Development of a reentrant arrhythmia model in human pluripotent stem cell-derived cardiac cell sheets

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Aims

Development of a human cell-derived reentrant arrhythmia model is needed for studying the mechanisms of disease and accurate drug response.

Methods and results

We differentiated human pluripotent stem cells (hPSCs) into cardiomyocytes, and then re-plated them into cell sheets that proved capable of forming electrically coupled assemblies. We monitored the function of these re-plated sheets optically with the Ca2+ sensitive dye Fluo-4, and found that they generated characteristic waves of activity whose velocity and patterns of propagation depended upon the concentration of sodium channel blockers; lidocaine and tetrodotoxin, and also the time after re-plating, as well as the applied stimulation frequency. Importantly, reentrant spiral-wave propagation could be generated in these sheets by applying high-frequency stimulation, particularly when cell-density in the sheets was relatively low. This was because cardiac troponin T-positive cells were more non-homogeneously distributed at low cell densities. Especially in such sheets, we could terminate spiral waves by administering the anti-arrhythmic drugs; nifekalant, E-4031, sotalol, and quinidine. We also found that in these sheets, nifekalant showed a clear dose-dependent increase in the size of the unexcitable ‘cores’ of these induced spiral waves, an important parallel with the treatment for ventricular tachycardia in the clinical situation, which was not shown properly in cardiac-cell sheets derived from dissociated rodent hearts.

Conclusions

We have succeeded in creating from hPSCs a valuable type of cardiomyocyte sheet that is capable of generating re-entrant arrhythmias, and thus is demonstrably useful for screening and testing all sorts of drugs with anti-arrhythmic potential.

Keywords
Reentrant arrhythmia • Human ES/iPS cells • Cardiac cell sheet • Spiral wave • Drug screening

Introduction

Human pluripotent stem cells (hPSCs), both in the form of human embryonic stem cells and human induced pluripotent stem cells (hiPSCs), are the logical candidates for future clinical applications, due to their unlimited differentiation capacity and the likelihood that they can be functionally integrated into the host cardiac tissue.1–3 Moreover, hPSCs are already well established research tools for disease modelling4–8 and for drug discovery and testing.9 Previous reports on hPSC-derived cardiomyocytes demonstrated that the cells did indeed develop cardiac-specific functional properties.10–14 However, these studies examined their electrophysiological function as single cells or as small cell-clusters; they did not attempt to define their functional properties in more complex multi-cellular networks. Thus, they could not study more complex phenomena such as the reentrant arrhythmias that are caused by spiral wave propagation in whole cardiac tissues. Optical mapping is a well-known method to monitor the progression of cell excitation in such whole tissues.15

This technique has been used to study intra- and inter-cellular Ca2+ transients in hPSC-derived cardiomyocytes,16,17 and recently

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there was a report that showed hPSC-derived cardiac myocyte monolayer was useful to monitor voltage and calcium mapping simultaneously. However, there have been no reports examining the mechanism of reentrant arrhythmias or the effects of anti-arrhythmic drugs systematically in hPSC-derived cardiac cell sheets.

We thus advanced the culturing and monitoring techniques used to investigate hPSC-derived cardiac cell sheets, in an effort to improve their functionality, and their utility for evaluating the mechanisms and therapeutics of reentrant arrhythmias. At first, we characterized the sheets structurally and biochemically, by light microscopic immunostaining, advanced techniques of electron microscopy (EM), and quantitative reverse transcription polymerase chain reaction (qRT–PCR). And then, we studied the excitation wave propagation on the sheets using optical mapping with a Ca²⁺ sensitive dye, Fluo-4. Specifically, we investigated the probability to induce spiral waves in the sheets as a model of reentrant arrhythmia. Finally, we studied the mechanisms of spiral wave termination by anti-arrhythmic drugs, especially IKr (rapidly activating component of delayed rectifier K⁺ current) blockers. In the Supplemental material, we show the detailed methods of efficient cardiac differentiation from hPSCs, structural and biochemical analyses, optical mapping with Fluo-4, and statistical analysis, as well as additional results, figures, and videos.

**Methods**

**Culture of human pluripotent stem cells and induction of cardiac differentiation**

Detailed culture methods are provided in the Supplementary material online. Here we used a novel small molecule to induce cardiac differentiation from hPSCs.

