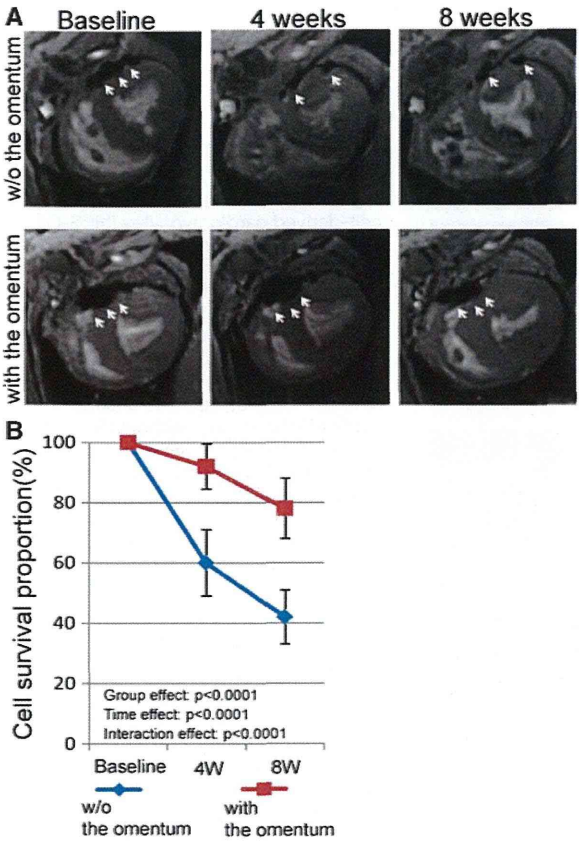

Figure 3. In vivo analysis of the survival of superparamagnetic iron oxide (SPIO)–labeled human induced pluripotent stem cell–derived cardiomyocytes (hiPS-CMs) after transplantation. **A**, Serial cardiac MRIs were examined at 1 week (baseline), 4 weeks, and 8 weeks after SPIO-labeled hiPS-CM cell-sheet 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/mm^2) than in those without it (9±5 U/mm^2; 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 AKT[24] 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

