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Enhanced Survival of Transplanted Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes by the Combination of Cell Sheets With the Pedicled Omental Flap Technique in a Porcine Heart

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Enhanced Survival of Transplanted Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes by the Combination of Cell Sheets With the Pedicled Omental Flap Technique in a Porcine Heart

Masashi Kawamura, MD; Shigeru Miyagawa, MD, PhD; Satsuki Fukushima, MD, PhD;
Atsuhiko Saito, PhD; Kenji Miki, PhD; Emiko Ito, PhD; Nagako Sougawa, PhD;
Takuji Kawamura, MD; Takashi Daimon, PhD; Tatsuya Shimizu, MD, PhD; Teruo Okano, PhD;
Koichi Toda, MD, PhD; Yoshiki Sawa, MD, PhD

Background—Transplantation of cardiomyocytes that are derived from human induced pluripotent stem cell–derived cardiomyocytes (hiPS-CMs) shows promise in generating new functional myocardium in situ, whereas the survival and functionality of the transplanted cells are critical in considering this therapeutic impact. Cell-sheet method has been used to transplant many functional cells; however, potential ischemia might limit cell survival. The omentum, which is known to have rich vasculature, is expected to be a source of blood supply. We hypothesized that transplantation of hiPS-CM cell sheets combined with an omentum flap may deliver a large number of functional hiPS-CMs with enhanced blood supply.

Methods and Results—Retrovirally established human iPS cells were treated with Wnt signaling molecules to induce cardiomyogenic differentiation, followed by superparamagnetic iron oxide labeling. Cell sheets were created from the magnetically labeled hiPS-CMs using temperature-responsive dishes and transplanted to porcine hearts with or without the omentum flap (n=8 each). Two months after transplantation, the survival of superparamagnetic iron oxide–labeled hiPS-CMs, assessed by MRI, was significantly greater in mini-pigs with the omentum than in those without it; histologically, vascular density in the transplanted area was significantly greater in mini-pigs with the omentum than in those without it. The transplanted tissues contained abundant cardiac troponin T–positive cells surrounded by vascular-rich structures.

Conclusions—The omentum flap enhanced the survival of hiPS-CMs after transplantation via increased angiogenesis, suggesting that this strategy is useful in clinical settings. The combination of hiPS-CMs and the omentum flap may be a promising technique for the development of tissue-engineered vascular-rich new myocardium in vivo. (*Circulation*. 2013;128[suppl 1]:S87-S94.)

Key Words: cell transplantation ■ induced pluripotent stem cells ■ regeneration

Stem cell therapy shows promise in the treatment of heart failure. However, the therapeutic benefits proven by clinical studies in the past decade were only modest, indicating that further investigations and refinements are required to establish this treatment in the clinical arena.^{1,2} The success of cell transplantation therapy for heart failure is dependent on the choice of cell source, cell delivery method, and target cardiac pathology. In these previous clinical trials, transplantation of somatic tissue–derived stem or progenitor cells has shown no or low cardiomyogenic differentiation capacity in vivo, but contributed to functional recovery via paracrine effects, potentially limiting the therapeutic effects, in particular, in

treating severe heart failure.^{1–4} In addition, it has been shown that direct intramyocardial or intracoronary injection of dissociated single cells, which was used in most of the clinical studies, yields <10% of engraftment rate of the cells immediately after transplantation, indicating that further refinement of the cell delivery method would be required to increase cell engraftment and enhance the consequent therapeutic effects.^{1,2}

Human induced pluripotent stem (hiPS) cells are initially established by nuclear reprogramming of somatic cells.^{5,6} hiPS cell carries a capacity of unlimited proliferation and differentiation to cardiomyocyte.⁷ Transplantation of hiPS-derived cardiomyocytes (hiPS-CMs) would have, thus, a potential to

From the Department of Cardiovascular Surgery, Osaka University Graduate School of Medicine, Suita, Osaka, Japan (M.K., S.M., S.F., K.M., E.I., N.S., T.K., K.T., Y.S.); Medical Center for Translational Research, Osaka University Hospital, Suita, Osaka, Japan (A.S.); Department of Biostatistics, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan (T.D.); Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan (T.S., T.O.).

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Correspondence to Yoshiki Sawa, MD, PhD, Department of Cardiovascular Surgery, Osaka University Graduate School of Medicine, 2-2(E1) Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail sawa-p@surg1.med.osaka-u.ac.jp

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increase the functional cardiomyocytes in damaged heart tissue to mechanically contribute to cardiac function. In addition, the recently developed scaffoldless tissue engineering technique of cell-sheet engineering is applicable to myocardial regeneration therapy.⁸ This technique preserves extracellular matrix without artificial scaffolds, which may prevent cell detachment-associated anoikis.⁹ In contrast to the needle injection technique, the cell-sheet technique can deliver a large number of cells to the damaged myocardium without loss of transplanted cells or injury to the host myocardium.^{10,11} Importantly, this method has already shown feasibility and safety in the clinical study.¹² On these bases, we studied the therapeutic efficacy of transplantation of hiPS-CMs with the cell-sheet method in a porcine chronic ischemic cardiomyopathy model.¹³ This study, however, showed that the transplanted cells rarely survived in the heart long-term, possibly because of poor vascular network support from the native tissue.