**Preparation of human pluripotent stem cell-derived cardiac cell sheets**

After around 1-month cardiac differentiation, floating cardiac colonies were collected and dispersed into single cells with stirring for 0.5–1 h in protease solution (0.1% collagenase type I (Wako, Japan), 0.25% trypsin, 1 U/mL DNase I (Applied Biosystems, Life Technologies), 116 mM NaCl, 20 mM HEPES, 12.5 mM NaH₂PO₄, 5.6 mM glucose, 5.4 mM KCl, and 0.8 mM MgSO₄, pH 7.35. After dispersion, the cells were filtered using a 35 μm cell strainer (BD Falcon, NJ, USA) and were plated at a density of 2.6 × 10⁴ cells/mm² on gelatin-coated 35 mm tissue culture dishes (BD Falcon) or 27 mm diameter glass plates (IWAKI, Japan) with an attached silicon ring (12 mm diameter, flexiPERM conA; Greiner Bio-One GmbH, Germany). When investigating the probability of spiral wave induction, cell density was changed to 0.65 × 10⁴ or 1.3 × 10⁴ cells/mm². In a small region of the obstacle model, a mould of polydimethylsiloxane was attached to prevent the attachment of cells on the first day. In the obstacle model and 35 mm culture dishes, cells were seeded at a cell density of 2.6 × 10⁴/mm². One day after seeding, the silicon ring and obstacle mould were detached from the culture dish, and the appropriate amount of cardiac differentiation medium was added. The medium was exchanged every 3–5 days.

**Optical mapping system**

Optical mapping was performed as described in detail in the Supplementary material online.

**Multi-electrode arrays recordings**

Multi-electrode arrays (MEA) recordings were performed as described in detail in the Supplementary material online.

**Immunostaining and flow cytometry**

Immunostaining and flow cytometry were performed as described in detail in the Supplementary material online.

**Quantitative reverse transcription polymerase chain reaction**

Quantitative reverse transcription polymerase chain reactions were performed as described in detail in the Supplementary material online.

**Electron microscopy**

Electron microscopy (EM) was performed as described in detail in the Supplementary material online.

**Culture of neonatal rat cardiac myocytes**

Detailed culture methods are provided in the Supplementary material online.

**Statistical analysis**

Statistical analysis was performed as described in detail in the Supplementary material online.

**Results**

**Characterization of a cardiac cell sheet**

First, we examined the properties of hPSC-derived cardiac cell sheets using immunostaining, EM, MEA and qRT–PCRs. Supplementary material online, Video S1 shows an example of a beating cardiac cell sheet. After seeding the cells, not only cardiac troponin T (cTnT) which is a highly cardiac-specific myofilament protein, but also alpha-actinin, which is present at the Z-line of the sarcomere, and NKX-2.5, cardiac specific transcription factor, were positive in approximately 90% of the cells in the sheets with immunostaining (Figure 1A–C). The gap junction (GJ) protein, connexin-43 (Cx43), was detected around the cTnT-positive cardiac cells (Figure 1B). More than 90% of the cells were positive for myosin light chain (MLC)-2v, ventricular specific protein, and less than 10% were positive for hyperpolarization-activated, cyclic nucleotide-gated (HCN)4, which is validated and earliest expressed nodal cell marker (Figure 1D). In Figure 1E, vimentin, a fibroblast marker, was also detected in around 10% of the cell sheet. Higher magnifications of immunostaining of cTnT and alpha-actinin show the clear cardiac muscle structures (Figure 1F and G). Before and after making a cell sheet, flow cytometry of differentiated cells showed that approximately 90% of the cells were positive for cTnT (Supplementary material online, Figure S1). The cTnT-positive ratio did not change for more than 50 days after seeding (n = 3 each, Figure 1H). We could, therefore, conclude that the sheets were composed of a relatively high percentage of cardiac muscle-like cells, but were not 100% pure cardiomyocytes.

