Original Article

Subcutaneous transplantation of engineered islet/adipose-derived mesenchymal stem cell sheets in diabetic pigs with total pancreatectomy

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A B S T R A C T

Introduction: Intraportal islet transplantation is a promising therapeutic approach for patients with type 1 diabetes mellitus (T1DM). However, despite being minimally invasive, the method has some limitations, such as short-term graft loss, portal venous thrombosis, and difficulty in collecting adequate amounts of islets. Subcutaneous islet transplantation on adipose-derived mesenchymal stem cell (ADSC) sheets has been suggested to overcome these limitations, and in this study, we have examined its feasibility in T1DM pigs.
Methods: Inguinal subcutaneous fat was harvested from young pigs and then isolated and cultured adequate ADSCs to prepare sheets. Islets were isolated from the pancreases of mature pigs and seeded on the ADSC sheets. T1DM pigs were generated by total pancreatectomy, and ADSC sheets with transplanted islets were administered subcutaneously to the waist ($n = 2$). The effects of the islets on the ADSC sheets and on blood glucose levels were evaluated. Insulin secretion was measured by insulin stimulation index.
Results: Islet viability was higher on ADSCs compared to islets alone (91.8 ± 4.3 vs. $81.7 \pm 4.1\%$). The insulin stimulation index revealed higher glucose sensitivity of islets on ADSC sheets compared to islets alone (2.8 ± 2.0 vs. 0.8 ± 0.3). After transplantation, the blood glucose levels of two pigs were within the normal range, and sensitive insulin secretion was confirmed by intravenous glucose tolerance tests. After graftectomy, decreased insulin secretion and hyperglycemia were observed.
Conclusions: Subcutaneous islet transplantation using ADSC sheets can regulate the blood glucose levels of T1DM pigs.

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1. Introduction

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease characterized by hyperglycemia caused by insulin deficiency induced through the destruction of pancreatic islets. Patients with T1DM are dependent on exogenous insulin to survive, either by daily injection or infusion [1]. Despite insulin replacement therapy, patients are continually at the risk of life-threatening hypoglycemia or hyperglycemia as well as serious complications such as retinopathy, neuropathy, and nephropathy because of unstable glycemic control [2]. The whole pancreas or islet transplantations are promising therapies to provide physiological glycemic control for T1DM. While the former is surgically invasive and associated with

significant comorbidity, the latter is a safe procedure with little comorbidity [3]. Patients receiving intraportal islet transplantation can maintain an insulin-independent state; however, the median duration of insulin independence is only 35 months, and the procedure requires pancreas from multiple donors [4]. Further, complications can occur at the intraportal transplantation site, including the risk of portal thrombosis and bleeding related to elevated portal pressures. Further, 60−80% of islets are lost immediately after transplantation through instant blood-mediated inflammatory reaction (IBMIR) [5]. Moreover, graft destruction can occur owing to the insufficient blood supply and innate and acquired immune reactions, despite the administration of immuno-suppressive regimens [6,7].

To avoid IBMIR-induced graft loss, several studies have examined extrahepatic islet transplantation sites, including the omentum [8], peritoneum [9], gastric submucosa [10], renal subcapsule [11], and bone marrow [12], as well as the intramuscular [13] and subcutaneous [14] spaces. Each site offers advantages and disadvantages, including various levels of transplant capacity, accessibility, vascularity, and invasiveness, but none have demonstrated practical and sufficient long-term glycemic control. In 2015, we reported that the subcutaneous transplantation of tissue-engineered sheets of islets and mesenchymal stem cells (MSCs) into severe combined immunodeficient mice with streptozotocin-induced diabetes induced long-term normoglycemia [15]. MSC sheets were used to induce anti-inflammatory, immunoregulatory, and angiogenic properties [16−18]. We have also examined the effective compatibility of MSC sheets and islets, showing that engineered MSC sheets provide an optimal substrate for islets, acting as a better source of cytokines and extracellular matrix than islets alone or islets cocultured with MSCs [19]. We have also clarified the best type of MSCs for the production of cell sheets with islets, reporting that adipose-derived mesenchymal stem cells (ADSCs) show significant advantages in islet viability, islet recovery rate, and insulin stimulation compared to fibroblasts and bone marrow-derived MSCs [20]. In this study, the investigation was further extended to testing the effects of islet-containing ADSC sheets on diabetic pigs, with an aim of eventually translating the method into clinical practice.

