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Creation of human cardiac cell sheets using pluripotent stem cells

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ABSTRACT

Although we previously reported the development of cell-dense thickened cardiac tissue by repeated transplantation-based vascularization of neonatal rat cardiac cell sheets, the cell sources for human cardiac cells sheets and their functions have not been fully elucidated. In this study, we developed a bioreactor to expand and induce cardiac differentiation of human induced pluripotent stem cells (hiPSCs). Bioreactor culture for 14 days produced around 8×10^7 cells/100 ml vessel and about 80% of cells were positive for cardiac troponin T. After cardiac differentiation, cardiomyocytes were cultured on temperature-responsive culture dishes and showed spontaneous and synchronous beating, even after cell sheets were detached from culture dishes. Furthermore, extracellular action potential propagation was observed between cell sheets when two cardiac cell sheets were partially overlaid. These findings suggest that cardiac cell sheets formed by hiPSC-derived cardiomyocytes might have sufficient properties for the creation of thickened cardiac tissue.

1. Introduction

Regenerative medicine is thought to be a promising therapeutic strategy for the treatment of severe heart failure. We previously developed an original scaffold-free tissue engineering technology, designated as "cell sheet-based tissue engineering", using temperature-responsive culture dishes covalently bonded to the temperature-responsive polymer poly(*N*-isopropylacrylamide) [1]. Lowering the culture temperature promotes a rapid surface transition from hydrophobic to hydrophilic, which enables collection of a viable monolayer cell sheet with full preservation of the cell-cell contacts and extracellular matrices [2]. Many studies have shown that cell sheet-based bioengineered tissue transplantation improves the cardiac function of various types of heart failure models [3–5]. However, recent evidences have suggested that paracrine mechanisms, including angiogenesis and cardioprotection mediated by the secreted factors of transplanted cells, mainly contribute to the improved cardiac function [3,6]. Furthermore, an adult human heart is reported to contain over a billion cardiomyocytes [7], indicating that creation of bioengineered thickened cardiac tissue *in vitro*, which directly contributes to contraction when transplanted, might be a prerequisite. Previously, we reported the development of cell-dense 1 mm thick cardiac tissue by repeated transplantation of triple-layered neonatal rat cardiac cell sheets [8]. Recently, we have also reported the development of cardiac cell sheets derived from mouse embryonic stem cells (ESCs) after three-dimensional suspension culture [9]. Mouse ESC-derived cardiac cell sheets have similar electrophysiological properties to those of neonatal rat cardiomyocytes, indicating that layered stem cell-derived cardiac cell sheets might show synchronous contraction. However, it remains unknown whether human pluripotent stem cell-derived cardiomyocytes are suitable for creating cell sheets in terms of their electrophysiological functions.

Many recent reports have suggested that human pluripotent stem cells, including ESCs and induced pluripotent stem cells (hiPSCs), differentiate into cardiomyocytes through embryoid body (EB) formation [10,11] and monolayer culture [12,13]. Although suspension culture of EBs is easy in terms of scale-up, advancements to overcome the limitation of EB size heterogeneity for efficient cardiac differentiation might be necessary. Conversely,

Abbreviations: hiPSCs, human induced pluripotent stem cells; ESCs, embryonic stem cells; EB, embryoid body; cTnT, cardiac troponin T; vWF, von Willebrand factor; MEFs, mouse embryonic fibroblasts; NEAA, nonessential amino acid; CM, conditioned medium; MED, multi-electrode array.

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the monolayer-based method is able to consistently produce cardiomyocytes with high efficacy, but scale-up might be a significant challenge. Recent methodological progress has enabled production of >80% cardiomyocytes in both methods [14,15]. However, the differences in cardiomyocyte properties from the viewpoint of cell sheet-based tissue engineering remain elusive.

The aims of this study were to establish easy and effective methods for collecting cardiomyocytes derived from hiPSCs to create cardiac cell sheets, and to elucidate the electrophysiological functions of hiPSC-derived cardiac cell sheets.

2. Materials and methods

2.1. Antibodies

The following antibodies were used for immunocytochemistry and/or flow cytometry: anti-sarcomeric α-actinin (Sigma–Aldrich, St. Louis, MO), anti-cardiac troponin T (cTnT; Thermo Scientific, Rockford, IL), anti-CD31 (BD Bioscience, San Jose, CA) and anti-Tra-1 60 (Millipore, Billerica, MA) mouse monoclonal antibodies, anti-SM22 (Abcam, Cambridge, UK), anti-connexin 43 (Enzo Life Sciences, Farmingdale, NY) and anti-von Willebrand factor (vWF, Dako, Japan) rabbit polyclonal antibodies, and an anti-Nkx2.5 goat polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Unless specified otherwise, reagents were purchased from Life Technologies, CA.

