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Nanowires and electrical stimulation synergistically improve functions of hiPSC cardiac spheroids

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Abstract

The advancement of human induced pluripotent stem cell-derived cardiomyocyte (hiPSC-CM) technology has shown promising potential to provide a patient-specific, regenerative cell therapy strategy to treat cardiovascular disease. Despite the progress, the unspecific, underdeveloped phenotype of hiPSC-CMs has shown arrhythmogenic risk and limited functional improvements after transplantation. To address this, tissue engineering strategies have utilized both exogenous and endogenous stimuli to accelerate the development of hiPSC-CMs. Exogenous electrical stimulation provides a biomimetic pacemaker-like stimuli that has been shown to advance the electrical properties of tissue engineered cardiac constructs. Recently, we demonstrated that the incorporation of electrically conductive silicon nanowires to hiPSC cardiac spheroids led to advanced structural and functional development of hiPSC-CMs by improving the endogenous electrical microenvironment. Here, we reasoned that the enhanced endogenous electrical microenvironment of nanowired hiPSC cardiac spheroids would synergize with exogenous electrical stimulation to further advance the functional development of nanowired hiPSC cardiac spheroids. For the first time, we report that the combination of nanowires and electrical stimulation enhanced cell-cell junction formation, improved development of contractile machinery, and led to a significant decrease in the spontaneous beat rate of hiPSC cardiac spheroids. The advancements made here address critical challenges for the use of hiPSC-CMs in cardiac developmental and translational research and provide an advanced cell delivery vehicle for the next generation of cardiac repair.

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ASSOCIATED CONTENT

Supporting Information

Supplementary figures and experimental details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Graphical Abstract

Keywords

silicon nanowires; electrical stimulation; cardiac spheroids; human induced pluripotent stem cell-derived cardiomyocytes

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) hold remarkable promise as an attractive source for cell transplantation to treat cardiovascular disease.^{1–3} During the past decade, significant efforts have been devoted to transplanting hiPSC-CMs into injured animal hearts to restore their functions.^{4–13} In particular, Murry and coworkers recently demonstrated the remuscularization of damaged hearts in a non-human primate model after transplantation of human embryonic stem cell-derived cardiomyocytes (hESC-CMs).⁵ Despite progress, the current hESC/hiPSC-CMs are composed of heterogeneous cardiomyocyte subpopulations (i.e., ventricular-like, atrial-like, nodal-like cardiomyocytes), and they possess a phenotype that is characterized by spontaneous beating and insufficient expression/development of contractile machinery. These lead to a potentially lethal arrhythmogenic risk and limits functional improvement of injured hearts after transplantation.^{5, 14–16}

To derive hiPSC-CMs suitable for cell transplantation, various tissue-engineering strategies have been utilized to mimic adult host ventricular myocardium to accelerate phenotypic development of hiPSC-CMs.^{13, 16–22} To this end, several groups have used electrical stimulation as an exogenous approach to recapitulate the natural pacemaker-initiated excitation of cardiomyocyte contraction to advance the function of hESC/hiPSC-CMs.^{13, 20, 23–28} Recently, Eng and others demonstrated that electrical conditioning of 3D cardiac embryoid bodies promotes cellular maturity of hiPSC-CMs and affects intrinsic beating characteristics.²⁵ Likewise, electrical nanomaterials have provided an endogenous means of improving electrical conductivities of cardiac microtissues.^{29–31} We have previously shown that the addition of electrically conductive silicon nanowires (e-SiNWs), a class of biodegradable nanomaterials,³² into scaffold-free 3D cardiac spheroids creates an electrically conductive microenvironment and leads to advancement in the function and structural development of hiPSC-CMs.³¹

