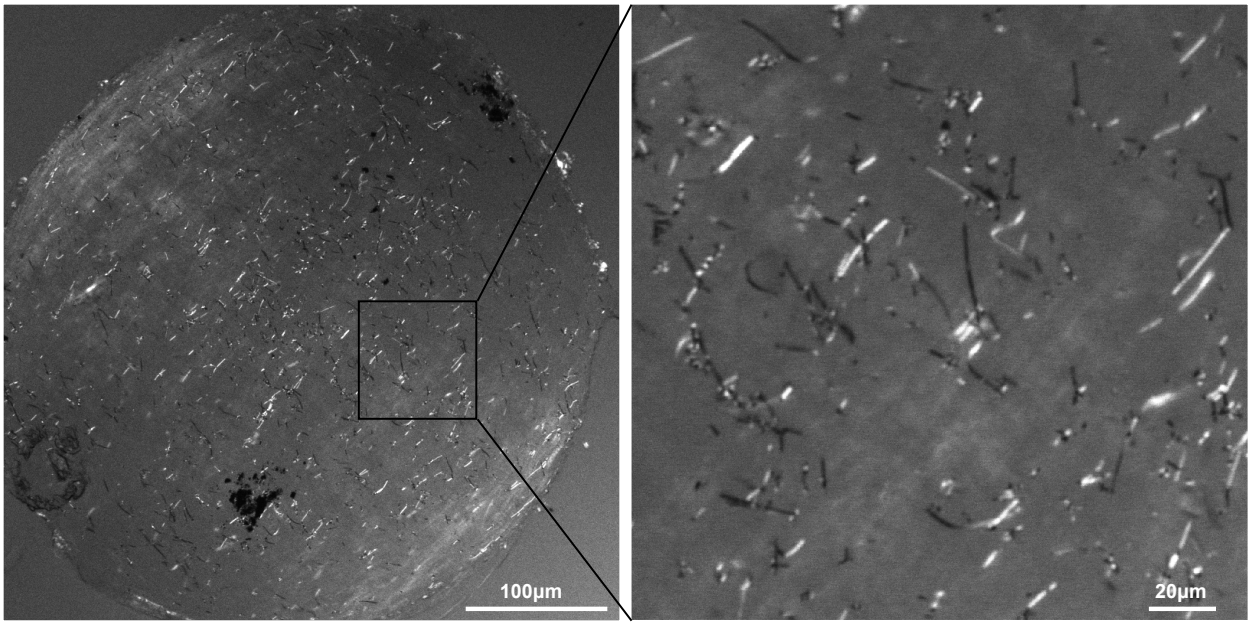
Transmission Electron Microscopy. SiNWs were gently sonicated in isopropyl alcohol (IPA) and dispersed onto lacey carbon grids (Ted Pella Inc.). TEM imaging was conducted using a 300kv FEI Tecnai G2 F30 Super Twin Transmission Electron Microscope.

Spheroids were fixed with 2.5% glutaraldehyde, postfixed in PBS buffered 1% osmium tetroxide with 1.5% K+ ferricyanide, dehydrated in graded ethanol and Acetonitrile, and embedded in PolyBed 812 (Polysciences). 70-nm thick spheroid sections were prepared by using a Leica UltraCut R and a diamond knife, stained with Hanaichi Pb citrate and uranyl acetate, and imagined using a JOEL 200 CX transmission electron microscope.