2.2. Human iPSC culture

Human iPSCs (253G1) were purchased from RIKEN (Tsukuba, Japan) and maintained in Primate ES Cell Medium (ReproCELL, Japan) supplemented with 5 ng/ml basic fibroblast growth factor (bFGF; ReproCELL) on mitomycin C-treated mouse embryonic fibroblasts (MEFs; ReproCELL). Cells were passaged as small clumps every 3–4 days using CTK solution (ReproCELL).

For monolayer cardiac differentiation, hiPSCs were adapted and maintained on Matrigel (growth factor reduced, 1:60 dilution) in MEF-conditioned medium (MEF-CM) supplemented with 10 ng/ml bFGF. Mitomycin-C treated MEFs were seeded at approximately 6×10^5 cells/cm^2 in DMEM (Sigma–Aldrich) supplemented with 10% FBS, 2 mM L-glutamine and 1% nonessential amino acid (NEAA) onto tissue culture dishes precoated with 0.5% gelatin. One day after seeding MEFs, the culture medium was exchanged with ESC medium (80% Knock-out DMEM, 20% Knock-out Serum Replacement, 1% NEAA, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol (Sigma–Aldrich) and 5 ng/ml bFGF). MEF-CM was collected everyday for 7 days and supplemented with an additional 5 ng/ml bFGF before use.

2.3. Bioreactor system

The culture process in the bioreactor system (Fig. 1A) is shown in Fig. 1B. Following CTK solution treatment, hiPSC aggregates (approximately 2×10^7 cells) from 10 culture dishes (10 cm diameter) were resuspended in 100 ml mTeSR1 (STEMCELL Technologies Inc., Canada) containing 10 μM Y27632 (Wako, Japan) and seeded into a 250 ml stirred bioreactor (Bio Jr. 8; ABLE Co., Japan). A 2-bladed delta-like paddle was used (Fig. 1A). The bioreactor was equipped with a temperature sensor, pH electrodes, as well as inoculation-, harvest- and sample-ports. Dissolved oxygen was monitored using a Fibox3 optical sensor (PreSens, Germany). Data acquisition and process control were performed with a digital control unit and process control software for the MiniJar8 100 ml bioreactor. Aeration was performed by headspace. The agitation rate was 40 rpm, dissolved oxygen was maintained at 40% with air, oxygen or nitrogen, pH was maintained at 7.2 by CO_2 addition, and the temperature was maintained at 37 °C for the entire process. After 1 day, cells were cultured in mTeSR1 without Y27632, and the medium was exchanged every day until day 3.

2.4. Cardiac differentiation in the bioreactor

Three days after starting cultures in the bioreactor system, EBs were cultured in StemPro34 medium containing 50 μg/ml ascorbic acid (Sigma–Aldrich), 2 mM L-glutamine and 400 μM 1-thioglycerol (Sigma–Aldrich). The following growth factors and small molecule were used at the corresponding days: days 3–4, 0.5 ng/ml BMP4 (R&D systems, Minneapolis, MN); days 4–7, 10 ng/ml BMP4, 5 ng/ml bFGF, 3 ng/ml activin A (R&D Systems); days 7–9, 4 μM IWR-1 (Wako); after day 9, 5 ng/ml VEGF (R&D Systems) and 10 ng/ml bFGF. At days 4, 7, 9, 11 and 14, the culture medium was exchanged.

2.5. Cardiac differentiation in a monolayer

Cardiac differentiation was induced as previously reported with a few modifications as shown Fig. 1C. hiPSCs cultured in MEF-CM on Matrigel were treated with versene for 7–10 min, and then the single cell suspension was seeded onto Matrigel-coated dishes at 1×10^5 cells/cm^2 in MEF-CM with an additional 5 ng/ml bFGF and 10 μM Y27632 at 3 days before cardiac induction. One day before cardiac induction, cells were covered with Matrigel (1:60 dilution). For cardiac induction, the medium was changed to RPMI 1640 containing B27 supplement without insulin. The following growth factors were used at the corresponding days; days 0–1, 100 ng/ml activin A; days 1–4, 10 ng/ml BMP4 and 10 ng/ml bFGF. After day 4, cells were cultured without any growth factors, and the culture medium was changed every other day.

2.6. Flow cytometric analysis

Cells at day 14 in the bioreactor and at day 12 in monolayer culture were dissociated with Accumax (Millipore) for 10 min. Tra-1 60 staining was performed according to the manufacturer's instructions for the antibody. For CD31 staining, the antibody was diluted in PBS containing 5% FBS. For cTnT staining, cells were fixed with 4% paraformaldehyde for 10 min, and then stained with the antibody diluted in PBS with 5% FBS and 0.2% Nonidet P 40 (Nacalai Tesque, Japan). Stained cells were analyzed using a Quanta (Beckman Coulter, Brea, CA) and Quanta SC software.