In this study, we reasoned that exogenous electrical stimulation could synergize with the endogenous benefits of nanowired spheroids to improve the functional development of hiPSC-CMs in cardiac spheroids. To this end, we have developed a viable strategy to electrically stimulate nanowired hiPSC cardiac spheroids and examined the effects of electrical stimulation and silicon nanowires on the functions of spheroids. Herein, for the

first time, we show that the synergy of exogenous electrical stimulation and nanowires to hiPSC cardiac spheroids 1) enhanced microtissue development through formation of functional cell-cell junctions, 2) improved development of contractile properties of hiPSC-CMs, and 3) decreased the endogenous spontaneous beat rate for reduced arrhythmogenic potential. Through tissue- and cell-level examination of cardiac spheroids, the progress here contributes to the fundamental understanding of hiPSC cardiac microtissue development and addresses the need for an advanced, functional cardiac cell therapy vehicle for future cardiac tissue repair.

In the adult heart, ventricular cardiomyocytes contract regularly due to the depolarizing electrical propagation originating in the sino-atrial node of the heart. To mimic this stimulation for contraction, cardiac tissue engineering strategies have utilized electrical pacing to develop and maintain cardiomyocyte function.^{13, 24–25, 27, 33–35} During our previous development of nanowired cardiac spheroids, electrical stimulation (3.75 V/cm, 1 Hz, 2 ms) successfully maintained contractile function when applied to scaffold-free spheroids composed of enriched neonatal rat cardiomyocytes (i.e., <25% fibroblasts).³¹ Based on electrical stimulation protocols for human cardiac tissue constructs established in previous studies and a spheroid size within the diffusional limit (i.e., 150 μ m radius), we empirically developed a viable electrical stimulation protocol (2.5 V/cm, 1 Hz, 5 ms) that supports optimal hiPSC cardiac spheroid viability and function (Figure 1, S1).^{26–27} Compared to unstimulated and unwired spheroids at Day 0 (NC-D0), treatment groups consisted of spheroids in prolonged culture (Day 19 - D19): unstimulated and unwired (NC) spheroids, unstimulated and wired (WC) spheroids, stimulated and unwired (NS) spheroids, and stimulated and wired (WS) spheroids. The nanowired hiPSC cardiac spheroids were prepared as described in our previous study.³¹ Briefly, n-type SiNWs (Diameter $\approx$ 100 nm; length $\approx$ 10 μ m; Silane/Phosphane = 500) were mixed with hiPSC-CMs at a ratio 1:1 and seeded into agarose microwells to prepare nanowired spheroids (Figure 1A–1C). The length of the e-SiNWs was selected to inhibit cell internalization (Figure 1D), and the doping ratio and diameter of the e-SiNWs were chosen to obtain a high conductivity (150 – 500 μ S/ μ m) to create highly electrically conductive microenvironments within spheroids.^{30, 36} While unstimulated (i.e., NC and WC) spheroids showed spontaneous beating, stimulated (i.e., NS and WS) spheroids displayed successful pacing throughout the entirety of the experiment. High viability validated the biocompatibility of silicon nanowires and electrical stimulation condition for prolonged spheroid culture (Figure S1A, S1B). Constant decrease in spheroid size and uniform laminin production indicated similar microtissue formation processes among each condition (Figure 1F, 1G, S1C).

Proper propagation of electrical and mechanical signals in the heart is a tightly regulated process dependent on functional cell-cell junctions between cardiomyocytes. Functionally mature human cardiomyocyte junctions develop throughout postnatal childhood and are characterized by localized expression of electrically conductive channel proteins, such as connexin-43 (Cx43), supported by mechanical junction proteins, such as N-cadherin (N-cad) and Zonula Occludens-1 (ZO-1).^{37–40} As shown in the Figure 2A–E, all the treatment groups showed significantly improved expression of cellular junctions (i.e., Cx-43 and N-cad) than NC-D0 spheroids, which indicates the benefits of prolonged microtissue culture. In addition, WC and NS spheroids showed the enhanced expression of phenotypic immunofluorescent