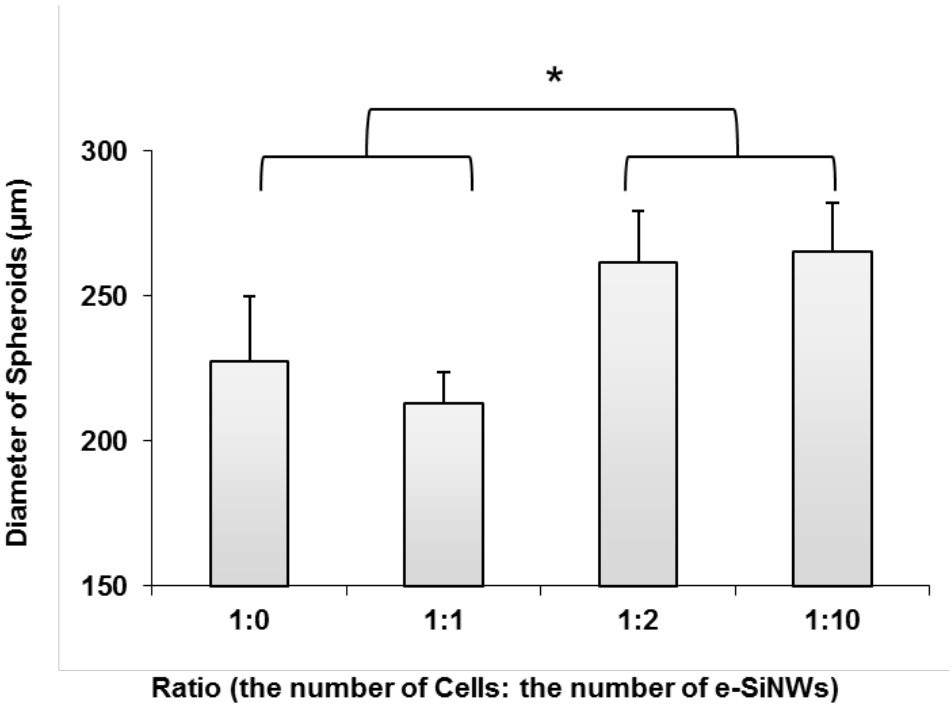
Statistics Analysis: Differences between experimental groups were analyzed using a independent Student T-tests and one-way ANOVA followed by Tukey's post-hoc test. $P < 0.05$ was considered significantly difference for all statistical tests.

Material and Methods References:

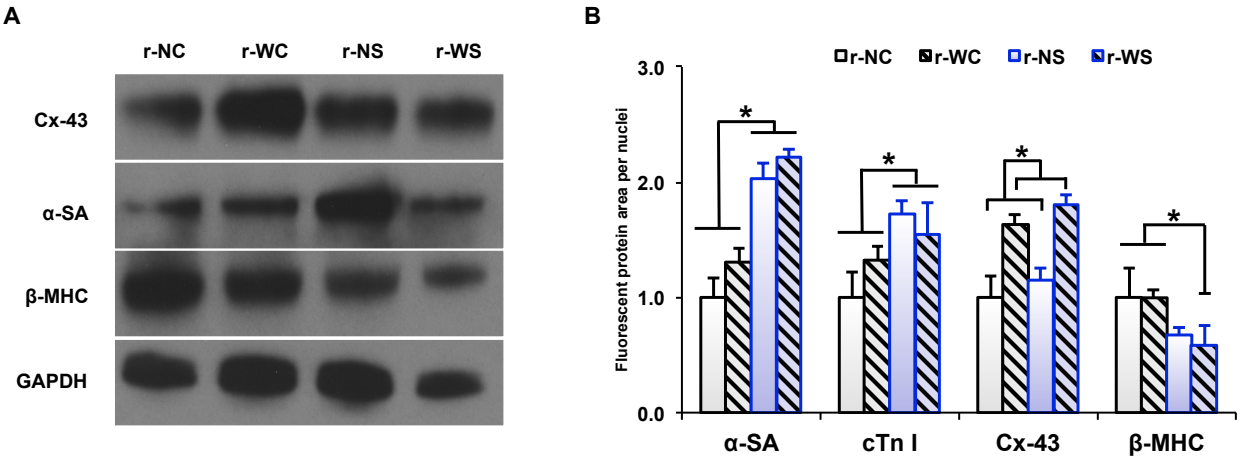
1. Wu, Y. et al. Controlled Growth and Structures of Molecular-Scale Silicon Nanowires. Nano Lett. 4, 433-436 (2004).
2. Fine, M. et al. Human-induced pluripotent stem cell-derived cardiomyocytes for studies of cardiac ion transporters. Am. J. Physiol. Cell Physiol. 305, C481–91 (2013).
3. Rodriguez, A. G., Han, S. J., Regnier, M. & Sniadecki, N. J. Substrate stiffness increases twitch power of neonatal cardiomyocytes in correlation with changes in myofibril structure and intracellular calcium. Biophys. J. 101, 2455–64 (2011).
4. Rezakhaniha, R. et al. Experimental investigation of collagen waviness and orientation in the arterial adventitia using confocal laser scanning microscopy. Biomech. Model. Mechanobiol. 11, 461–73 (2012).