The omentum has been historically used in surgical revascularization for patients with ischemic heart disease^{14–16} and is also known to have rich vasculature and angiogenic factors.^{17,18} Importantly, we reported that a pedicle omentum flap covering the transplanted skeletal myoblast cell sheets enhanced angiogenesis over the cell-sheet–transplanted territory, survival of cells, and therapeutic effects.¹⁹ We herein hypothesized that covering with an omentum flap may enhance the survival of transplanted hiPS-CM cell sheets via the promotion of angiogenesis over the transplanted territory. In this study, we compared the survival of hiPS-CMs, with or without a pedicle omentum flap, after transplantation to the mini-pig heart, and we examined whether the omentum enhanced the angiogenic capacity of hiPS-CM sheets *in vivo*.

Materials and Methods

All experimental procedures were approved by the institutional ethics committee. Animal care was conducted humanely in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Animal Resources and published by the National Institutes of Health (publication no. 85-23, revised 1996).

Preparation of SPIO-Labeled hiPS-CM Cell Sheets

The hiPS cell line 201B7 that was generated using the 4 transcription factors Oct4, Sox2, Klf4, and c-Myc was used in this study.⁵ Culture of the hiPS cells, formation of the embryoid bodies, and subsequent cardiomyogenic differentiation and purification were performed as described previously to generate hiPS-CMs.¹³ The purified hiPS-CMs were then labeled with the superparamagnetic iron oxide (SPIO) ferucarbotran (Resovist; Bayer Pharma, Berlin, Germany) using the hemagglutinating virus of Japan envelope vector (GenomOne-Neo; Ishihara Sangyo, Osaka, Japan).^{20,21} Subsequently, human mesenchymal stem cells (Lonza, Basel, Switzerland) were seeded at a density of 5×10^6 cells/dish onto 10-cm UpCell dishes, on which the SPIO-labeled hiPS-CMs were grown. The next day, the dishes were incubated at room temperature, which induced the cells to detach spontaneously to form scaffold-free hiPS-CM cell sheets.

Flow Cytometry

Dissociated cells after hiPS cell differentiation were fixed, permeabilized, and labeled with anticardiac isoform of troponin T (cTNT; clone 13211; Thermo Fisher scientific, Runcorn, UK) conjugated with Alexa-488 using Zenon technology (Invitrogen), followed by

analysis on BD FACSCanto II (BD Biosciences) with BD FACSDiva Software (BD Biosciences).

Study Protocol

Normal 16 female mini-pigs (Japan Farm Co Ltd, Kagoshima, Japan) weighing 20 to 25 kg were randomly divided into 2 groups (n=8 each) to perform hiPS-CM cell-sheet transplantation either with or without the pedicle omentum translocation. All animals were immunosuppressed by daily administration of tacrolimus (0.75 mg/kg; Astellas, Tokyo, Japan), mycophenolate mofetil (500 mg; Teva Czech Industries s.r.o, Opava, Czech), and prednisolone (20 mg; Takeda Pharmaceutical Co Ltd, Osaka, Japan) daily from 5 days before transplantation until euthanasia. Cardiac MRI scans were taken on the same mini-pigs at 1 week, 4 weeks, and 8 weeks after transplantation. After the final scan, the mini-pigs were humanely euthanized for analysis (Figure 1A).

Transplantation of SPIO-Labeled hiPS-CM Cell Sheets Covered With the Pedicle Omentum

All animals were preanesthetized with ketamine hydrochloride (20 mg/kg; Daiichi Sankyo, Tokyo, Japan) and xylazine (2 mg/kg; Bayer HealthCare, Leverkusen, Germany), intubated endotracheally, and maintained by continuous infusion of propofol (6 mg/kg per hour; AstraZeneca K.K., Osaka, Japan) and vecuronium bromide (0.05 mg/kg per hour; Daiichi Sankyo). Seven SPIO-labeled hiPS-CM sheets were placed on the epicardium via the median sternotomy. In the case of transplantation of the cell sheet covered with the pedicled omentum, the omentum was mobilized to the mediastinal space via additional small upper midline laparotomy, preserving both gastroepiploic arteries and their arcade. Initially, 4 hiPS-CM cell sheets were placed on the epicardium and covered with the omentum. The remaining 3 hiPS-CM cell sheets, then, were placed on the covering omentum and covered with the omentum again (Figure 1B). The omentum was stitched and fixed on the excised pericardium (Figure 1C). Mini-pigs were then allowed to recover and were later humanely euthanized.

Cardiac MRI

ECG-gated cardiac MRI (CMR) was performed under general anesthesia with an 8-channel cardiac coil wrapped around the chest wall.²² CMR images were acquired on a 1.5-T MR scanner (Signa EXCITE XI TwinSpeed; GE Medical Systems, Milwaukee, WI). To assess SPIO-labeled hiPS-CM detection, animals were imaged 1 week after transplantation. In addition, 1 animal was reimaged at 4 and 8 weeks after transplantation to detect SPIO-labeled hiPS-CM retention. Short-axis images with 8-mm slice thickness, including the entire heart, were obtained by pulse parameters for cardiac-gated, fast gradient-recalled echo. The SPIO-labeled hiPS-CM hypointense area was measured using planimetry of fast gradient-recalled echo images on a workstation (Virtual Place Lexus64; AZE, Tokyo, Japan). The survival proportion of hiPS-CMs was determined using the hypointense area at 4 and 8 weeks after transplantation divided by the area at 1 week after transplantation as the baseline.