2.7. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, and the immunostaining methods have been described previously [3]. Samples were imaged by laser confocal microscopy (Carl Zeiss, Germany) and Image Express (Molecular device, Sunnyvale, CA) with MetaXpress and AcuityXpress software (Molecular device).

2.8. Cell sheet preparation

Prior to seeding cells, the surface of temperature-responsive dishes (UpCell; CellSeed, Japan) was coated with FBS for 2 h. After cardiac differentiation, cells were dissociated with 0.05% trypsin/EDTA, cell aggregates were removed using a strainer (BD Bioscience), and single cells were plated onto the UpCell at 2.1×10^5 cells/cm^2 in DMEM supplemented with 10% FBS and 10 μM Y27632 at 37 °C in a humidified atmosphere with 5% CO_2. At days 1 and 3, the medium was exchanged with prewarmed DMEM supplemented with 10% FBS. After 5 days in culture, cell sheets were

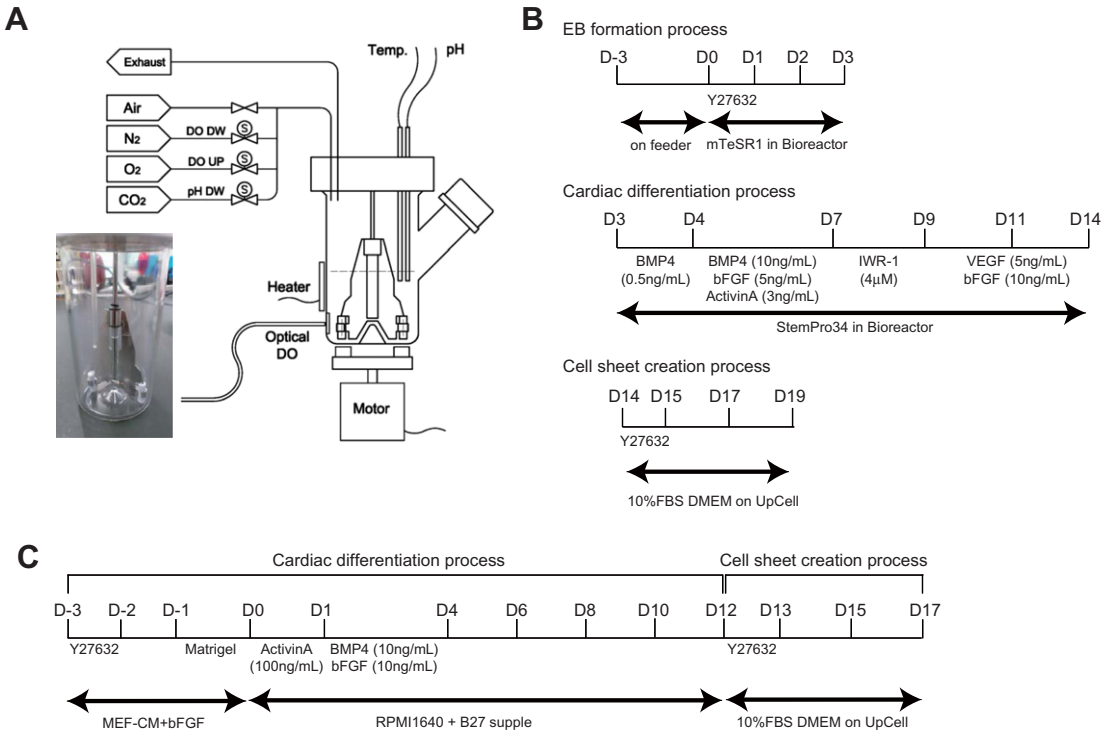

Fig. 1. Scheme of the bioreactor and culture process (A) Schematic of the bioreactor system. The photograph shows the impeller. (B) Schematic of the culture process for cardiac differentiation in the bioreactor system. (C) Schematic of the culture process for cardiac differentiation in monolayer culture.

harvested and layered using the cell sheet stacking technique as described previously[9].

2.9. Electrophysiological analysis

Electrical activities of cardiomyocyte sheets were obtained from extracellular potentials measured by a multi-electrode array (MED) system (Alpha MED Sciences, Osaka, Japan) as described previously[9]. Intracellular calcium transient of cardiomyocytes in the cell sheet was visualized using a Rhod-3 imaging kit (Life Technologies) according to the manufacturer's instructions. Fluorescence and phase contrast images were obtained by a CCD camera (HiSCA, HAMAMATSU, Japan) connected to the fluorescence microscope (ELIPSE TE2000-U, Nikon, Japan).

2.10. Statistics

Data were presented as the means ± standard deviation.