Cx43 puncta per nuclei when compared with unwired, unstimulated NC spheroids at D19 (Figure 2A, 2B), indicating the beneficial role of nanowires and electrical stimulation in the formation of electrically conductive cellular junctions. Further, significantly more Cx43 expression was found in the WS spheroids than both WC and NS spheroids and demonstrated the synergy between nanowires and electrical stimulation. Also, the WS spheroids showed a significant increase in N-cad expression per nuclei when compared with the remaining groups (Figure 2C, 2D). The expression of Cx43 and N-cad per nuclei allowed for the development of a cellular junction index that supports the synergy of electrical stimulation and nanowires in WS spheroids for cardiomyocyte junction formation (Figure 2E). Colocalized staining of Cx43 with N-cad and ZO-1 in WS spheroids showed not only expression of key junction proteins but also suggests functional cardiomyocyte junctions for enhanced electrical and mechanical coupling (Figure 2F). Enhanced junction formation seen in WS spheroids may facilitate their stable integration upon coupling with the host myocardium after transplantation.

Improved junction formation supports cardiomyocyte coupling for contractile signal propagation. Spheroids were sectioned and stained with alpha sarcomeric actinin (α SA) to visualize the sarcomeric organization, the structural units that support cardiomyocyte contraction. Consistent with the cell-cell junction staining, all the treatment groups showed significantly more α SA-stained structures than that of NC-D0 spheroids (Figure 3). In addition, WC and WS spheroids showed enhanced α SA-positive structures compared to NC and NS spheroids, while electrical stimulation alone (i.e., NS) did not change the amount of α SA-positive structures significantly from NC spheroids (Figure 3A-F). Further, high magnification α SA images showed visible formation of sarcomeric banding in WC and WS spheroids and a lack of sarcomeric banding in NS spheroids (Figure 3G, 3H). This finding supported the critical role of e-SiNWs to improve contractile protein organization in electrically stimulated hiPSC-CM spheroids. This was further supported by the significant increases in expression of the sarcoplasmic reticulum ryanodine receptor (RYR2) in the WC and WS spheroids (Figure S2), which regulates intracellular calcium release required for cardiomyocyte contraction.

The benefits of nanowires for improved contractile characteristics were consistent with the genotypic and phenotypic characterization of treated hiPSC-CM spheroids. Genetically, the nanowired spheroids (i.e., WC, WS) exhibited increased ratio of beta myosin heavy chain (β -MHC; MYH7) to alpha myosin heavy chain (α -MHC; MYH6) (Figure 4A). Individually, β -MHC expression increases significantly compared to Day 0 in nanowired groups, while α -MHC did not change significantly (Figure S2). This isoform trend is consistent with in vivo maturation of human cardiomyocytes and the nonfailing adult human heart.^{15, 41} The increase in β -MHC/ α -MHC gene ratio has been reported to translate to the myosin protein shift and favors lower cellular ATPase activity.^{42–44} This property may be an energetically favorable condition for transplanting cells into the energy-limited conditions of a damaged heart. In contrast, the NS group showed significantly lower β -MHC/ α -MHC ratio compared to the other groups. This suggests a limitation of electrical stimulation alone for contractile development of hiPSC cardiac spheroids. These findings were supported by immunofluorescent analysis of cardiac troponin I (cTnI), a key indicator of contractile protein maturation (Figure 4B, 4C). While nanowired spheroids showed the improvement in

cTnI expression when compared with their unwired spheroids, the electrical stimulation alone lead to the reduced cTnI expression. The significantly higher density of cTnI in the WS spheroids indicated a critical role of nanowires in promoting contractile development of hiPSC cardiac spheroids under electrical stimulation.^{45–46}

While current hiPSC-CMs are composed of heterogeneous subpopulations (i.e., ventricular-like, atrial-like, nodal-like cardiomyocytes), the observed improved contractile machinery suggests a shift towards a working myocardial phenotype. Specifically, electrical stimulation of hiPSC cardiac spheroids significantly increased ventricular myosin light chain (MYL2) over unstimulated spheroids (Figure 5A, left). No change was observed in atrial myosin light chain (MYL4), indicating an increase in overall ventricular-like contractile genotype of stimulated spheroids (Figure 5A). This was further supported with ventricular myosin light chain immunofluorescent staining (MLC-2v), showing significantly higher density of MLC-2v expression per spheroid in the WS condition (Figure 5B, 5C). This suggests that exogenous electrical stimulation may override the endogenous spontaneous electrical signals in hiPSC-CMs to regulate contraction in a pacemaker-like manner and promote the ventricular-like shift.