Histology and Immunohistolabeling

The hiPS-CM cell sheets and the excised heart specimens were either embedded in paraffin or optimal cutting temperature compound (Tissue Tek; Sakura Finetek, Torrance, CA) for frozen section. The paraffin-embedded sections were stained with hematoxylin–eosin or Prussian blue that visualizes iron contents. Ten different fields were randomly selected. The number of spindle-shaped cells with a nucleus and iron in the cytoplasm in each field was counted with a light microscopy under high-power magnification ($\times 400$). Cells from 10 fields were averaged, and the results are expressed as cell density (per high-power field). In addition, the paraffin-embedded sections were immunolabeled with anti-human von Willebrand factor antibody (Dako, Glostrup, Denmark) and visualized with the horseradish peroxidase-based EnVision kit (Dako). Ten different fields were randomly selected, and the number of von Willebrand factor–positive

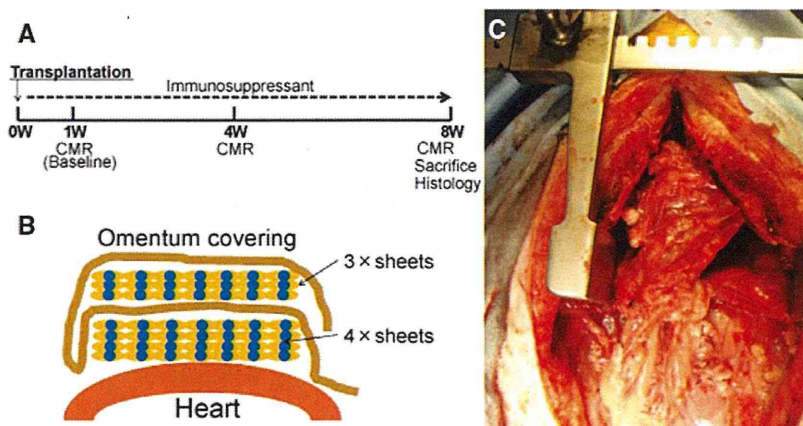


Figure 1. Study protocol of the mini-pig experiment and operative procedure. **A**, Schedule of cardiac MRI (CMR) and histological evaluations. **B**, Procedural scheme of cell-sheet transplantation with the omentum. **C**, Image taken after treatment. The omentum is mobilized and transplanted with the cell sheets on the heart through median sternotomy with an additional upper midline laparotomy.

cells in each field was counted using a light microscope under high-power magnification ($\times 200$). The stained blood vessels from the 10 fields were averaged and the results expressed as vascular density (per square millimeter). The frozen sections were immunolabeled with anti-cTNT antibody (1:100 dilution; Abcam, Cambridge, UK) and anti-CD68 antibody for macrophages (1:100 dilution, Abcam) as primary antibodies and visualized with AlexaFluor488-conjugated goat anti-mouse (Invitrogen) and AlexaFluor555-conjugated goat anti-rabbit (Invitrogen) as secondary antibodies. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Dojindo, Tokyo, Japan) and assessed using the Biorevo BZ-9000 (Keyence) or confocal microscopy (Olympus Japan, FV1000-D IX81, Tokyo, Japan). SPIO particles of Prussian blue staining were visualized by differential interference contrast of confocal microscopy.

Real-Time Polymerase Chain Reaction

Total RNA was extracted from cardiac tissue and reverse transcribed using Omniscript reverse transcriptase (Qiagen, Hilden, Germany) with random primers (Invitrogen), and the resulting cDNA was used for real-time polymerase chain reaction with the ABI PRISM 7700 (Applied Biosystems, Stockholm, Sweden) system using pig-specific primers (Applied Biosystems) for vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and stromal-derived factor-1 (SDF-1). Each sample was analyzed in triplicate for each gene studied. Data were normalized to GAPDH expression level. For relative expression analysis, the delta-delta Ct method was used, and values of the cell-sheet transplantation without the omentum were used as reference values.

Statistical Analysis

Data are expressed as means \pm SDs. Comparisons between 2 groups were made using Welch *t* test. Cell survival proportion over time was assessed by repeated-measures ANOVA with group, time, and group \times time interaction effects. All *P* values are 2-sided, and values of *P*<0.05 were considered to indicate statistical significance. Statistical analyses were performed using JMP 9.02 (SAS Institute, Cary, NC).

Results

Generation of SPIO-Labeled hiPS-CM Cell Sheets

Cardiomyogenic differentiation of hiPS cells was induced by treatment of the embryoid bodies formed from cultured hiPS cells with Wnt3a and R-spondin-1. Subsequently, the differentiated hiPS cells were purified by culture in glucose-free medium to yield ≈ 1 to 2×10^7 hiPS-CMs. Approximately 80% (83.6 \pm 8.1%) of the hiPS-CMs were positive for cTNT, as determined by flow cytometry (Figure 2A). After SPIO labeling to the hiPS-CMs, human mesenchymal stem cells were added

to the hiPS-CM culture. Subsequently, culture in the thermo-responsive dishes yielded round-shaped hiPS-CM cell sheets (Figure 2B). The hiPS-CMs on the sheet continued to beat before and after detaching from culture surface (Movies I and II in the online-only Data Supplement). Immunohistolabeling showed that the large number of cells in the hiPS-CM cell sheets were homogeneously positive for cTNT (Figure 2C). Prussian blue staining confirmed that the hiPS-CMs contained iron in the cytoplasm (Figure 2D).

