ABSTRACT

Functional and Electrical Integration of Induced Pluripotent Stem Cell-Derived Cardiomyocytes in a Myocardial Infarction Rat Heart

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In vitro expanded beating cardiac myocytes derived from induced pluripotent stem cells (iPSC-CMs) are a promising source of therapy for cardiac regeneration. Meanwhile, the cell-sheet method has been shown to potentially maximize survival, functionality and integration of the transplanted cells into the heart. It is thus hypothesized that transplanted iPSC-CMs in a cell-sheet manner may contribute to functional recovery *via* direct mechanical effects on the myocardial infarction (MI) heart.

: F344/NJcl-rnu/rnu rat were left coronary artery-ligated (n=30), followed by transplantation of Dsred-labeled iPSC-CMs cell-sheets of murine origin over the infarct heart surface. Effects of the treatment were assessed, including *in vivo* molecular/cellular evaluations using a synchrotron radiation scattering technique. Ejection fraction and activation recovery interval were significantly greater from day 3 onwards after iPSC-CMs transplantation compared to those after sham operation. A number of transplanted iPSC-CMs were present on the heart surface expressing cardiac myosin or connexin43 over two weeks, assessed by immunoconfocal microscopy, while mitochondria in the transplanted iPSC-CMs gradually showed mature structure as assessed by electronmicroscopy. Of note, X-ray diffraction identified 1,0 and 1,1 equatorial reflections attributable to myosin and actin-myosin lattice planes typical of organized cardiac muscle fibers within the transplanted cell-sheets at 4 weeks, suggesting cyclic systolic myosin mass transfer to actin filaments in the transplanted iPSC-CMs.

Transplantation of iPSC-CM cell-sheets into the heart yielded functional and electrical recovery with cyclic contraction of transplanted cells in the rat MI heart, indicating that this strategy may be a promising "cardiac muscle replacement" therapy.

Keywords: iPS cell; regeneration therapy; cell-sheet; synchrotron imaging

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INTRODUCTION

To increase the number of functional cardiomyocytes in the heart is a goal of regenerative therapy for advanced cardiac failure (14). It has been shown that induced pluripotent stem cells (iPSCs) differentiate into functional cardiomyocytes *in vitro* by specific culture regimens, suggesting that replacement of damaged cardiac tissue might be achieved by transplantation of iPSCs-derived cardiomyocytes (iPSC-CMs) into the damaged area using an appropriate cell-delivery method. Further, it has been shown that the cell-sheet method, in which a scaffold-free sheet-shaped cultured cell-cluster is placed on the surface of the heart, delivers a large number of the cells while preserving the functionality of the cells and the myocardium, indicating that the cell-sheet method may be an ideal delivery method of iPSC-CM to replace the damaged cardiac area (8,9,15). In fact, we reported that transplantation of iPSC-CMs into the heart by the cell-sheet method improves functional performance of the infarcted heart in pigs (5). However, in that study (5), synchronous contraction of the transplanted iPSC-CMs as "cardiac myocytes" that express the regular, cyclic actin-myosin cross-bridge motion, which is the aim of this treatment, was not demonstrated due to the limitations of current image analysis methods *in vivo*.

Shiba et al. reported that the fluorescent signal of calcium sensor, GCaMP3, which has been genetically encoded in the cells prior to transplantation into the heart, was useful for visualizing spontaneous contraction of the transplanted cells in the heart *in vivo* (16). However, the calcium sensor signal does not necessarily correlate with normal cyclic actin-myosin cross-bridge motion. On the other hand, third generation synchrotron radiation (SPring-8, Hyogo, Japan) has been utilized to quantify actin-myosin cross-bridge dynamics in cardiac fibres of localized regions *in vivo* (12,17). We herein hypothesized that transplanted iPSC-CMs in a cell-sheet manner may contribute to functional recovery *via* direct mechanical

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effects on myocardial infarction (MI) heart. We therefore explored functional and electrical integration of the transplanted iPSC-CMs in the acute MI rat heart using the latest imaging modality utilising synchrotron radiation from a third generation facility.

METHODS

Studies were performed with the approval of the ethics committee of Osaka University Graduate School of Medicine and the Animal Experiment Review Committee of the Japan Synchrotron Radiation Research Institute. All animals used in this study received care in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No 85-23, revised 1996).