The observed shift in cardiomyocyte subpopulation characterization was further explored using beat rate analysis to examine hiPSC-CM spheroid function. Each spheroid condition was characterized by spontaneous beating throughout the entirety of the experiment. While the newly formed NC-D0 spheroids had an average beat rate ($\pm$ s.e.m.) of 35.5 ± 3.0 beats per minute (bpm), all the treatment groups showed a reduced spontaneous beat rate. With the 1 Hz stimulation treatment, the spontaneous beat rate of NS and WS spheroids significantly decreased to 24.5 ± 2.4 and 25.6 ± 2.8 bpm in comparison with NC and WC spheroids (30.4 ± 3.4 and 29.2 ± 3.9 bpm, respectively) (Figure 5D). The potential mechanism of this functional change due to an exogenous electrical field was explored using genetic analysis of ion channels known to be related to the spontaneous beating of hiPSC-CMs.^{15, 47} HCN4 is the dominant form of the HCN protein in nodal cells and contributes to the pacemaking mechanism of the sino-atrial node.¹⁵ HCN4 is expressed during fetal ventricular expression but is down-regulated in the working myocardium during development and virtually absent in adults.⁴⁸ When compared with unstimulated spheroids, electrical stimulation significantly reduced HCN4 expression, suggesting a shift away from a pacemaker-like genotype (Figure 5E, left). This decrease in HCN4 expression combined with a functional decrease in beat rate indicates a critical role of electrical stimulation in the regulatory mechanism governing hiPSC-CM spheroid electrical properties. Furthermore, the lack of inward rectifying channel (KCNJ2) expression has been shown to contribute to the spontaneous beating phenomenon in hiPSC-CMs.^{49–50} Notably, under electrical stimulation, the addition of e-SiNWs to hiPSC cardiac spheroids was critical to preserve inward rectifying channel (KCNJ2) gene expression (Figure 5E, right). Interestingly, the significant decrease in KCNJ2 gene expression in NS is in line with the limited contractile development of NS discussed above. Nevertheless, electrical stimulation can synergize with e-SiNWs to provide exogenous and endogenous cues to affect the cardiomyocyte subpopulation specification and significantly decrease the spontaneous beat rate of hiPSC-CM spheroids. Notably, HERG (KCNH2) showed no significant changes with electrical stimulation at 1 Hz, which is consistent with the recent studies conducted by Eng and coworkers (Figure S2). They reported an up-

regulated HERG expression with a 2 Hz stimulation treatment and un-changed HERG expression with 1 Hz stimulation.²⁵

To date, transplantation of hiPSC-CMs into damaged hearts have led to limited improvements in heart functions and increased risk of arrhythmia.^{12, 51–53} These results alongside recent research showing enhanced cell retention using cellular aggregates indicate that hiPSC-CM microtissues with advanced cellular development provide an attractive cell delivery system for transplantation.^{11, 54–55} Our previous research showed that addition of e-SiNWs to hiPSC cardiac spheroids creates an electrically conductive microenvironment and significantly improves the structural and functional development of hiPSC cardiac spheroids.³¹ In this study, we demonstrated that the addition of nanowires to the spheroids is essential for the utilization of electrical stimulation to improve the functions of hiPSC cardiac spheroids. With nanowires, exogenous electrical stimulation increases cell-cell junction formation (i.e., Cx-43 and N-cad), improves development of contractile machinery (i.e., sarcomeric structure, β -MHC/ α -MHC gene ratio and cTnI expression), and preserves KCNJ2 gene expression. In contrast, electrical stimulation alone (i.e., NS) showed increased Cx43 yet lower N-cad along with reduced sarcomere development, cTnI expression, β -MHC/ α -MHC gene ratio, and KCNJ2 gene expression. Although it has been reported that electrical stimulation alone can advance cardiomyocyte development in vitro, the data presented here suggests potential limitations of the use of electrical stimulation without nanowires in hiPSC cardiac spheroids for ventricular applications.