In Vivo Analysis of Survival of Transplanted SPIO-Labeled hiPS-CMs by Serial CMR

Transplantation of the same number of hiPS-CM cell sheets with or without the omentum covering was successfully performed via median sternotomy in 16 normal mini-pigs. There was no mortality related to the procedure or otherwise before the planned euthanasia. In addition, the omentum was attached to the surface of the heart in all mini-pigs with the omentum. CMRs were performed to assess the survival of transplanted SPIO-labeled hiPS-CMs at 1 week (baseline), 4 weeks, and 8 weeks after cell transplantation.

SPIO signals were clearly identified as the hypointense area in the surface of the left ventricle by CMR in all mini-pigs throughout the study period (Figure 3A). SPIO-positive hypointense area was gradually decreased in both the groups during the 8 weeks, whereas the SPIO-positive area was larger and thicker in mini-pigs with the omentum compared with those without the omentum during the study period. The survival proportion of the SPIO-labeled hiPS-CMs was determined by the formula that the hypointense area at 4 and 8 weeks after transplantation was divided by the area at 1 week after transplantation as baseline. Both groups showed steady decrease in the cell survival during the 7 weeks, whereas the proportion of decrease was significantly less in mini-pigs with the omentum than in those without it at 4 weeks (92 \pm 10% versus 60 \pm 10%) and 8 weeks (78 \pm 10% versus 42 \pm 9%) after treatment (*P*<0.0001 for interaction effect of time and group in the repeated ANOVA; Figure 3B).

Histological Evaluation of Transplanted hiPS-CMs With or Without the Omentum

Excised heart tissues at 8 weeks after transplantation were assessed by histology. The transplanted hiPS-CMs and the

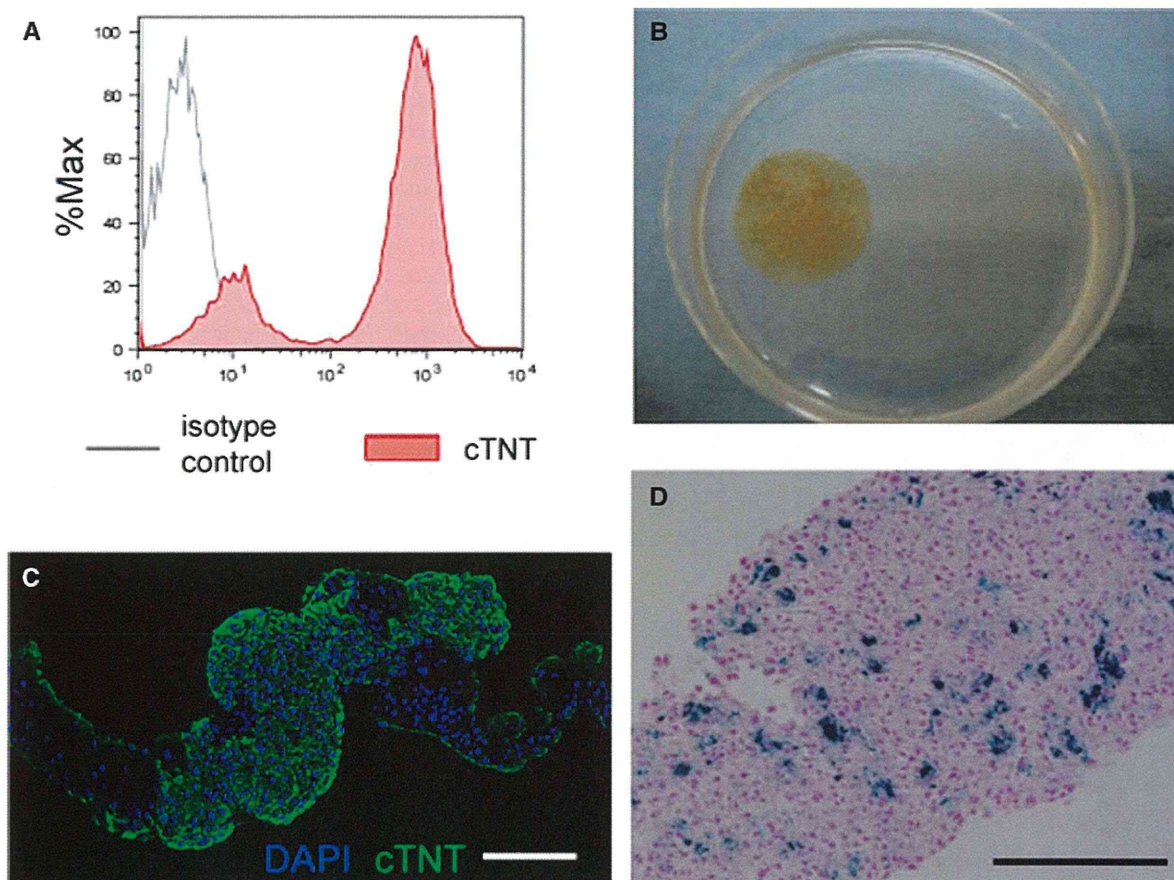


Figure 2. Histological characteristics of the human induced pluripotent stem cell-derived cardiomyocyte (hiPS-CM) cell sheet. **A**, Expression of cardiac troponin T (cTNT) after differentiation and purification of hiPS-CMs. **B**, A superparamagnetic iron oxide (SPIO)-labeled hiPS-CM cell sheet in a 10-cm dish. **C**, Immunostaining of the hiPS-CM cell sheet with cTNT antibody (green). The cell nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI; blue). **D**, Prussian blue staining of the SPIO-labeled hiPS-CM cell sheet. Scale bar, 50 μ m in **C** and **D**.