Electron microscopy study showed the development of a few attachment zone (AZ) and GJs between cells 2 days after seeding (Figure 2A). After 7 days, however, the GJs, which presumably support the propagation of excitation, developed not only laterally...
between adjacent cells but also abutting ends of these cells (Figure 2B). The adherence junctions and desmosomes were clearly seen in the extracellular space (Figure 3A). The structural architecture of the sarcomeres in the hPSC-derived cardiac cell was visualized in both in the 2 and 7 days after seeding (Figure 2A and 3B). Moreover, after 1-month culturing, cardiac intracellular organelles as well as intercalated disks including AZ and GJ were much more matured (Figure 4).

The data of MEA recordings and qRT–PCR are described in the Supplementary material online (Supplementary material online, Figures S2 and S3).

**Optical mapping**

We used optical mapping to determine whether the hPSC-derived cardiac cell sheets generate functional ‘syncytia’. Excitation wave propagation was initiated in the cell sheet either spontaneously or by application of electrical stimulation (Figure 5A and B). The general applicability of cardiac cell sheets was examined with a variety of hPSC lines (253G1, IMR90-1, and KhES3). Table 1 shows the comparison of cardiac cell sheets derived from three hPSC lines and neonatal rat ventricular myocytes (NRVM). There was no significant difference in the conduction velocity (CV) and calcium transient duration (CaT80) between three hPSC lines; therefore, we mainly examined 253G1 hiPSC-derived cells. In Figures 5C, D and 6A, IMR90-1 and 253G1 hiPSC-derived cells were used and in Supplementary material online, Figures 2B–D and 4A–B, KhES3 and 253 G1 hiPSC-derived cells were used. Excitation wave propagation was detected in the cell sheet from 2 to 28 days after seeding. The CV slowed down when the stimulation frequency was increased (Figure 5C). On the other hand, the CV increased as time passed during 1 month after seeding (Figure 5D). Administration of lidocaine (Figure 5E) and tetrodotoxin (Figure 5F) slowed down the CV.
Specifically, we determined whether the sheets could display spiral wave propagation and could thus serve as reentrant tachyarhythmia models. When the cell sheets were stimulated with high-frequency electric pulses, propagating waves would often break apart and start to create rotating reentrant spiral waves (Supplementary material online, Video S3). We compared three groups with varying density of cells used for the initial seeding to determine the propensity to initiate spiral waves (Group 1: $0.65 \times 10^3$ per mm$^2$, Group 2: $1.3 \times 10^3$ per mm$^2$, and Group 3: $2.6 \times 10^3$ per mm$^2$). Interestingly, the induction rates of stationary spiral waves were 7/11 (64%), 21/25 (84%), and 23/70 (33%) for Groups 1, 2, and 3, respectively (Figure 6A). A decreased cell density increased the inducibility of spiral waves, but the wave could not propagate as stationary spiral waves when the density of the cells was too low. In the NRVM-derived cell sheets also, low cardiac cell density ($<0.65 \times 10^3$/mm$^2$) made it difficult to rotate as stationary spiral waves ($n=4$, Supplementary material online, Video S4), as indicated in the reference. When the density was more than $2.6 \times 10^3$ cells/mm$^2$, the cells in the periphery peeled up and did not maintain the flat sheet structure. The difference of the cTnT-positive area was significant between Groups 1 and 2 ($P<0.001$), but not between Groups 2 and 3.

**Figure 2** Adherence and gap junctions in 2- and 7-day cardiac cell sheets visualized by EM anaglyph. (A) Two-day sheets are generally two layers thick, with a characteristic, very unique layer of myofilaments that lack Z-bands in the lower-most cells that are attached right up against the plastic substrate (flat green on lower left), and more normal myofilament bundles punctuated by Z-bands in the upper layers, plus lots of adhesive junctions in-between the layers, holding them together (attachment zone; AZ). The higher-magnified insets to the right show a couple of the few gap junctions (GJs). (B) Examples of GJs, between adjacent cells in 7-day sheets, either at their abutting ends (left panel) or along their lateral margins (right panel), showing that they occur typically adjacent to adherence junctions, as in mature cardiac tissues.
Figure 3 Characteristics of desmosomes and sarcomeric bundles organized in 7-day cell sheets. (A) Adherence junctions and desmosomes are beautifully differentiated in these sheets, with a magnified inset (not in three-dimensional) superimposed on a three-dimensional image of the junction between two cells deep within a 7-day sheet, plus three more optimal examples shown on the right. These would be the images of the dense lamina that exists in the extracellular space between cells. AZ: attachment zone. (B) The sarcomeric bundles of myofilaments between adjacent cells as in mature cardiac tissue. White scale bars: 500 nm (A and B).