3. Results

3.1. Cardiac differentiation of hiPSCs

Various types of methods to induce cardiac differentiation of human pluripotent stem cells have been reported [10–12]. In this study, we used two types of methods; (1) suspension culture in the bioreactor, and (2) monolayer culture. Small aggregates of hiPSCs (approximately 2×10^7 cells) were seeded in the bioreactor containing mTeSR1 medium, resulting in the formation of many EBs, and the cell number increased up to around 4×10^7 cells at day 3 (Fig. 2A and B). Then, EBs were treated with several growth factors and a Wnt inhibitor as shown in Fig. 1B. At day 14, the cell number was slightly increased to around 8×10^7 cells (Fig. 2B), and almost all of the remaining EBs showed spontaneous beating (Supplementary Video 1). Z-stack images of immunocytochemical analysis showed that numerous areas of EBs were composed of cTnT-positive cardiomyocytes (Fig. 2C). Furthermore, almost all cTnT-positive cells expressed Nkx2.5 and SM22 (Fig. 2D), suggesting that differentiated cardiomyocytes showed a fetal phenotype. On the other hand, vWF-positive endothelial cells were distributed in minor areas (Fig. 2C), but showed a microvascular network (Fig. 2E). Flow cytometric analysis revealed that about 80% of cells were positive for cTnT, 5% of cells were positive for CD31 and 1% of cells were positive for Tra-1 60 (Fig. 2F). These findings indicate that bioreactor-based EB suspension culture with suitable growth factor and small molecule treatments might sufficiently induce cardiac differentiation of hiPSCs to create cell sheets.

Next, we induced cardiac differentiation of hiPSCs in monolayer culture (Fig. 1C). Spontaneously beating cells were observed from day 10, and robust pulsation was observed macroscopically all over the culture dish at day 12 (Video 2). Flow cytometric analysis showed that about 80% of cells were positive for cTnT (Fig. 2G). Furthermore, cardiac differentiated cells expressed cTnT and sarcomeric α-actinin with a fine striated pattern (Fig. 2H). The cell number was increased from 1×10^5 to 3.5×10^5 cells/cm^2 at day 12 (Fig. 2I). These findings suggest that the monolayer cardiac induction method might also be suitable to collect sufficient cardiomyocytes to create cardiac cell sheets.

3.2. Creation of cardiac cell sheets

Next, we created cardiac cell sheets using hiPSC-derived cardiomyocytes and UpCell temperature-responsive culture dishes. After cardiac differentiation in the bioreactor, cells were dissociated with trypsin and seeded onto the UpCell. One day after seeding (D15 in Fig. 1B), some spontaneously beating cells were observed (Video 3). Moreover the number of spontaneously beating cells increased over time, and synchronous beating over the entire dish