pedicle omentum were attached over the epicardium of the left ventricle without any histological gaps in all mini-pigs, as assessed by hematoxylin–eosin staining (Figure 4D). The hearts without the omentum showed cellular and fibrous components over the anterior wall of the ventricles (Figure 4A), whereas the hearts with the omentum showed thick cellular, fibrous, and fat-rich components covering the anterior and lateral wall of the ventricles (Figure 4D).

Prussian blue staining revealed cells containing iron on the surface of the ventricles, corresponding to the area seen on CMR in both groups (Figure 4B and 4E). A larger number of cells with iron contents were identified in mini-pigs with the omentum compared with those without (Figure 4B, 4C, 4E, and 4F). In fact, the density of iron-containing cells in the transplanted site, assessed semiquantitatively by Prussian blue staining at 8 weeks after treatment, was significantly greater in the mini-pig with the omentum (27 ± 6 cells/high-power field) than in those without it (5 ± 2 cells/high-power field; $P < 0.0001$; Figure 4G). Immunohistolabeling showed that a larger number of cells are positive for cTNT in the area where cells with iron inclusions are present in mini-pigs with the omentum compared with those without it (Figure 4H). The distribution of the SPIO particles was visualized by

differential interference contrast of confocal microscopy. Grafted hiPS-CMs were identified and confirmed as double-positive for cTNT and SPIO and negative for CD68, which is a specific marker for macrophages, by immunohistolabeling (Figure 4I–4N). In addition, no teratomas were formed in the heart or other thoracic organs at 8 weeks after the transplantation of the hiPS-CM cell sheets with or without the omentum (data not shown).

Capillary Density in the Transplanted Area

Vessels and capillaries in the transplanted cell sheets at 8 weeks after transplantation were visualized and assessed by immunohistochemistry for von Willebrand factor. The transplanted cell sheets without the omentum contained a large number of capillaries and a small number of vessels in a homogeneous manner (Figure 5A), suggesting that vascular network was created possibly to support the survival and function of the cell sheets. Of note, the number of capillaries and vessels were markedly greater in the cell sheets covered by the omentum compared with those without it (Figure 5B). In fact, capillary density in the transplanted cell sheets, assessed semiquantitatively by immunohistochemistry for von Willebrand factor at 8 weeks after treatment, was significantly and markedly

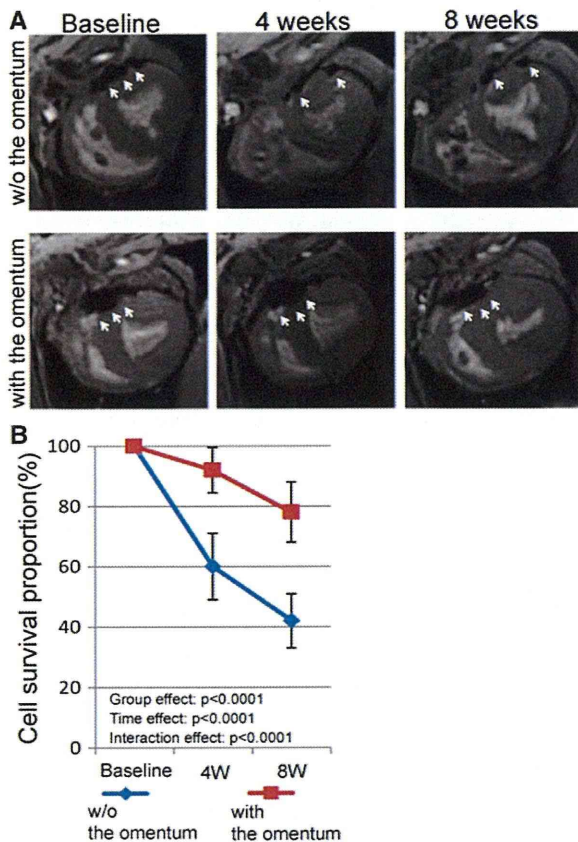


Figure 3. In vivo analysis of the survival of superparamagnetic iron oxide (SPIO)-labeled human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) after transplantation. **A**, Serial cardiac MRIs were examined at 1 week (baseline), 4 weeks, and 8 weeks after SPIO-labeled hiPS-CM cell-sheet transplantation, with or without the omentum. Representative hypointense area of the SPIO-labeled hiPS-CMs is indicated by white arrows. **B**, Cell survival proportion was estimated by the SPIO-labeled area at 4 and 8 weeks, corrected by cell survival at 1 week.

greater in mini-pigs with the omentum (64 ± 21 U/mm²) than in those without it (9 ± 5 U/mm²; $P < 0.0001$; Figure 5C).