Figure 4 Characteristics of myofilaments and intercalated disks organized in 1-month cardiac cell sheets. (A) In overview of 1-month culture, myofibrils with Z-bands, sarcoplasmic reticulum around myofibrils, mitochondria, desmosomes, lipid droplets, and glycogen granules were observed by ultra-thin section EM. (B) In three-dimensional magnified views, the intercalated disk architecture with nice adherence junctions at the ends of the myofilament bundles, flanked by gap junctions that run parallel to the myofilaments, was visualized just as in mature cardiac tissue by EM anaglyph.

(Figure 6B). Figure 6C shows samples of sheets made from three different densities of seeding cells. Regional differences of cTnT-positive cells were seen between the enlarged fields in Groups 1 and 2. In contrast, when compared with Group 2, cTnT-negative spots were much smaller in any enlarged fields in Group 3. Spiral waves were induced in Groups 1 and 2 as...
shown in the isochronal maps, but the waves were easily broken in Group 1. In Group 3, spiral waves were not inducible frequently. These findings showed that an increased cardiac cell number and even distribution of the cells are important for preventing spiral wave generation.

Figure 6D shows the comparison between sheets where the spiral waves were inducible and not inducible, irrespective of the densities of seeding cells. There was much higher percentage of cTnT-positive cells in the un-inducible cell sheets compared with the spiral-inducible sheets, either in the total and cTnT-low areas. Additionally, there was more non-homogenous distribution of cTnT-positive cells in the spiral-inducible sheets because there was significant difference between areas.

Administration of several anti-arrhythmic drugs, especially $I_{Kr}$ blockers, during spiral wave propagation was effective for its termination (Supplementary material online, Videos S5 and S6). Figure 7 shows the dynamics of spiral wave propagation before and after treatment with nifekalant. The merged images in the right panels of Figure 7A–C show the dose-dependent increase in the size of unexcitable area. The change of the unexcitable area (spiral core) was statistically significant ($n = 4$, Figure 7D).

Figure 8 shows increases of the unexcitable areas by sotalol, quinidine, and E-4031. The meandering of the spiral wave tip was enhanced after sotalol administration; that is sotalol treatment resulted in destabilization of the spiral core and enhancement of the meandering pattern (Figure 8A). We administered quinidine in larger cell sheets (35 mm diameter), which showed a decrease in the rotational rates; that may be caused by the blockade of sodium channels (Figure 8B). The observed spiral core size changed with the application of E-4031 in an obstacle model, in which a small region of the substrate was left cell-free, to induce spiral wave rotation around an anatomical non-homogeneity.
As a negative control, aspirin (100 μM) had no effect against the spiral waves (n = 3). The mean effective doses of drugs terminating spiral waves are summarized in Figure 8D. In the obstacle model obtained from the NRVM, E-4031 did neither change the spiral core size nor terminate the spiral waves (Supplementary material online, Videos S7 and S8). Our previous report showed no effect of nifekalant in the sheets of NRVM; therefore, it is important to use human-derived cells to investigate the effect of I\textsubscript{Kr} blockers. Additional data are described in the Supplementary material online, Figure S4 and Video S9.

**Discussion**

The present report represents to our knowledge the first study showing reentrant arrhythmias and the effects of anti-arrhythmic drugs in hPSC-derived cardiac tissue. Our main findings are (i) that human stem cell-derived cardiac cell sheets can mimic cardiac tissue with appropriate electrophysiological and biophysical properties, (ii) that human stem cell-derived cardiac cell sheets can serve as a model for investigating arrhythmogenesis in hPSC-derived cardiac tissues, (iii) that alterations in cell density and distribution can affect the induction of spiral waves in characteristic ways, and (iv) that such hPSC-derived cardiac cell sheets can respond to several anti-arrhythmic drugs, and thus serve as valuable platforms for drug screening.