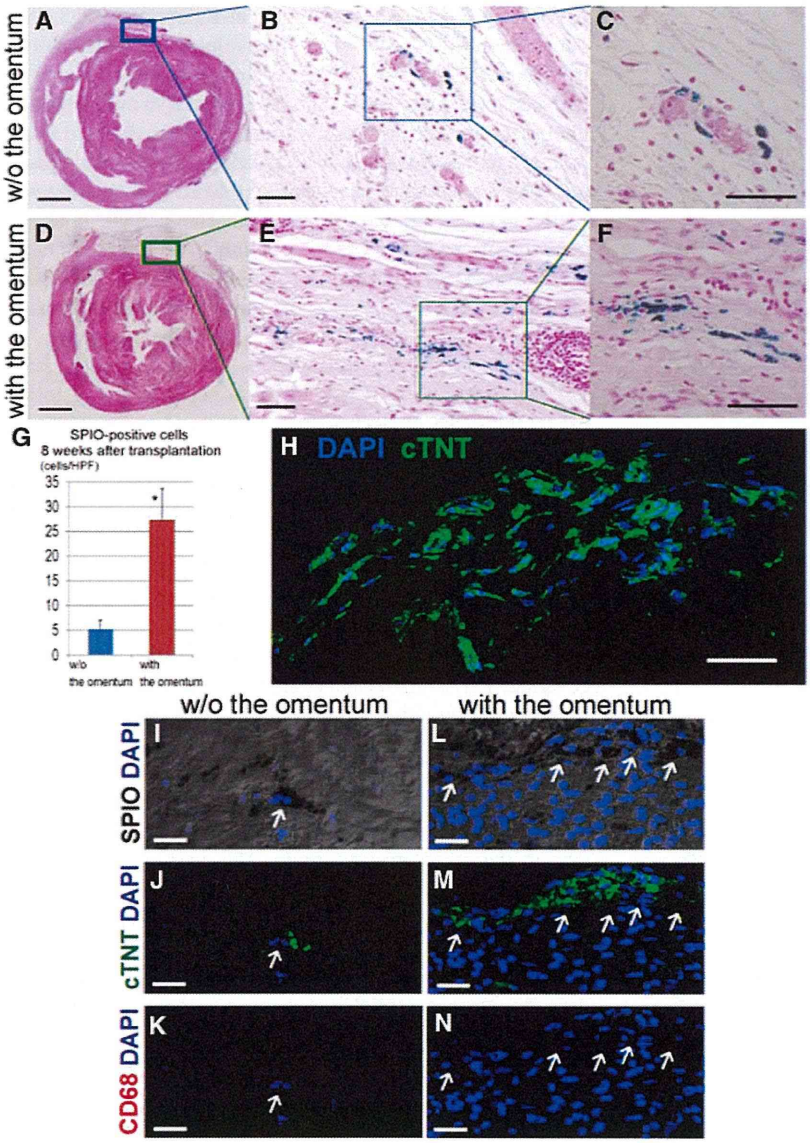

Figure 4. Human induced pluripotent stem cell–derived cardiomyocytes (hiPS-CMs) after transplantation. Macroscopic images of the whole heart by hematoxylin–eosin staining at the mid level in the mini-pig without (**A**) or with (**D**) the omentum; scale bar, 1 cm in **A** and **D**. Cells containing 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 μm 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 immunohistolabeling (green). The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue); scale bar, 50 μm 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 indentified by immunohistolabeling. The cell nuclei were counterstained with DAPI (blue). Arrows indicate SPIO particles, referred to DIC images in **I** and **L**; scale bar, 20 μm 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.

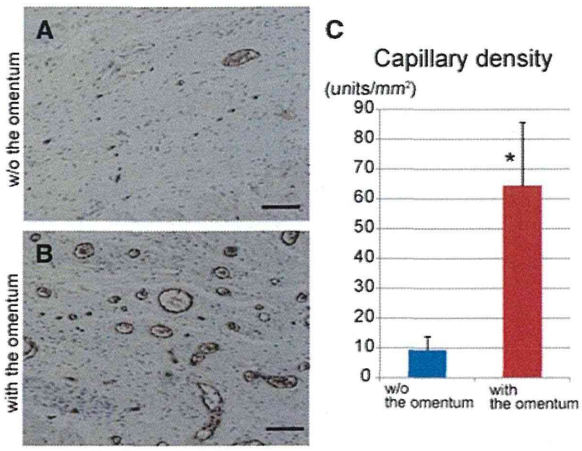

Figure 5. Capillary density in the transplanted area. Photomicrographs of immunostaining for von Willebrand factor are shown in **A** and **B**; scale bar, 50 μm. **C**, The capillary density in the transplanted area was significantly greater in the mini-pigs with the omentum than in those without it.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

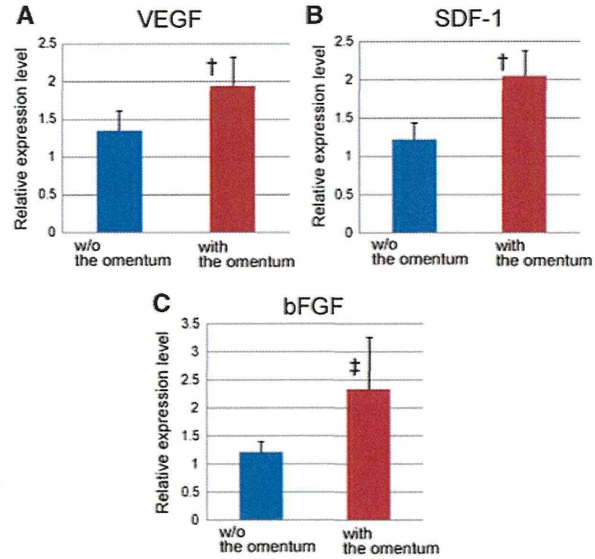

Figure 6. Angiogenesis-related mRNA expression in the transplanted area, as measured by real-time polymerase chain reaction. Relative expression of angiogenesis-related factors at the transplanted area was significantly greater in mini-pigs with the omentum than in those without it (**A**, vascular endothelial 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).

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 ≈200 μm^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 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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