Cell-culture and cell-sheet generation

Germline-competent mouse iPSC-line 256H18 was established by introducing only *Oct3/4*, *Sox2*, and *Klf4* (without *c-Myc*), constitutively expressing red-fluorescent protein (Dsred) (generously contributed by Professor S Yamanaka, Kyoto University, Japan) (11). Maintenance of the 256H18 iPSCs and induction of cardiomyogenic differentiation was performed following the protocol established in Professor Yamanaka's and our laboratories, respectively (7,23). Briefly, the iPSCs were maintained on feeder layers of mitomycin C-treated mouse embryonic fibroblasts (Chemicon, Billerica, MA). Embryoid bodies (EBs) were then generated under the presence of 6-bromoindirubin-3′-oxime (Calbiochem, Darmstadt, Germany) (23).

The EBs were plated on 12-well temperature-responsive culture dishes (CellSeed, Tokyo, Japan) at 37°C with the EB number adjusted to 20 per well, thereafter the EBs were differentiated in the serum free medium with insulin-transferin-selenium-X (Invitrogen,

Carlsbad, CA). Subsequently, the dishes were removed to refrigerator set at 20°C, while scaffold-free iPSC-CM cell-sheets detached spontaneously from the dish surfaces (23). Cardiac troponin T positivity in this preparation assessed by immunohistolabelling was consistently 70-80% (7,23).

Generation of acute myocardial infarction (MI) model and cell-sheet transplantation

Female F344/NJcl-rnu/rnu rats of 6 weeks of age (Crea, Tokyo, Japan) were anaesthetized by inhalation of isoflurane (1.5%) and endotracheally intubated for mechanical ventilation. The left coronary artery was then permanently occluded under thoracotomy (3). Two weeks after the ligation, the cell-sheet generated by iPSC-CMs or mitomycin C-treated mouse embryonic fibroblasts were simply placed on surface of the left ventricle (LV, n=6 each) (6). Bupivacaine (1% in saline, 250 μl) was subcutaneously injected near the incision line to minimize the postoperative pain and the rats were then recovered in a temperature-controlled individual cage.

Transthoracic echocardiography analysis

Transthoracic echocardiography was performed using a system equipped with a 12-MHz transducer and SONOS5500 (Agilent Technologies, Palo Alto, CA) under isoflurane inhalation (1%). Diastolic/systolic dimensions (Dd/Ds), and ejection fraction (EF) of the LV were measured (n=6 each) (3).

Telemetry study

A telemetry transmitter with two electrodes (Data Sciences International) was implanted prior to the treatment (day 14 post-MI) under inhalation of 1.5% isoflurane (n=4 each) (3). Electrocardiogram was then continuously monitored over 7 days. Ventricular premature

contractions (VPC) were detected and the frequency of the VPC was expressed as the number of VPC per day divided by the daily cumulative heart beats.

Electrical potential mapping analysis

Electrical potential mapping study was performed under the repeated left thoracotomies in the same animal at day 2, 3, 4, 7, and 14 (n=4 each group). There was no repeated procedures-related morbidity or mortality. A sixty-four electrical potential mapping system (Alpha MED Scientific, Osaka, Japan) was directly placed on surface of the LV *via* the thoracotomy under general anaesthesia and endotracheal intubation with mechanical ventilation as above. The electrical potential was recorded as the calculated activation recovery interval (ARI) in the same animals at the indicated time-points (n=4 each group).

Synchrotron small-angle scattering study

The fundamentals of synchrotron small-angle scattering techniques for the investigation of cross-bridge dynamics in the intact heart are presented in detail elsewhere (17). In brief, total thoracotomy was performed under general anaesthesia and endotracheal intubation with mechanical ventilation as above at the synchrotron radiation facility SPring-8 (n=4 in iPSC-CMs treated rats and in MI-only rats) as described in detail elsewhere (13). Cardiac catheterisation was performed to allow continuous LV pressure-volume recordings simultaneous with all SAXS and arterial pressure recordings. Pressure-volumetry was used to establish the timing of the cardiac cycle in all treatment periods and to permit assessment of actin-myosin contributions to global LV function. Heart rate (HR) was determined from the interval between end-diastolic (ED) events in the pressure-volume loops. Hemodynamic data were recorded using CHART (v5.5.6, ADInstruments, NSW) at a sampling rate of 1000/s.