Upregulation of VEGF, Basic Fibroblast Growth Factor, and SDF-1 Expression in the Transplanted Area

The expression level of cardioprotective and angiogenic factors in the transplanted area at 8 weeks after treatment was quantitatively assessed by real-time polymerase chain reaction for VEGF, basic fibroblast growth factor, and SDF-1. The relative expression of all the factors in the transplanted area was significantly greater in mini-pigs with the omentum than in those without it (VEGF, 1.94 ± 0.38 versus 1.35 ± 0.26 ; $P < 0.05$; basic fibroblast growth factor, 2.33 ± 0.92 versus 1.21 ± 0.19 ; $P < 0.05$; SDF-1, 2.05 ± 0.33 versus 1.22 ± 0.21 ; $P < 0.01$; Figure 6A–6C).

Discussion

It is herein demonstrated that our differentiation protocol yielded hiPS-CMs with $>80\%$ purity, and hiPS-CM cell sheets were transplanted over the anterior wall of the ventricle,

covered by the pedicle omentum, in a porcine model without procedural failure or procedure-related morbidity/mortality. The number of surviving cTNT-positive hiPS-CMs on the native myocardium was significantly greater in mini-pigs with the omentum than in those without it, although there was a steady decrease in the surviving cell number, regardless of the omentum support, as assessed by SPIO cell labeling with CMR and by immunohistolabeling. The pedicle omentum covering markedly increases the number of vessels and capillaries, associated with the upregulation of VEGF, hepatocyte growth factor, and SDF-1, at the transplanted area compared with the cell-sheet transplantation without the omentum.

In the present study, SPIO-labeled hiPS-CMs were clearly visualized in vivo by CMR, corresponding to the histological findings that confirmed iron contents in the transplanted hiPS-CMs that were positive for cTNT, as reported by previous publications.^{22,23} Using this method, the distribution and survival of the transplanted hiPS-CMs were serially evaluated in this study. As a result, it was proved that the unique technique in which transplanted cell sheets were covered by the pedicle omentum elicited a greater survival of the transplanted hiPS-CMs over the ventricular epicardial surface at 4 weeks compared with cell-sheet transplantation without the omentum covering. This suggests that pedicle omentum covering the cell sheets promptly induced angiogenesis to improve the hypoxic environment at the transplanted area, compared with the omentum-free method. In addition, although the size of the graft was decreased in both groups during the 8 weeks, trend in the size reduction was significantly milder in the omentum group than in the omentum-free group. This was consistent to the increased vascular network and upregulated angiogenic factors at the transplanted area in the omentum group at 8 weeks after the cell-sheet transplantation. These findings indicate that covering the cell sheet with the pedicle omentum that carries abundant angiogenic potentials^{17–19} enhanced neovascular formation at the transplanted area promptly after transplantation and that vascular-rich structure at the transplanted area persisted long-term. In previous studies, antiapoptotic treatments on the transplanted cells, including upregulation of AKT²⁴ or overexpression of Bcl-2,²⁵ have been shown to improve survival after cell transplantation. We achieved to improve cell survival after transplantation by modifying the cell delivery method. The pedicled omental flap is frequently and safely applied for the treatment of mediastinitis after cardiovascular surgery. As cell transplantation is indicated to the patients with severe heart failure, we need to establish a minimally invasive approach to mobilize the omentum. Besides, we expect our unique combination method to be a feasible and safe treatment option in clinical settings. However, in this study, transplanted hiPS-CMs produced by our protocol may be immature, although they were spontaneously contractile. In the specimen 8 weeks after transplantation with the omentum, there were few surviving hiPS-CMs with organized sarcomeres in the cytoplasm, whereas there were many cTNT-positive cells (data not shown). In recent studies, mechanical load of hiPS-CMs in vitro controlled their alignment, proliferation, and hypertrophy,²⁶ and spontaneous and synchronous beating cardiac cell sheets were created by a bioreactor culture, which expanded and induced cardiac differentiation of hiPS cells.²⁷ It is necessary to modify

