Supplementary Data

Supplementary Materials and Methods

All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD). The Institutional Ethics Review Committee for Animal Experimentation approved all study protocols.

Engineered cardiac tissue preparation from human-induced pluripotent stem cells

Human-induced pluripotent stem cells (iPSCs), 253G1 (RIKEN BRC Cell Bank, Tsukuba, Japan), were maintained on mitomycin C-treated mouse embryonic fibroblasts (ReproCELL, Kanagawa, Japan) in Primate ES Cell Medium (ReproCELL). Cardiomyogenic differentiation was induced by using a bioreactor system as previously reported.[11] Briefly, embryoid bodies were generated from undifferentiated iPSCs and suspended in StemPro34 medium containing 50 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine, and 400 μM 1-thioglycerol (Sigma-Aldrich). Bone-morphologic protein 4 (R&D Systems, Minneapolis, MN), basic fibroblast-growth factor (R&D Systems), activin A (R&D Systems), vascular endothelial growth factor (VEGF; R&D Systems), and Wnt 1 inhibitors (Wako, Osaka, Japan) were added at the defined concentrations and times. Cardiomyocytes were isolated from the differentiated cell preparation by using a magnetic-activated cell-sorting (MACS) system (Miltenyi Biotec, Teterow, Germany) based on expression of the cardiac-specific cell surface marker CD172a.[12] For MACS, cells were stained with antibodies for CD172a conjugated with phycoerythrin (PE), followed by incubation with anti-PE microbeads (Miltenyi Biotec). Isolated cardiomyocytes and non-cardiomyocytes were mixed at ratios of 25%, 50%, 70%, or 90% of cardiomyocytes and plated in thermoresponsive culture dishes (Cellseed, Tokyo, Japan) at 4.5×10^5 cells/cm^2 in DMEM supplemented with 10%FBS for 5 days to produce scaffold-free engineered cardiac tissues (ECTs).

Flow cytometry and cell sorting

Cultured cells were dissociated with 0.05% trypsin-EDTA for 5–10 min at 37°C and labeled with fluorescence-conjugated antibodies for CD31 (1:10; Becton Dickinson, East Rutherford, NJ), CD144 (1:10; Becton Dickinson), or CD172a (1:20; BioLegend, San Diego, CA) for 30 min at 4°C and then assessed by using the FACScantoII system (Becton Dickinson). Cells were labeled with antibodies for cardiac troponin T (cTnT, 1:200; Thermo Fisher Scientific, Waltham, MA), TE-7 (1:50; Merk Millipore, Billerica, MA), or alpha-smooth muscle actin (αSMA, 1:100; Abcam, Cambridge, United Kingdom), caldsemon (1:100; Abcam), calponin (1:100; Abcam), vimentin (1:00; Abcam), Nkx2.5 (1:50; Abcam), alpha-actinin (αacitnin, 1:50; Abcam), or cardiac myosin heavy chain (cMHC; 1:100; Abcam) after fixation by using BD Cytofix Fixation Buffer (Becton Dickinson), followed by incubation with fluorescence-conjugated secondary antibodies. To analyze gene expression in ECT cardiomyocytes, CD172a-positive cardiomyocytes were isolated with an FACSAria Fusion Sorter (Becton Dickinson). Data were analyzed by using Diva (Becton Dickinson) or FlowJo software (TreeStar, Ashland, OR).