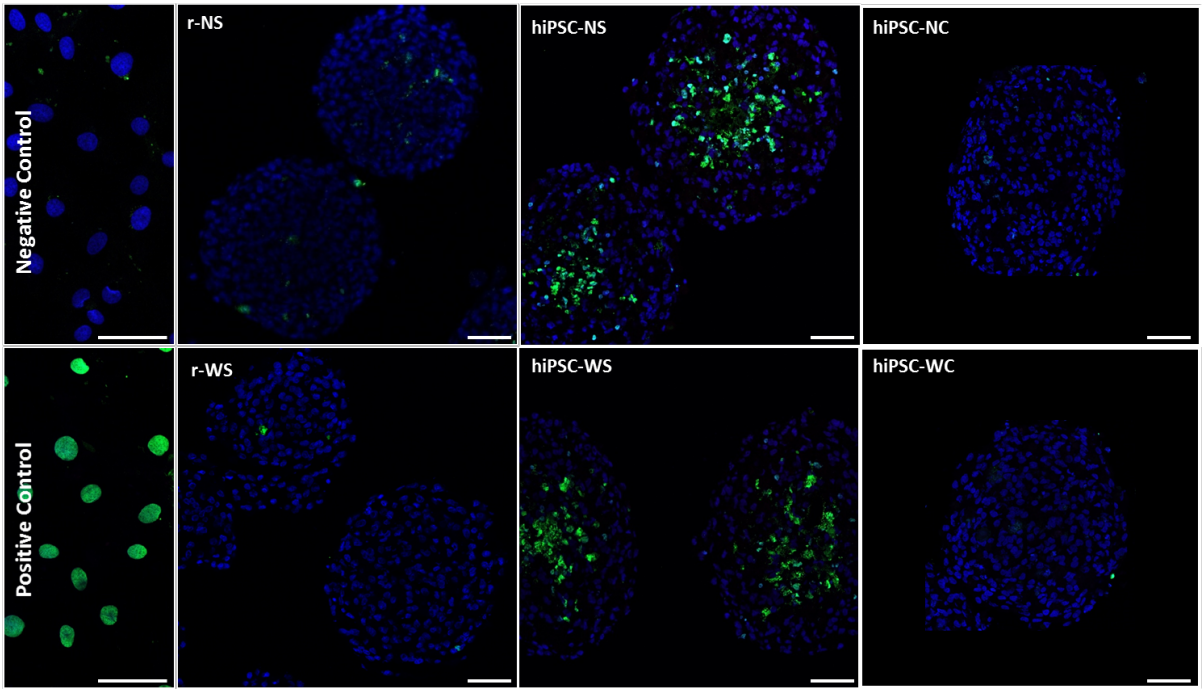
Supplementary Figure 1. DIC image of the e-SiNW-reinforced human cardiac spheroids shows the uniform distribution of e-SiNWs within the spheroids at a 1:1 ratio (number of cells/number of e-SiNWs).