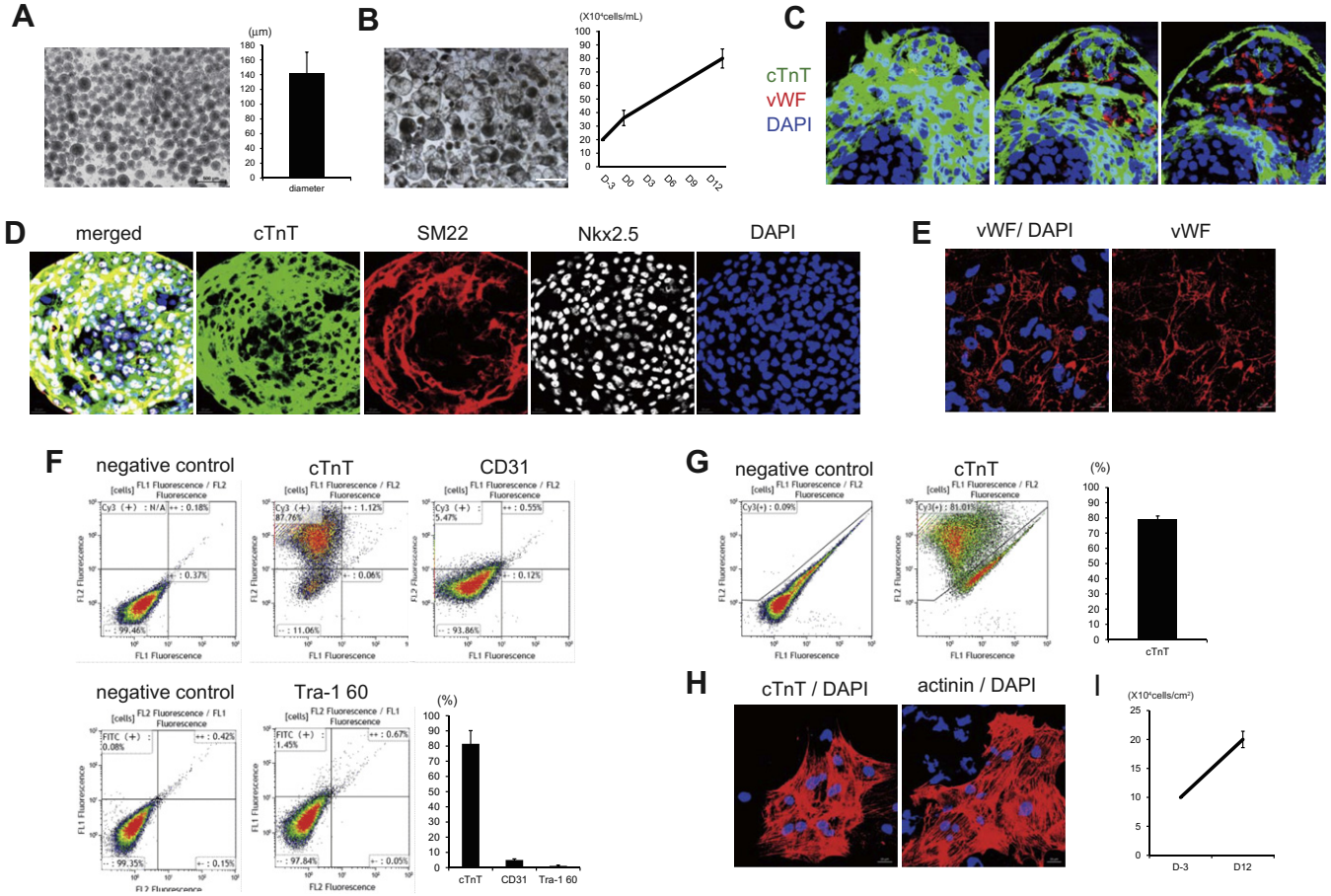

Fig. 2. Cardiac differentiation of hiPSCs (A–F) Results of the bioreactor experiments. (A) Representative image (left) and the diameter (right) of EBs at day 3. Bar, 500 μm. (B) Representative image of EBs at day 15 (left). Bar, 500 μm. Right, the cell number during bioreactor culture. (C) Z-stack images of EBs stained for cTnT (green) and vWF (red). The interval between images is 80 μm. Nuclei were stained with DAPI. Bar, 20 μm. (D) Almost all cells in EBs consisted of cTnT-positive cells that co-expressed Nkx2.5 and SM22. Nuclei were stained with DAPI. Bars, 20 μm. (E) Endothelial microvascular networks in EBs. Endothelial cells were stained for vWF. Nuclei were stained with DAPI. Bars, 20 μm. (F) Flow cytometric analyses at day 15. The percentage of cells expressing each protein was calculated and shown in the graph ($n = 2$). (G–I) Results of monolayer culture experiments. (G) Flow cytometric analyses at day 15. The percentage of cTnT-positive cells was calculated and shown in the graph ($n = 3$). (H) Differentiated cells expressed cTnT (left) and sarcomeric α-actinin (right). Nuclei were stained with DAPI. Bars, 20 μm. (I) The cell number during monolayer culture.

was observed at day 5 (D19 in Fig. 1B) (Video 4). Cells were cultured in a 20 °C incubator to create a cell sheet (Fig. 3A) that showed synchronous and spontaneous contraction when floating (Video 5). To examine the cell distribution and components in the cell sheets, immunocytochemical analysis was performed. High content confocal image analysis revealed that about 80–90% of cells were positive for cTnT and sarcomeric α-actinin and these cells also expressed Nkx2.5 and SM22 (Fig. 3C and E), indicating that the percentage of cardiomyocytes in the cell sheets was consistent with the results of flow cytometric analyses of EBs (Fig. 2F). On the other hand, almost all of the non-cardiomyocyte area was filled with SM22-positive cells without co-expression of cTnT (Fig. 3B, C and E), suggesting the presence of mural cells. We also observed a very small number of vWF-positive endothelial cells in cell sheets (Fig. 3D). These findings indicate that cardiac cell sheets were mainly composed of cardiomyocytes and mural cells.

We also created cardiac cell sheets using cardiomyocytes obtained by differentiation in monolayer culture. Cells were dissociated with trypsin and seeded onto the UpCell, which showed spontaneous and synchronous beating cells after 3 days of culture (D15 in Fig. 1C) (Video 6). However, cardiomyocytes showed colony formation (Fig. 3F and G) that led to an unequal distribution of cardiomyocytes in the cell sheets. These findings suggest that different cardiomyocyte characteristics result from bioreactor- and monolayer-based cardiac inductive methods, and cardiomyocytes obtained from bioreactor-based cardiac differentiation are more suitable for creating cell sheets in the current protocol.