Remarkably, electrical stimulation of nanowired spheroids also resulted in a significant reduction in endogenous, spontaneous beat rate and shifts in cardiomyocyte subpopulation characterization. These phenomena point to the beneficial functional and genomic effects of exogenous electrical stimulation to affect the endogenous electrical properties of nanowired hiPSC cardiac microtissues. The observed reduction of spontaneous beat rate and pacemaker-like genotype clearly demonstrates tissue level functional development that would be beneficial for reduced pacemaking/arrhythmic risk after transplantation.

In conclusion, we demonstrated, for the first time, that the combination of e-SiNWs and electrical stimulation synergistically improves cell junction formation, increases contractile properties, and reduces the endogenous spontaneous beat rate of hiPSC-CM spheroids. Enhancing the cell-cell junctions of hiPSC cardiac spheroids in vitro may lead to improved cellular integration and functional contribution after transplantation. The increased expression of contractile machinery in vitro indicates an increased cardiomyocyte developmental age, which has been shown to be advantageous for transplantation.^{17, 56} Given that many cardiac cell therapies target ventricular myocardial repair, the observed expression shift towards the ventricular isoform of myosin light chain shows promise for improved functional integration. Likewise, by decreasing the spontaneous beat rate in hiPSC cardiac spheroids and directly affecting genes that regulate the spontaneous beating of hiPSC-CMs, we propose that this in vitro conditioning model will reduce the arrhythmogenic risk of hiPSC-CMs. In addressing these underdeveloped properties of hiPSC-CMs with the combination of endogenous nanomaterials and exogenous stimuli, we hope to contribute to the next generation of cell therapy approaches for cardiac repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

hiPSC	human induced pluripotent stem cell
CM	cardiomyocyte
e-SiNW	electrically conductive silicon nanowires
NC-D0	unwired/unstimulated hiPSC-CM spheroids at Day 0
NC	unwired/unstimulated hiPSC-CM spheroids at Day 19
WC	wired/unstimulated hiPSC-CM spheroids at Day 19
NS	unwired/stimulated hiPSC-CM spheroids at Day 19
WS	wired/stimulated hiPSC-CM spheroids at Day 19

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Figure 1.

Development of electrical stimulation for nanowired hiPSC cardiac spheroids. (A) The schematic diagram of the stimulation setup shows cardiac spheroids cultured in non-adhesive agarose molds between carbon electrodes. (B) Transmission electron micrograph details the structure of n-type doped silicon nanowires. (C) Nanowired hiPSC cardiac spheroids assembly by Day 0 in non-adhesive agarose molds. (D) Transmission electron micrograph of spheroid section depicts nanowire location in extracellular space; asterisk - nanowire location, triangle - cell membrane. (E) The timeline of in vitro conditioning indicates specified electrical stimulation parameters and time points. Electrical stimulation and nanowires do not significantly change spheroid assembly indicated by consistent (F) spheroid size and (G) laminin expression; dotted line represents spheroid edge; red-laminin, yellow-silicon nanowires. NC-D0 -unwired and unstimulated at Day 0; NC - unwired and unstimulated at Day 19; WC - wired and unstimulated at Day 19; NS - unwired and stimulated at Day 19; WS - wired and stimulated at Day 19.

Figure 2.