Real-time polymerase chain reaction

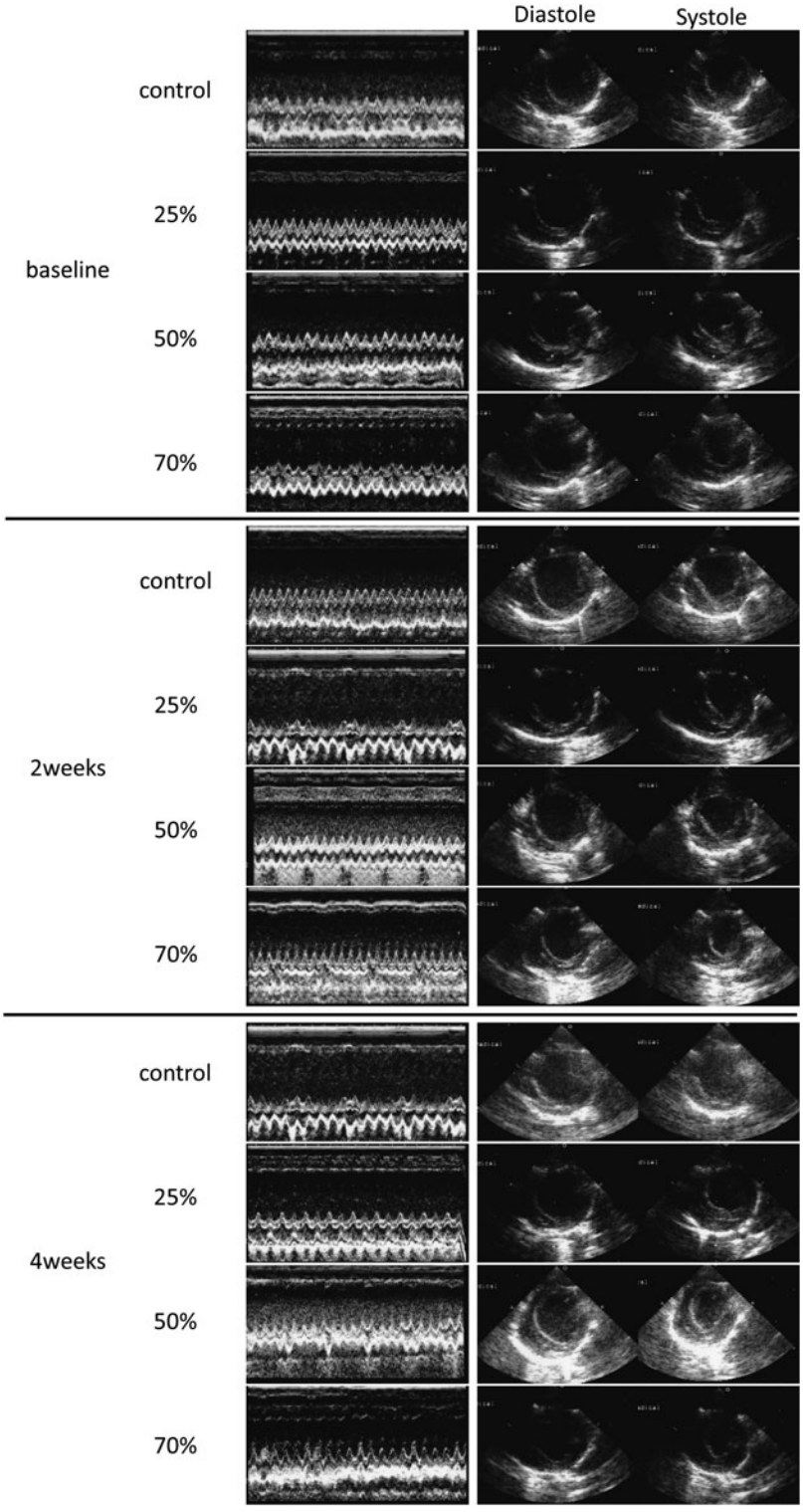
Total RNA was extracted by using an RNAeasy kit (Qiagen, Hilden, Germany), and cDNA was synthesized by using a SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific). Extraction of total RNA and cDNA synthesis from fluorescence-activated cell sorting-derived cells was conducted by using Cell-to-CT kits (Thermo Fisher Scientific). Real-time polymerase chain reaction (PCR) was performed by using TaqMan PCR master mix on a Viia7 Real-time PCR system (Thermo Fisher Scientific).

