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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