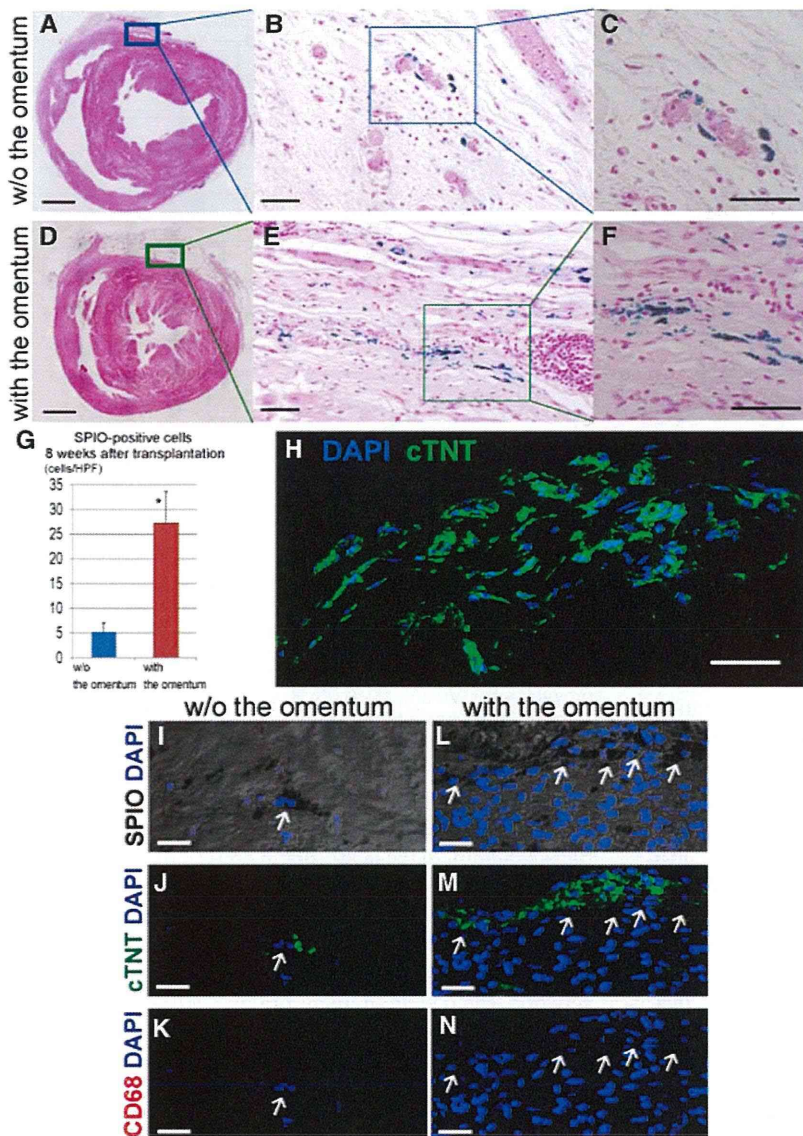


Figure 4. Human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) after transplantation. Macroscopic images of the whole heart by hematoxylin-eosin staining at the mid level in the mini-pig without (A) or with (D) the omentum; scale bar, 1 cm in A and D. Cells containing iron, indicative of superparamagnetic iron oxide (SPIO)-labeled hiPS-CMs, were detected by Prussian blue staining of sections of mini-pigs without (B and C) or with (E and F) the omentum at the transplanted area; scale bar, 50 μ m in B, C, E, and F. G, The density of SPIO-positive cells in the transplanted site was semiquantitatively assessed at 8 weeks after treatment. * $P < 0.0001$ vs without the omentum. H, In the transplanted regions of mini-pigs with the omentum, cardiac troponin T (cTNT)-positive cells were also demonstrated by immunohistochemical labeling (green). The cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue); scale bar, 50 μ m in H. I–N, In the transplanted regions of mini-pigs, SPIO particles were visualized by differential interference contrast (DIC), and grafted hiPS-CMs, which were double-positive for cTNT (green) and SPIO (DIC) and negative for CD68 (red), were identified by immunohistochemical labeling. The cell nuclei were counterstained with DAPI (blue). Arrows indicate SPIO particles, referred to DIC images in I and L; scale bar, 20 μ m in I–N.

our hiPS-CM preparation protocols referred to in these studies to yield the amount of contracting hiPS-CMs contributing to the mechanical function of the injured heart. In addition, we previously demonstrated that maturation of iPS-CMs progressed after iPS-CMs were transplanted in nude rat heart.²⁸ Therefore, we also expect that improving environments after cell transplantation, such as avoiding delivered cell ischemia, inflammation, and immunogenic rejection, will promote in vivo differentiation of iPS-CMs and their therapeutic effects. The combination of hiPS-CM sheets and the omentum is a promising delivery method to differentiate hiPS-CMs in vivo, because the omentum at least prevents cell ischemia after transplantation and provides better environments.

The cause of reduction in the graft size during the 8 weeks after the cell-sheet transplantation in both groups was not fully addressed in this study. However, one may consider that this reduction was caused by host immune rejection. We used a combined 3 immunosuppressant regimen, consisting

of tacrolimus, mycophenolate mofetil, and corticosteroid, because our experiment was a xenotransplantation model, in which human tissue-derived cells were transplanted in a porcine. In addition, mesenchymal stem cells, which have the potential to induce immunologic tolerance,²⁹ were involved in creating hiPS-CM cell sheets, and recent studies have reported that the omentum has not only angiogenic cytokines and growth factors but also anti-inflammatory properties and thus can facilitate tissue healing of injured tissue or organs.³⁰ With our cell delivery method that combines the cell-sheet method with the pedicled omental flap, the 3-drug immunosuppressant regimen, and a mixture of mesenchymal stem cells, it would be difficult to permanently maintain a large number of delivered cells in this xenotransplantation model. Future clinical study of hiPS-CM transplantation for treating heart disease might be performed as allogeneic transplantation.³¹ Further studies related to immunologic tolerance are needed to maintain the delivered cells long-term or permanently in this treatment.