3.3. Electrophysiological evaluation of hiPSC-derived cardiac cell sheets

Consistent with the results of cardiomyocytes in cell sheets showing spontaneous and synchronous beating, connexin 43 expression was observed at the edge of adjacent cardiomyocytes (Fig. 4A). To confirm the electrophysiological connections among cardiomyocytes in cell sheets, extracellular action potentials were measured using the MED system. As shown in Fig. 4B, synchronous extracellular action potential excitation was observed in all areas. Furthermore, to clarify the electrophysiological connections between cell sheets, a cell sheet was seeded onto one side of the MED system. After 30 min, another cell sheet was seeded onto the other side of the MED system. Under this condition, the two cell sheets were partially overlaid and synchronous beating in the two cell sheets was observed (Fig. 4C). Furthermore, the synchronous beating of two and three overlaid cardiac cell sheets was confirmed by the results of intracellular calcium transient images (Videos 7 and 8). These findings indicate that electrical communication was established between hiPSC-derived cardiac cell sheets.

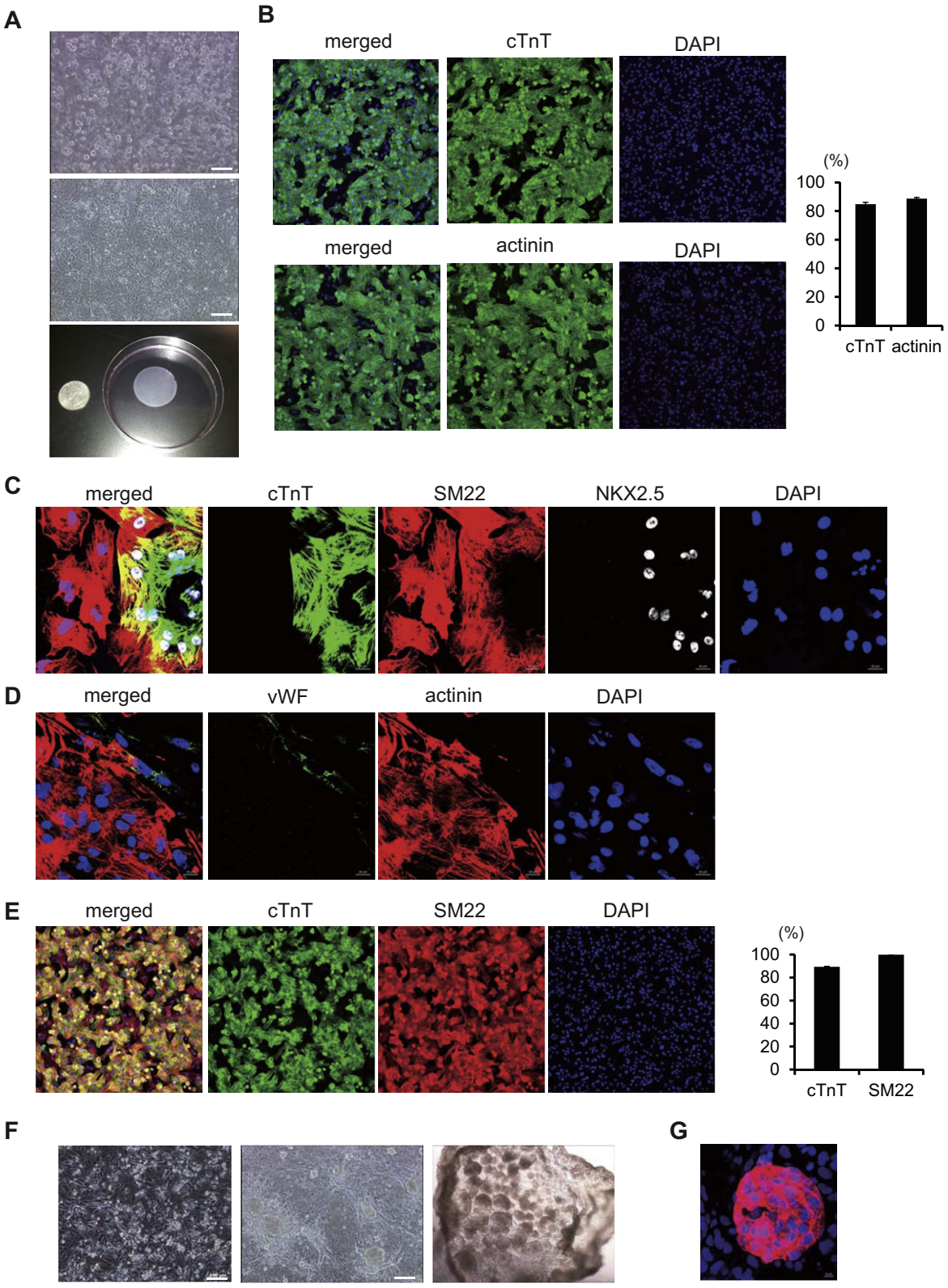

Fig. 3. Immunocytochemical evaluation of cardiac cell sheets (A–E) Results using cells from the bioreactor. (A) Representative images of cells after re-seeding on the UpCell, and a cell sheet. Upper, day 1. Middle, day 5. Bars, 100 μm. Lower, a cell sheet from a 10 cm dish. (B) Confocal image analysis of the percentage of cardiac protein-expressing cells in cell sheets. Left panels are representative images. Upper, cTnT. Lower, actinin. The percentage of cells expressing each protein was calculated and shown the graph (right). Nuclei were stained with Hoechst 33258. Original magnification, 20×. (C) The non-cardiomyocyte area was composed of SM22-positive cells that did not express cTnT or Nkx2.5. Nuclei were stained with DAPI. Bar, 20 μm. (D) Endothelial cells (vWF) in the cell sheet. Nuclei were stained with DAPI. Bar, 20 μm. (E) Confocal image analysis of the percentage of cTnT- and SM22-positive cells in cell sheets. Left panels show representative images. Nuclei were stained with Hoechst 33258. Original magnification, 20×. The percentage of cells expressing each protein was calculated and shown the graph (right). (F and G) Results using cells from monolayer culture. (F) Representative images of cells after re-seeding on the UpCell, and a cell sheet. Left, day 1. Middle, day 5. Bars, 100 μm. Right, a cell sheet from a 3.5 cm dish. Original magnification, 2×. (G) Representative confocal microscopic image of cardiomyocytes (cTnT) in a cell sheet. Nuclei were stained with DAPI. Bar, 20 μm.