Synergistic contribution of electrical stimulation and nanowires to cellular junction formation. (A and B) Connexin-43 (Cx43) immunofluorescent staining shows an increased number of phenotypic puncta per nuclei in stimulated conditions; red-Cx43, yellow-nanowires; + = significant difference with NC-D0, * = significant difference with unstimulated spheroids ($p < 0.05$). (C and D) N-cadherin (N-cad) expression shows significantly higher staining per nuclei at the intercellular border in WS spheroids; green-N-cad, yellow-nanowires; + = significantly different from other groups, * -significant

difference ($p < 0.05$). (E) The junction index is the product (i.e., size of the circle) of the normalized (to NC-D0) Cx43 and N-cad expression per nuclei and represents the synergistic benefit in WS for cellular junction formation. (F) Representative staining for WS spheroids shows colocalization of Cx43 with N-cad and ZO-1 (Zonula Occludens-1), suggesting formation of functional cardiomyocyte junctions. NC-D0 -unwired and unstimulated at Day 0; NC – unwired and unstimulated at Day 19; WC – wired and unstimulated at Day 19; NS – unwired and stimulated at Day 19; WS – wired and stimulated at Day 19.

Figure 3.

Nanowires influence sarcomeric organization in hiPSC cardiac spheroids. (A) αSA immunofluorescent staining shows minimal sarcomeric organization at Day 0. (B-F) All conditions showed significantly increased αSA-stained structures at Day 19 compared to NC-D0. The addition of nanowires further increases αSA expression based on αSA-positive area per spheroid area. green-αSA, blue-DAPI nuclear stain. + = significantly different from other groups; * = significantly different from unwired groups ($p < 0.05$). (G and H) High magnification αSA-stained images and qualitative scoring show highest quality of sarcomere structures in WC and WS spheroids. NC-D0 -unwired and unstimulated at Day 0; NC – unwired and unstimulated at Day 19; WC – wired and unstimulated at Day 19; NS – unwired and stimulated at Day 19; WS – wired and stimulated at Day 19.

Figure 4.

Nanowires and electrical stimulation affect development of contractile machinery in hiPSC cardiac spheroids. (A) Nanowires increase the ratio of beta myosin heavy chain (MYH7) to alpha myosin heavy chain (MYH6) expression, and NC, WC, and WS show statistical significance compared to NS. (B and C) Immunofluorescent expression of cardiac troponin I (cTnI) shows significant increases in nanowired conditions, indicating signs of contractile machinery maturation; red-cTnI. * = significant difference from marked or remaining groups ($p < 0.05$). NC-D0 -unwired and unstimulated at Day 0; NC – unwired and unstimulated at Day 19; WC – wired and unstimulated at Day 19; NS – unwired and stimulated at Day 19; WS – wired and stimulated at Day 19.

Figure 5.

Electrical stimulation and nanowires directly affect subpopulation specification and beating properties in hiPSC cardiac spheroids. (A) Electrical stimulation significantly improves gene expression of ventricular myosin light chain (MYL2), while atrial myosin light chain (MYL4) is unchanged. (B and C) Immunofluorescent staining of the MYL2 protein, MLC-2v, supports genetic trend with significantly more expression per area in WS; red-MLC-2v. (D) Electrical stimulation significantly reduced the spontaneous beat rate of hiPSC cardiac spheroids compared to unstimulated and NC-D0 spheroids. + = significantly different from NC-D0; * = significantly different from unstimulated groups ($p < 0.05$). (E) Electrical stimulation significantly reduced the mRNA expression of HCN4, a pacemaker gene in the adult heart. The presence of nanowires preserved KCNJ2 expression when under stimulation, an important gene to prevent spontaneous contraction in adult ventricular cardiomyocytes. * = significantly different from unmarked groups. NC-D0 -unwired and unstimulated at Day 0; NC – unwired and unstimulated at Day 19; WC – wired and unstimulated at Day 19; NS – unwired and stimulated at Day 19; WS – wired and stimulated at Day 19.