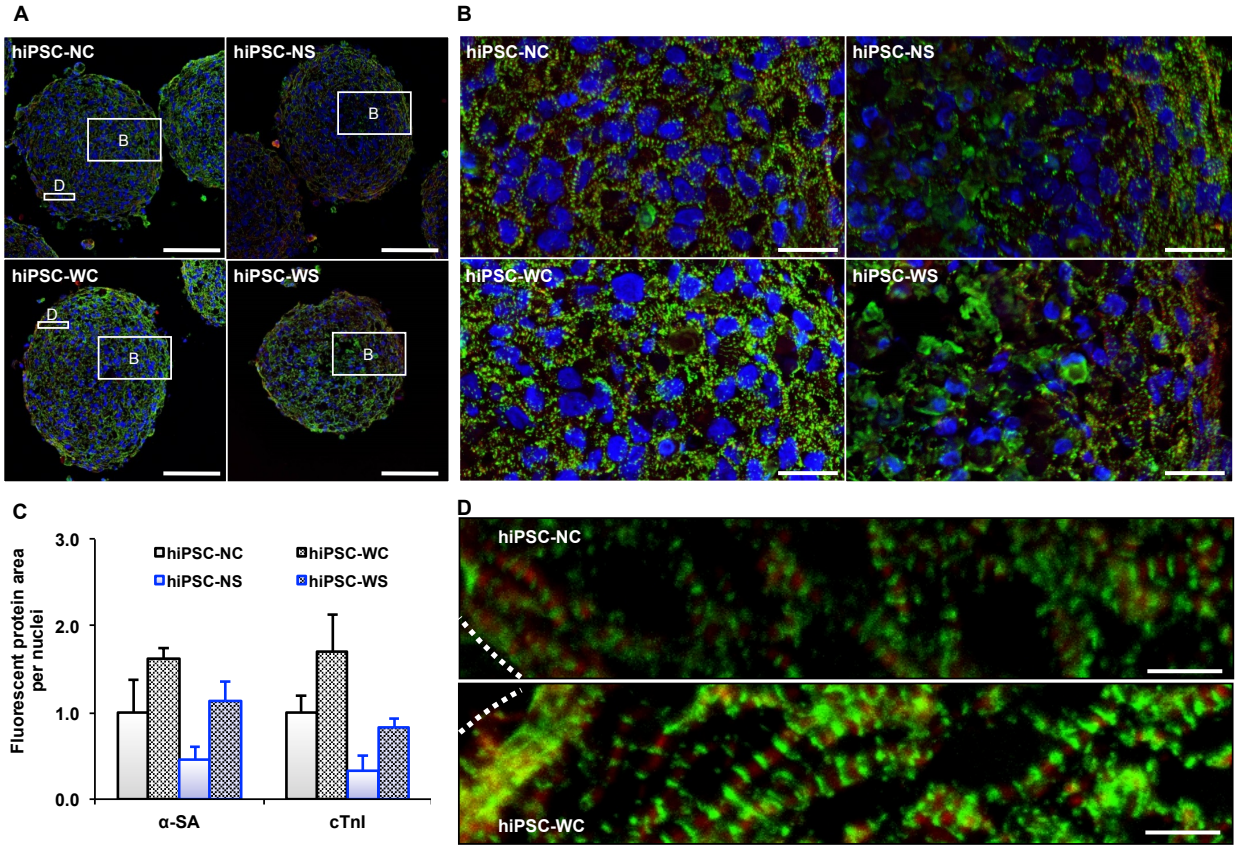
Supplementary Figure 2. Changes in diameter of rat-neonatal cardiac spheroids using different ratios of cells to e-SiNWs on Day 0; n = 6 spheroids per condition. Asterisks (*) represent statistical significance with $p<0.05$; error bar represents standard deviation.



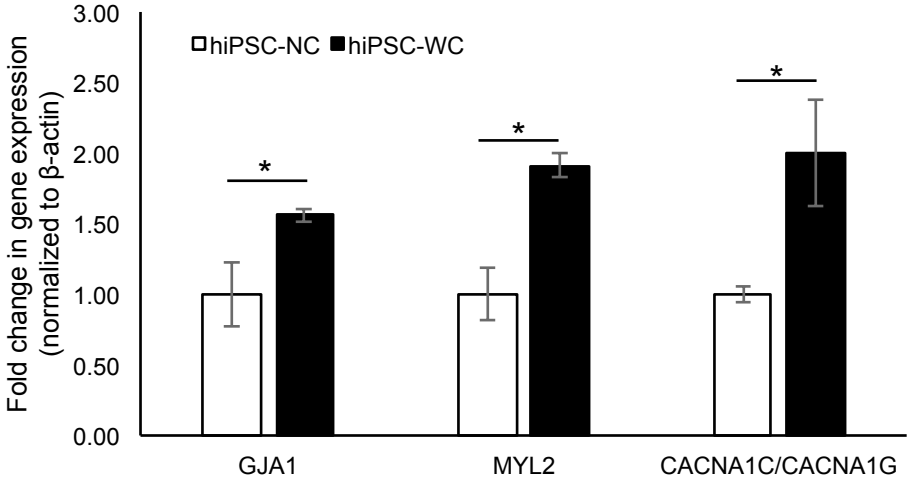
Supplementary Figure 3. Protein expression analysis of rat-neonatal cardiac spheroids after 7 days of treatment. (A) Western blot of cardiac-specific proteins after 7 days for all 4 groups. (B) Protein expression levels based on fluorescent signal-covered area per nuclei normalized over r-NC expression; n = 3 picture regions; 50 μm x 80 μm picture regions, at least containing >24 nuclei. r-NC= rat-neonatal cardiac spheroids, no e-SiNWs, no stimulation; r-NS= rat-neonatal cardiac spheroids, no e-SiNWs, with stimulation; r-WC= rat-neonatal cardiac spheroids, with e-SiNWs, no stimulation; r-WS= rat-neonatal cardiac spheroids, with e-SiNWs, with stimulation. Asterisks (*) represent statistical significance with $p<0.05$; error bar represents standard deviation.