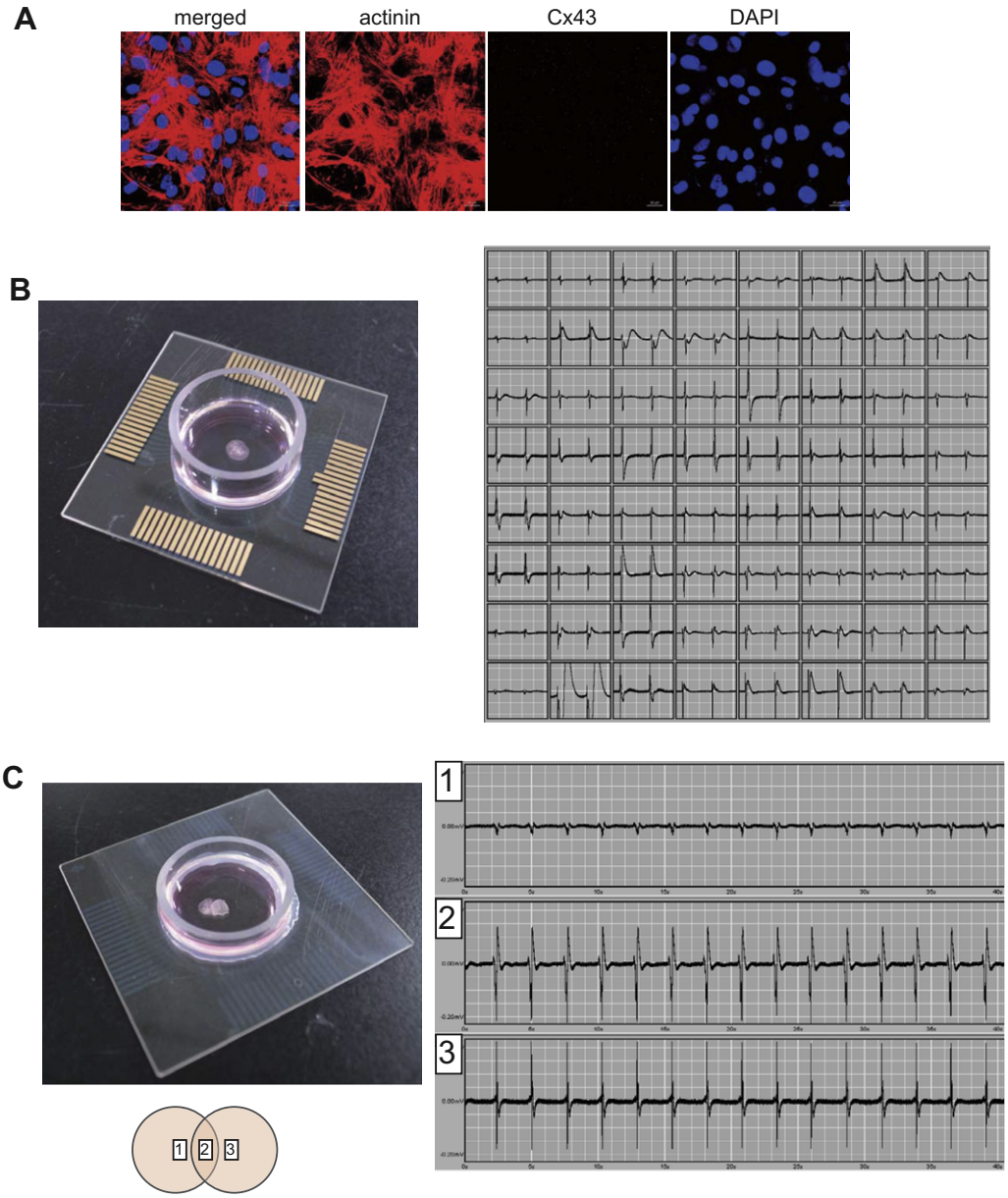

Fig. 4. Electrophysiological evaluation of cardiac cell sheets (A) Connexin 43 expression between cardiomyocytes (actinin). Nuclei were stained with DAPI. Bar, 20 μm. (B) Extracellular action potential evaluation of a monolayer cardiac cell sheet. Left panel shows a macroscopic image of the cell sheet on the MED system. Synchronous extracellular action potential excitation was observed in all areas. (C) Electrophysiological connections between cell sheets. The left panel shows a macroscopic view of partially overlaid cell sheets on the MED system. The right panel shows the extracellular action potential at each electrode position. The number in the lower panel shows the position of the electrode under the cell sheets. Number 2 is under the overlaid area.

4. Discussion

The present study shows that a bioreactor-based method sufficiently induces cardiac differentiation of around 80% of hiPSCs. Cardiomyocytes in the cell sheets showed spontaneous and synchronous beating, and the cell sheet itself showed macroscopic contraction. Furthermore, electrophysiological connections were observed between cardiac cell sheets. Therefore, human pluripotent stem cell-derived cardiac cell sheets might be used to construct functional thickened cardiac tissue.

Many reports have indicated that human pluripotent stem cells can differentiate into cardiomyocytes. EB formation is wildly used for differentiation of pluripotent stem cells, and stage-specific treatments with specific growth factors enhance cardiac differentiation [16]. Moreover, some methods, including forced aggregation, have been reported to overcome the limitation of EB size heterogeneity in terms of cardiac differentiation efficacy [14]. However, these methods are technically complex and time consuming. In this study, we have developed a suspension culture bioreactor system for cardiac differentiation. The suitable agitation system enabled formation of homogeneously sized EBs, to a certain extent, simply by seeding cell aggregates. Furthermore, because it has been reported that hypoxia is important to enhance cardiac differentiation [10], the system was capable of strict regulation of a low dissolved oxygen concentration. Collectively, the integration of mainly homogeneously sized EBs by modest agitation, a hypoxic condition and appropriate treatments with growth factors and a small molecule enabled us to successfully induce the differentiation of cardiomyocytes with high efficacy.