The following genes were analyzed by using TaqMan gene-expression assays (Thermo Fisher Scientific): laminin α2 (Hs01124081_m1), laminin α4 (Hs00935293_m1), laminin α5 (Hs00966585_m1), collagen type I (Hs00164004_m1), collagen type III (Hs00943809_m1), fibronectin (Hs00365052_m1), myosin light chain (*MLC*)*2v* (Hs00166405_m1), *MLC2a* (Hs00221909_m1), connexin 43 (Hs00748445_s1), sarcoplasmic reticulum Ca^{2+} ATPase (*SERCA)2a* (Hs00544877_m1), cardiac calsequestrin *(CASQ*; Hs00154286_m1), ryanodine receptor 2 (*RYR-2*; Hs00181461_m1), *VEGF* (Rn01511602_m1), hepatocyte growth factor (*HGF*; Rn00566673_m1), and stromal cell-derived factor (*SDF*)*-1* (Rn00573260_m1). Relative gene expression was calculated by using the ΔΔCt method, normalizing with glyceraldehyde-3-phosphate dehydrogenase (Rn01775763_g1) or 18 s ribosomal RNA (Hs99999901_s1).

Immunohistolabeling and fluorescence intensity analysis

The ECT or dissociated single cells were fixed with 4% paraformaldehyde (PFA) and labeled with primary antibodies for MLC2a (Synaptic Systems GmbH, Goettingen, Germany), MLC2v (Proteintech, Rosemont, IL), laminin (1:400; Sigma-Aldrich), fibronectin (1:100; Abcam), collagen type I (1:100; Abcam), collagen type III (1:100; Abcam), cTnT (1:200; Thermo Fisher Scientific), TE-7, αSMA, caldesmon, vimentin, nkx2.5, α-actinin, or cMHC followed by incubation with fluorescence-conjugated secondary antibodies or fluorescence-conjugated isolectin IB4 (1:25; Thermo Fisher Scientific); then, they were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst33258. The stained tissue sections were then visualized by confocal microscopy (Carl Zeiss, Jena, Germany). Alternatively, labeled cells were captured based on their fluorescence intensity by using a high-content imaging system (Operetta; PerkinElmer, Waltham, MA) and assessed by using Harmony software (PerkinElmer).

Harvested hearts were fixed with 4% PFA, frozen in liquid nitrogen, and cryosectioned. Sections were labeled with primary antibodies for cTnT (1:50; R&D Systems), human nuclear-specific antigen (HNA, 1:50; Merck Millipore), human-specific lamin A/C (1:250Abcam), connexin 43 (1:50;



SUPPLEMENTARY FIG. S1. Echocardiography images. Representative two-dimensional echocardiographic view. *Left*: M-mode; *right*: B-mode.

Sigma-Aldrich), TE-7, αSMA, caldesmon, vimentin, a-actinin or, followed by incubation with fluorescence-conjugated secondary antibodies or fluorescence-conjugated Isolectin IB4. The slides were then counterstained with DAPI before analysis by confocal microscopy. Sirius red staining was performed to examine myocardial fibrosis in the peri-infarct region. The fibrotic region was calculated as the percentage of myocardial area using Metamorph software (Sunnywale, CA). To assess capillary density, sections were labeled with primary antibody for von Willebrand factor (vWF, 1:50; DAKO, Glostrup, Denmark), and they were visualized by using the Universal LSAB2 System, HRP kit (DAKO).

Multi-electrode array recording

Cells were plated on 0.1% gelatin-coated multi-electrode array probes (Alpha MED Scientific, Ibaraki, Japan) containing platinum black electrodes (50 μm in diameter) arranged in an 8 × 8 electrode grid, spaced 300 μm apart, and cultured for 5 days. Analogue field potential signals were acquired through a 1 Hz low-pass filter and digitized at 20 kHz by using MED64 system in a 5% CO_2 incubator at 37°C ($n = 6$). Field potential wave forms were extracted and beating rate, inter-beats interval, and peak time of the beats were analyzed by using Mobius software (Alpha Med Scientific). Conduction velocity of field potential was calculated by using peak time of the beats and electrode spacing.