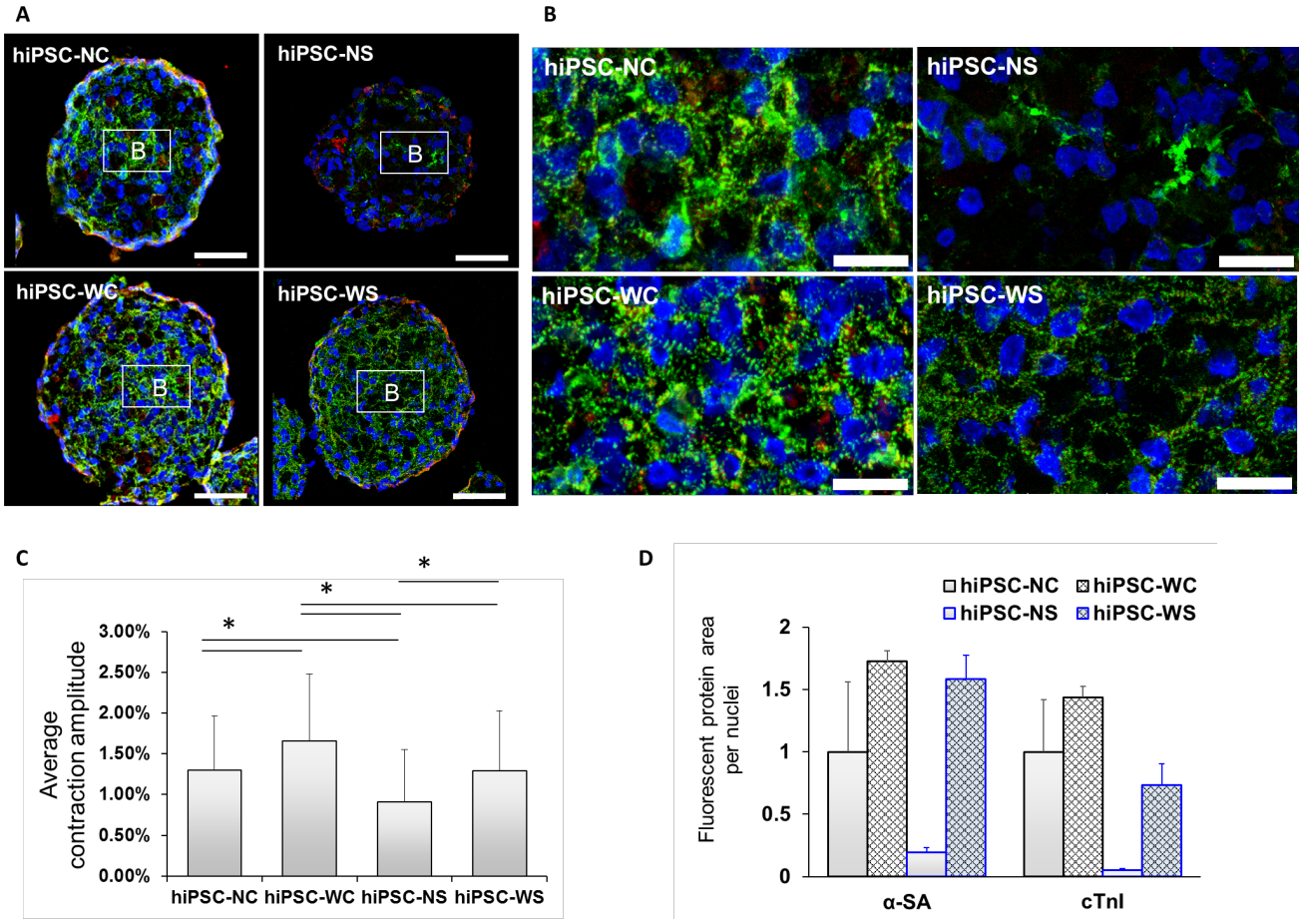
Supplementary Figure 4. TUNEL staining for the frozen sections of spheroids. Blue color is DAPI staining for nuclei, which indicates the viable cells in spheroids. Green color is TUNEL staining for fragments of DNA, which indicates the apoptosis of cells in spheroids. Scale bars: 50μm.