A collimated quasi-monochromatic beam with 0.08 nm wavelength (15 keV),

dimensions 0.2 × 0.1mm (horizontal × vertical) and beam flux ~10^{12} photons/s (ring current 90–100 mA) was focused on the surface myocardium at an oblique tangent (rat ~3m from the detector). SAXS sequences (12 bit, 144 × 150 pixels) each lasting <2.1s were collected at a sampling interval of 15 ms with the aid of an image intensifier (V5445P, Hamamatsu Photonics, Hamamatsu, Japan) and a fast charge-coupled device camera (C4880-80-24A, Hamamatsu Photonics). Patterns were then digitally recorded using HiPic32 software (v5.1.0 Hamamatsu Photonics). With rats in a supine position, X-ray diffraction profiles were recorded vertically through the iPSC-CMs cell-sheet grafts and infarcted myocardium of the anterior LV wall of the exposed *in situ* beating hearts (12). Periodically between diffraction recordings a laser aligned with the X-ray beam was used to determine the point of path trajectory at which the beam also passed through normal myocardium. Diffraction patterns obtained from in situ iPSC sheets were of lower intensity compared to normal myocardium (remote regions) and easily distinguished on the basis of established cardiac fibre-intensity peak orientations (21).

Using custom software the average radial line profile around the centre of the spectrum was calculated using a three point background curve fitting process with manual definition of peak spectra limits. Background subtraction was then performed between user-defined inner and outer limits on either side of the 1,0 and 1,1 reflections. The integrated intensity of the 1,0 and 1,1 reflection intensities was then determined from the areas under the reflection peaks, defined as $I_{1,0}$ and $I_{1,1}$ respectively.

Enzyme-linked immunosorbent assay (ELISA)

Culture supernatants of cell-preparation were centrifuged to remove debris and cells. Content of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in the undiluted culture supernatants was determined with an ELISA kit (R&D Systems, MN, USA).

Immunohistolabelling analysis

Under anaesthesia by 5% isoflurane inhalation, heart was promptly excised, immersed in 4% paraformaldehyde, cut transversely and then frozen (n=5 each group). Ten μm-cryosections were labeled with monoclonal anti-cardiac myosin (Molecular Probes, Eugene, OR, USA), or monoclonal anti-connexin(Cx)43 (Millipore, Billerica, MA, USA) antibodies. The labelled sections were again labelled by the secondary antibodies (AlexaFluor488, or AlexaFluor594 phalloidin, Invitrogen), counterstained with 6-diamidino-2-phenylindole (DAPI, Invitrogen) and then assessed by immunoconfocal microscopy (FV1000D, Olympus, Tokyo, Japan)

Electron microscopy analysis

Sliced cardiac tissues were fixed with 2% glutaraldehyde in 0.1 mM phosphate buffer (pH 7.4) for 60 minutes at 4°C, washed and immersed overnight in PBS at 4°C, and fixed in 1% buffered osmium tetroxide, then dehydrated through graded ethanol and embedded in epoxy resin. Ultrathin sections (85 nm) were double-stained with uranyl acetate and lead citrate, and were observed under electron microscopy (H-7600; Hitachi, Tokyo, Japan).

Statistical analysis

All values were expressed as mean±standard deviations. Contents of VEGF and HGF in the supernatant of the four different cultures were compared by one-way ANOVA followed by Bonferroni's test for individual significant difference. Frequency of the VPC, ARI and LVEF were compared by two-way ANOVA followed by Bonferroni's tests for individual significant differences. $P < 0.05$ was considered to be statistically significant.

RESULTS

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iPSCs-derived cardiomyocytes as a source of potential paracrine effects

It has been shown that cell transplantation into the heart produces "paracrine effects", in which the transplanted cells release a variety of protective factors into the adjacent native cardiac tissue to enhance native regenerative process, such as neo-angiogenic, anti-fibrotic, or anti-apoptotic effects (2). Capacity to release protective factors, such as VEGF and HGF, which have been shown to be the most important factors in the paracrine effects, were investigated here *in vitro*. Supernatant of the culture dishes of iPSC-CMs and fibroblasts was collected to measure concentration of VEGF and HGF by ELISA, showing that concentration of VEGF and HGF was not significantly different in the conditioned medium of the iPSC-CMs and the fibroblasts, suggesting potential "paracrine effects" of the iPSC-CM transplantation therapy.

Electrical integrity and functional recovery after cell-sheet transplantation in vivo

Cell-sheet method has been shown to transplant abundant somatic-tissue derived cells into the heart, which can be integrated into the cardiac tissue with minimal damage to the transplanted cells and to the myocardium (9). Functional integration of cell-sheets generated by iPSC-CM into the heart is, however, poorly understood. We speculate that similarity of the phenotype and/or character of the iPSC-CM to the native CM might result in a better integration into the native cardiac tissue compared to somatic tissue-derived cells.