Analysis of Ca^{2+} transients and membrane potential

Dissociated cells were plated on 0.1% gelatin-coated plates and cultured for 5 days. For Ca^{2+}-transient or membrane-potential analysis, cells were loaded with 2 μM Fluo-8 AM (ABD Bioquest, Sunnyvale, CA) or 4 μM FluoVlot (Thermo Fisher Scientific) at 37°C for 30 min, respectively. Intracellular fluorescence was recorded under pacing at 1 Hz at 37°C by using an FDSS/μCELL system (Hamamatsu Photonics, Hamamatsu, Japan) ($n = 6$). Data were analyzed by using FDSS software U8524-12 (Hamamatsu Photonics). For motion analysis, beating cells were monitored at a frame rate of 150 fps for 10 s at 37°C with a high-speed camera-based, motion analysis system (SI8000 View; Sony, Tokyo, Japan) ($n = 5$). Data were analyzed by using SI8000C Analyzer (Sony).

Cytokine measurement

ECT culture supernatant was collected and analyzed by fluorescence-dyed microsphere-based immunoassay for cytokine/chemokine production by using the Bio-Plex suspension array system (Bio-Rad, Hercules, CA).

Epicardial ECT transplantation into a chronic myocardial infarction rat model

The left anterior-descending artery of athymic nude rats (F344/NJcl-rnu/rnu; CLEA Japan, Tokyo, Japan) was permanently ligated through left thoracotomy under general anesthesia administered with endotracheal intubation ($n = 52$). After 2 weeks, rats underwent re-thoracotomy for the exposed pericardial space ($n = 48$) and were then randomly divided into a sham group or three ECT-transplanted groups designated as containing 25%, 50%, or 70% cardiomyocytes ($n = 12$/group). ECTs were transplanted over the epicardium of the anterior and lateral left-ventricular walls. Transplanted ECTs were spread manually to cover both infarct and border areas of the heart and fixed with fibrin glue. After surgery, all rats were allowed to recover in individual temperature-controlled cages.

Transthoracic echocardiography

Transthoracic echocardiography was performed by using a SONOS 7500 machine (Philips Medical Systems, Eindhoven, Netherlands) and a 12-MHz annular-array transducer on rats under general anesthesia that was administered without endotracheal intubation 2 weeks after the coronary artery ligation (just before transplantation) and 2 and 4 weeks after transplantation ($n = 12$ for each group). The hearts were imaged in short-axis, two-dimensional views at the level of the papillary muscles, and the left-ventricular end-systolic and -diastolic dimensions (LVESD and LVEDD, respectively) were determined. The left-ventricular ejection fraction (LVEF) was calculated by Pombo's method: $[(LVEDD^3 - LVESD^3)/LVEDD^3]$.[13]

Optical mapping of the ECT-transplanted whole heart

To visualize the excitation-conduction properties of transplanted ECTs exerted on native myocardium using the whole-heart optical imaging technique, the differentiated cardiomyocytes used to engineer the ECTs were transfected with a plasmid encoding a fluorescent calcium indicator, GCAMP2 (kindly provided by Junichi Nakai, Saitama University Brain Science Institute, Japan), by using an adenoviral vector. The ECT-transplanted hearts were excised and mounted on a Langendorff-perfusion system with modified Tyrode solution at 37°C. The hearts were loaded with 10 μM RH237 (Thermo Fisher Scientific) and the excitation-contraction uncoupler 2,3-butanedione monoxime (15 mM; Sigma-Aldrich) to avoid the effects of motion artifacts. Images were recorded by using two charge-coupled device cameras simultaneously with electrocardiogram monitoring (Labchart; ADInstruments, Dunedin, New Zealand) of the isolated perfused hearts under external fixed-pacing rates of 100 beats/min (Electronic stimulator SEN-8203; Nihon Koden, Tokyo, Japan). Recorded images were analyzed by using BV_Ana software (BrainVision, Tokyo, Japan).

Statistical analysis

All statistical analyses were performed by using GraphPad Prism version 6.07 (GraphPad Software, La Jolla, CA). Data are expressed as mean ± standard error of the mean. Differences between groups were assessed by using one-way analysis of variance (ANOVA). Comparisons among different time points and between experimental groups were performed with two-way ANOVA, followed by Tukey's *post hoc* testing.