Supplementary Figure 5. Cellular organization of hiPSC-derived cardiomyocyte spheroid cross-sections after 7 days of treatment. (A) Low and (B) high magnification confocal images (green, α-sarcomeric actinin (α-SA); red, troponin I; blue, DAPI nuclear stain) that display the difference in the sarcomere expression and organization within spheroids. (C) Protein expression analysis based on fluorescent signal-covered area per nuclei normalized over hiPSC-NC expression; n = 3 picture regions; 75 μm x 130 μm picture regions, at least containing >50 nuclei. (D) Characteristic images of hiPSC-NC and hiPSC-WC treatments to reveal differences in sarcomere alignment of each whole spheroid (dotted line = spheroid border). hiPSC-NC= human induced pluripotent stem cell cardiac spheroids, no e-SiNWs, no stimulation; hiPSC-NS= human induced pluripotent stem cell cardiac spheroids, no e-SiNWs, with stimulation; hiPSC-WC= human induced pluripotent stem cell cardiac spheroids, with e-SiNWs, no stimulation; hiPSC-WS= human induced pluripotent stem cell cardiac spheroids, with e-SiNWs, with stimulation. Error bars represent standard deviation. Scale bars: (A) = 100 μm; (B) = 20 μm; (D) = 5 μm.



Supplementary Figure 6. qPCR analysis of mRNA expression of conductive and contractile genes in hiPSC-NC and hiPSC-WC spheroids. GJA1 – connexin-43; MYL2 – myosin light chain ventricular isoform; CACANA1C – calcium L-type channel; CACNA1G – calcium T-type channel. Asterisks (*) represent statistical significance with $p<0.05$; error bar represents standard deviation, $n = 3$.



Supplementary Figure 7. Analysis of hiPSC-derived cardiomyocyte spheroids after 3 weeks culture. (A) Low and (B) high magnification confocal images (green, α-sarcomeric actinin (α-SA); red, troponin I; blue, DAPI nuclear stain) that display the difference in the sarcomere expression and organization within spheroids after 21 days in culture. (C) Average contraction amplitude (i.e., fractional area change) of spontaneously beating spheroids with and without e-SiNWs and/or electrical stimulation after 21 days in culture; n=6 spheroids per condition. (D) Protein expression analysis based on fluorescent signal-covered area per nuclei normalized over hiPSC-NC expression; n = 3 picture regions; 40 μm x 40 μm picture regions. hiPSC-NC= human induced pluripotent stem cell cardiac spheroids, no e-SiNWs, no stimulation; hiPSC-NS= hiPSC cardiac spheroids, no e-SiNWs, with stimulation; hiPSC-WC= hiPSC cardiac spheroids, with e-SiNWs, no stimulation; hiPSC-WS= hiPSC cardiac spheroids, with e-SiNWs, with stimulation. Asterisk (*) represents statistical difference between groups with $p<0.05$; error bar represents standard deviation. Scale bars: (A) = 50 μm; (B) = 20 μm.