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,

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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

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

iPSC-CMs showed myosin-positive sarcomeres at 14 days after transplantation (Figure 6A and B). The sarcomeres consisted of myosin and sarcomeric actin. Although the transplanted iPSC-CMs expressed Cx43 at 14 days, the distribution of Cx43 was scarce, and did not clearly show the typical intercalated disks between the transplanted iPSC-CMs (Figure 6C).

In vitro iPSC-CMs showed typical sarcomeric structures in cardiac myocytes with immature, less dense mitochondria (Figure 6D). On the other hand, clear desmosomes were generated between the iPSC-CMs at 3 days after transplantation, while the mitochondria showed more mature structure compared to that prior to the transplantation. At 7 days, mitochondria showed mature structure, whereas the sarcomeric structure or the number of mitochondria was not as dense as those in the native CMs (Figure 6E and F). This indicates that the transplanted iPSC-CMs by the cell-sheet method might have established electrical/mechanical integrity with the native heart. In addition, maturity in the structure and functionality of the iPSC-CMs progressed after the transplantation into the heart.

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DISCUSSION

We demonstrated here that the amount of VEGF and HGF released by iPSC-CMs was not significantly different from that of the same number of fibroblasts, *in vitro*. Transplantation of the iPSC-CM cell-sheets of mouse origin into the nude rat heart that was subjected to MI better preserved LV function and ARI, compared to that of the fibroblasts. Daily electrical mapping of the heart surface uncovered that transplantation of the iPSC-CM induced multiple ectopic excitations over the cell-sheet implanted area for the initial 2 days, and subsequently ectopic excitations disappeared. In *in vivo* synchrotron radiation small-angle scattering studies, the transplanted iPSC-CMs displayed regularly contracting actin-myosin cross-bridge interactions, similar to that recorded in the native cardiomyocytes of the remote myocardium of the same hearts. Immunohistologically, the transplanted iPSC-CMs, which were equipped with myosin-positive sarcomeres in the cytoplasm, formed Cx43-gap-junction with the native cardiomycytes, while electronmicroscopically, the transplanted iPSC-CMs were equipped with immature sarcomeres and mitochondria, compared to the native cardiomyocytes.

The mechanisms underlying the global functional recovery by iPSC-CM transplantation include 1) that transplanted iPSC-CMs survived and showed synchronized contraction *in vivo* as proven by the diffraction analyses, 2) that transplanted iPSC-CMs were equipped with fully developed sarcomeres *in vivo* and these cells might connect with the recipient myocardium, and 3) that there is functional integration of the transplanted iPSC-CMs into the native myocardium to produce direct mechanical contribution to cardiac function. The direct effects of the transplanted cells were studied chiefly by histological assessment of the excised cardiac tissues, which were examined for the presence and integration of the transplanted cells into the native cardiac tissue, but not the functionality of the transplanted cells. For the first time, *in vivo* scans dissected contractile motion of the

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