Cultured cardiac cells derived from animal tissue, for example NRVM, serve as the current model for basic study of wave propagation and cardiac arrhythmias. They form a synthetic tissue system under the investigators’ control that is structurally and functionally intermediate between a single cell and the native tissue. However, animal-derived cultured tissues are different from intact human tissues, mainly because there is a difference in the ion channel types between the species, such as the HERG (human ether-a-go-go related gene) channel. In our case, terminations of spiral waves by I\textsubscript{Kr}-blockers were seen in the hPSC-derived cell sheets but not in NRVM-derived cardiac
sheets. We expect that hPSC-derived cultured tissue will serve as a versatile, alternative research tool because they are derived from human tissues and can be generated consistently and in adequate amounts.

Cardiac cells derived from hPSCs have already been reported to mature during differentiation. Mechanical loading induced the hypertrophy of engineered hPSC-derived cardiac tissue; however, there was no report that showed the maturation after making the cell sheets. The cardiac cells after 1-month differentiation by our protocol were mainly ventricular type as analysed by FACS and they showed prolongations of action potential duration as well as suppressions of voltage-dependent K⁺ current with E4031 by the whole cell patch-clamp method. In this study, the CV gradually increased during an additional month of tissue culturing. The gene expression patterns changed from a relatively premature type (alpha-MHC, HCN4) to a mature type (beta-MHC, SERCA2, and HERG). Differentiated cardiomyocytes developed well-organized sarcomeres, and they showed appropriate responses to sodium and Iₖr channel blockers. Both cardiac cell count and percentage of cTnT-positive cells as analysed by FACS did not change between 2 and 7 days, indicating our hPSC-derived cardiac cells were post-mitotic and functionally matured. Moreover, we showed the development of GJs in EM studies as well as up-regulation of connexin-43 with RT–PCR after 1-month culture; therefore, we think these developments of GJs could be a reason for the increase in CV during 1 month after seeding. In Table 1, the CV was slower and CaT 80 was longer than a previous report; however, the difference may be caused by the observing temperature, because the CV and CaT 80 significantly changed after heating as shown in Supplementary results.

Figure 7 Reentrant spiral wave propagation and the effects of nifekalant. (A–C) Dynamics of spiral wave propagation with Fluo-4 before (A) and after administration of 0.5 μM (B) or 1.0 μM (C) of nifekalant. Numbers at the lower right of the panels indicate the time on the second time scale. Merged activation time maps are showed in the right panels. The unexcitable spiral core area (mm²) increased in a dose-dependent manner. The red lines are the trajectories of the spiral wave tips. Numbers at bottom of each merged image show the rotational rates (Hz). The colours in the merged images denote different phases of the excitation, with white colour showing the excitation wave front. White line interval: 400 ms. Scale bar: 2.5 mm. (D) The change of the unexcitable area before and after treatment with nifekalant. *P < 0.05, **P < 0.01 vs. 0 μM.
waves because cardiac-scarce spots may cause lowering of the CV, leading to breaks in the wave. Therefore, the cardiac-scarce spots are necessary for an arrhythmogenic model. We suggest that our tissue culture model may serve as a good pathophysiological model of cardiac arrhythmogenic diseases and as a tool for the evaluation of arrhythmogenic potential.

The evaluation of QT prolongation is important because the HERG-encoded $I_{Kr}$ current has been known as a common cause of drug-associated torsade de pointes. In our experiments, the hPSC-derived cell layer could show not only the drug-induced QT prolongation but also termination of spiral waves by several anti-arrhythmic drugs (nifekalant, E-4031, sotalol, and quinidine); the latter effect is clinically relevant to the acute management of ventricular tachyarrhythmia. Using the cell sheet resulted in a more precise mechanism of the effect of $I_{Kr}$ blockers against tachycardia model. We observed that the excitation front of the rotating wave bumped into the prolonged refractory tail and was forced to rotate around a larger core. Although there were reports using animal models to investigate the anti-tachycardia effects of nifekalant, this is the first report to show these drugs promote termination of tachycardia through destabilization of spiral waves using a human cell model. Sotalol and quinidine are known to modulate a number of ionic currents; therefore, the response was not the same as for nifekalant and E-4031, which are specific blockers of $I_{Kr}$. In summary, our hPSC-derived cardiac cell sheet was seen as a miniaturized model of anti-tachycardia management and a platform for drug discovery and testing for the treatment of arrhythmias.

Supplementary material

Supplementary material is available at European Heart Journal online.

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