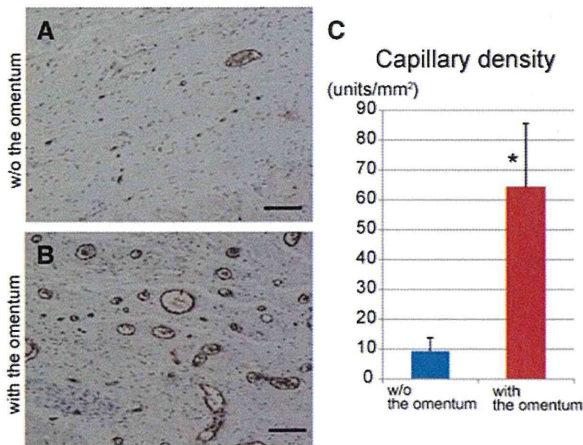


Figure 5. Capillary density in the transplanted area. Photomicrographs of immunostaining for von Willebrand factor are shown in **A** and **B**; scale bar, 50 μm . **C**, The capillary density in the transplanted area was significantly greater in the mini-pigs with the omentum than in those without it. * $P<0.0001$ vs without the omentum.

In addition, more importantly, hiPS-CM cell sheets were transplanted over the normal epicardium, in which the tissue structure is well organized. New vascular network formation between the native myocardium and the transplanted cell sheets is thus insufficient to support the survival of the transplanted cells, leading to reduction of surviving transplanted cells long-term. In the clinical scenario, however, cell sheets will be transplanted over the diseased heart surface, in which epicardial structure is impaired. Conditions of the host myocardium possibly influence the survival of the transplanted cells. Our results indicate that transplanted cell sheets may provide sufficient blood supply, not from the host myocardium but from the omentum tissue. Thus, we consider that the omentum flap technique could provide a well-organized vascular network, regardless of conditions of the host myocardium, to enhance the survival of the transplanted cells. Further studies are needed to explore the mechanisms underlying integration of the transplanted cells sheets into the heart and to develop methods to enhance the survival and functionality of the transplanted cells.

Cardiac tissue engineering is another strategy that uses stem cells for the treatment of heart failure. One of the major challenges of in vitro engineering techniques is to overcome the limited thickness of the construct because the maximum oxygen diffusion is limited to $\approx 200 \mu\text{m}^2$. A few recent methodologies have successfully yielded thicker engineered cardiac tissues. Cardiomyocytes in the Matrigel matrix were implanted with an arteriovenous blood vessel loop in vivo, and spontaneously contracting, thick, 3-dimensional constructs with extensive vascularization were thus attained.³² The cell-sheet method, which is a scaffold-free system, is also an in vitro engineering technique. A cell sheet, itself, has a potential to induce angiogenesis quickly after implantation, and cell-dense 1-mm thick cardiac tissue was developed by repeated transplantation of triple-layered rat neonatal cardiac cell sheets.³³ This cardiac graft generated by this method, however, would be limited in use as a

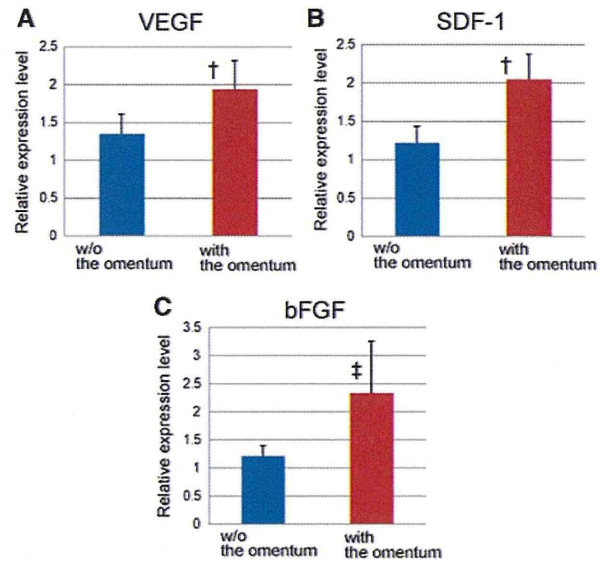


Figure 6. Angiogenesis-related mRNA expression in the transplanted area, as measured by real-time polymerase chain reaction. Relative expression of angiogenesis-related factors at the transplanted area was significantly greater in mini-pigs with the omentum than in those without it (**A**, vascular endothelial growth factor [VEGF], † $P<0.05$; **B**, stromal-derived factor [SDF]-1, † $P<0.05$; **C**, basic fibroblast growth factor [bFGF], ‡ $P<0.01$ vs without the omentum).

graft transplanted to the heart because of the lack of responsible large arteries and veins that can be revascularized after transplantation to the heart. In the present study, we used the omentum as a blood supply source after cell transplantation and demonstrated that the omentum enhanced angiogenesis and survival of the delivered cells. In addition, the omentum can easily be handled and mobilized, preserving its vascular network. The omentum, therefore, is a promising tool for in vivo vascularization in cardiac tissue engineering, although further studies with technological development would be needed for this strategy.

In conclusion, covering of the omentum flap over the transplanted hiPS-CM cell sheets on the myocardium effectively promoted angiogenesis, leading to enhanced survival of the hiPS-CMs. These results warrant further investigations as a clinically relevant strategy to enhance hiPS-CM transplantation therapy for heart failure.

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Disclosures

Dr Shimizu is a consultant for CellSeed, Inc. Dr Okano is an Advisory Board Member in CellSeed, Inc, and an inventor/developer designated on the patent for temperature -responsive culture surfaces. The other authors report no conflicts.

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