On the other hand, we also used a monolayer-based method for cardiac differentiation. Matrigel was overlaid on cells at 1 day prior to differentiation, and bFGF was added from day 1–4, resulting in robust cardiac differentiation at day 12. However, these cardiomyocytes showed colony-like formation after re-seeding onto the UpCell (Fig. 3F), which might lead to an unequal cardiomyocyte distribution in cell sheets. These differences in the phenotypes of cardiomyocytes between bioreactor-based and monolayer-based methods might be explained by the difference in cardiomyocyte maturation levels and the phenotype of mural cells. The optimized timing of cardiomyocyte harvesting after differentiation might overcome this limitation.

In this study, the cardiac cell sheets were mainly composed of cardiomyocytes and partially composed of mural cells. The ratio of cardiomyocytes to mural cells in a cell sheet was approximately 8:2. We previously reported that purified cardiomyocytes do not form cell sheets, and a certain level of fibroblasts is necessary to create mouse ESC-derived cardiac cell sheets with an optimized ratio of cardiomyocytes/fibroblasts of 8:2 [9].The existence of non-cardiomyocytes potentially expressing extracellular matrix components might be important to form human cardiac cell sheets. Recently, several methods have been reported for the purification of cardiomyocytes from human pluripotent stem cells using an antibody [13,17], mitochondrial labeling [18] and genetic modification [19]. These cardiomyocyte purification methods will enable us to optimize the ratio of cardiomyocytes/non-cardiomyocytes for creating more functional cardiac tissue.

Competing interests statement: Tatsuya Shimizu and Masayuki Yamato are consultants for CellSeed, Inc., Teruo Okano is an investor in CellSeed, Inc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.089.

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