Scaffold-free cell-sheets generated by Dsred-labeled iPSC-CMs of mouse origin were transplanted into the nude rat heart that had been subjected to permanent occlusion of the left coronary artery prior to the cell-sheet transplantation. The cell-sheet generated by Dsred-labelled fibroblasts, were used as controls. Electrical integrity and arrhythmogenicity were assessed by daily Holter ECG monitoring and 64-channel electrical potential mapping. In addition, global cardiac function was serially assessed by transthoracic echocardiography.

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Electrical potential mapping identified multiple ectopic excitations over the cell-sheet transplanted area in the iPSC-CM group until day 2 (day 16 post-MI), which gradually disappeared from day 3 onwards (day 17 post-MI) (Figure 1A). In contrast, transplantation of the cell-sheet derived from fibroblasts, or MI-only rarely induced ectopic excitations over the study period. Ventricular premature contractions more frequently occurred post-iPSC-CM cell-sheet transplantation than those post-sham operation (Figure 1B). In addition, ARI was significantly less in the iPSC-CM group than the other groups from day 3 onwards (day 17 post-MI) (Figure 2A). Moreover, LVEF was significantly greater in the iPSC-CM group than the other groups between day 3 and day 14 (day 17 and day 28 post-MI) (Figure 2B). These findings indicate that the transplanted cell-sheets of iPSC-CMs generated functional and electrical integration into the acute MI rat heart more rapidly than those of fibroblasts.

In vivo recording of actin-myosin cross-bridge activity in the transplanted iPSC-CMs in the heart

While transplantation of iPSC-CM into the rat infarcted heart was shown to induce functional and electrical recovery, mechanical or functional behavior of each transplanted iPSC-CMs in the infarcted heart remains unclear. Actin-myosin cross-bridge interactions in the transplanted iPSC-CM in the rat infarcted heart was therefore investigated using fast synchrotron small-angle X-ray scattering.

At 4 weeks after the transplantation of iPSC-CM cell-sheets on the surface of the infarcted heart (6 weeks post-MI), the rats were subjected to removal of thoracic wall for the synchrotron study. X-ray diffraction profiles were recorded vertically through the iPSC-CM grafts and infarcted myocardium of the anterior LV wall of the *in situ* beating hearts. It was found that 1,0 and 1,1 equatorial reflections attributable to myosin and actin-myosin lattice

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planes typical of cardiac muscle fibres were detected within the iPSC-CM grafts, but not in the infarcted myocardium of the sham-treated rats (Figure 3); albeit reflection intensity was generally much less than that obtained from the remote myocardium. Furthermore, in iPSC-CM hearts cyclic changes in myosin mass-transfer to actin with regular changes in myofilament lattice spacing were evident (Figure 4); similar to that previously reported by us for *in situ* beating rat hearts (20). Importantly, the shift in myosin mass to actin of the iPSC-CMs was synchronous with LV pressure increase during the start of systole on a beat-by-beat basis (Figure 5). The decrease in intensity ratio from end-diastole through early-systole was approximately linearly related to LV pressure development (Figure 5, lower panel). Hence, significant cyclic systolic myosin head transfer to actin filaments, and therefore force-developing cross-bridges were detected within the grafts. Notably, implanted iPSC-CM sheets produced consistent reflections, but the same sheets fresh from culture did not reveal any reflections.

Consistent with the findings in the synchrotron study, myosin and actin were well aligned in the cytoplasm of the Dsred-positive transplanted iPSC-CMs, which were present in the surface of the rat heart, assessed by immunohistolabeling (Figure 6A). In contrast, Dsred-negative native CMs were rarely found in the border and infarct areas which were assessed in the synchrotron study. These findings indicate that the *in situ* X-ray diffraction pattern originated from the transplanted iPSC-CMs, suggesting that regular actin-myosin cross-bridge motion had occurred in the transplanted iPSC-CMs in the rat heart.

Phenotypic and morphological fate of the transplanted iPSC-CM in the heart

Phenotype, morphology and microstructure of the transplanted iPSC-CM, which might be modulated following transplantation into the cardiac tissue, were then histologically analyzed by using immunoconfocal microscopy and electron microscopy. Dsrred-positive transplanted