2. Materials and methods

2.1. Animals and experimental protocol

Two-month-old young female pigs (10−20 kg) were reared in the same farm according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Nagasaki University (1604111298).

The transplantation protocol is shown in Fig. 1. First, the inguinal subcutaneous fat was harvested from a young pig, and ADSCs were isolated and cultured. After confirming that a sufficient amount of cultured ADSCs was obtained, the ADSC sheets were prepared using the tissue engineering technique described below. The pancreases were harvested from five-year-old mature edible pigs, and islet isolation was performed. Next, the islets were seeded on the ADSC sheets and cultured. T1DM model pigs were then prepared by the total pancreatectomy of young pigs, and the sheets were subcutaneously transplanted into the waist of T1DM pigs.

2.2. ADSC isolation and culture

A young pig was anesthetized by inhalation of sevoflurane 2−3 weeks before islet isolation. Inguinal subcutaneous adipose tissues were carefully collected under local anesthesia using lidocaine and

briefly stored in phosphate-buffered saline (PBS; Wako, Osaka, Japan) at 4 °C. The adipose tissue was decontaminated with povidone-iodine and rinsed with PBS. Approximately 8 g of adipose tissue was minced into pieces <1 mm^3 using sterile surgical scissors. The minced tissues were split into two 4 g samples, which were immersed in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 IU/mL penicillin (Gibco), and 100 μL/mL streptomycin (Gibco) in a centrifuge tube and digested with collagenase NB (Crescent Chemical Co., Inc, NY, USA) for 1 h at 37 °C with shaking. Then, the tubes were centrifuged at 400×g at 4 °C for 5 min, gently shaken for 30 s, and centrifuged again. The top layer was removed, and the remaining solution was sequentially filtered through 100 and 40 μm filters. The filtrate was then centrifuged at 1500×g at 4 °C for 5 min, and the supernatant was aspirated. The cell pellet was resuspended in 24 mL DMEM and seeded into two 10-cm cell culture dishes (Corning® Primaria™, Corning Inc., Corning, NY, USA). Nonadherent cells were removed after 24 h of culture. The culture medium was replaced with fresh medium every 2−3 d. Cultures were maintained in a 5% CO_2 air-humidified atmosphere at 37 °C.

2.3. Characterization of ADSCs by flow cytometry

ADSCs were identified using the following markers: CD31, CD44, CD45, and CD90. First, ADSCs were incubated with fluorescence-conjugated primary antibodies against these markers for 1 h. Then, the cells were washed thrice in PBS and incubated with secondary antibodies for 1 h. After three washes, ADSCs were analyzed on a FACScanto II (Becton Dickinson, Lincoln Park, NY, USA) flow cytometer using FACSDiva software (Becton Dickinson). The following antibodies were used: allophycocyanin (APC)-conjugated mouse anti-CD31 (GeneTex Inc., CA, USA), phycoerythrin-conjugated mouse anti-CD44 (Abcam, Cambridge, MA, USA), APC-conjugated mouse anti-CD45 (BioLegend, San Diego, CA, USA), and fluorescein isothiocyanate-conjugated mouse anti-CD90 (Abcam).

2.4. Construction of ADSC sheets

The isolated ADSCs were cultured for approximately 2 weeks, harvested at passages 1−2, and seeded on 35-mm temperature-responsive cell culture dishes (UpCell®; CellSeed, Tokyo, Japan) prepared with cell culture medium. Cells were seeded at densities of 1.0×10^6 and 2.0×10^6 and comparatively examined for strength, vascularization, and oxygenation. The sheet strength was evaluated by measuring size and thickness. Vascularization was compared by examining interleukin (IL) 6 and vascular endothelial growth factor A (VEGFA) expression by quantitative real-time polymerase chain reaction (qRT-PCR). Oxygenation was evaluated by examining hypoxia-inducible factor 1α (HIF1α) expression and anaerobic metabolism.

2.5. qRT-PCR analysis

As previously mentioned, the vascularization of ADSC sheets was compared by examining IL6 and VEGFA expression. Total RNA was extracted from ADSC sheets seeded at 1.0×10^6 and 2.0×10^6 on 35-mm temperature-responsive cell culture dishes using a spin column (NucleoSpin RNA II; Macherey−Nagel, Düren, Germany) following the manufacturer's instructions. The cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo, Japan) following the manufacturer's instructions. The samples were then stored at −20 °C until use. PCR was performed using an Applied Biosystems StepOnePlus Real-
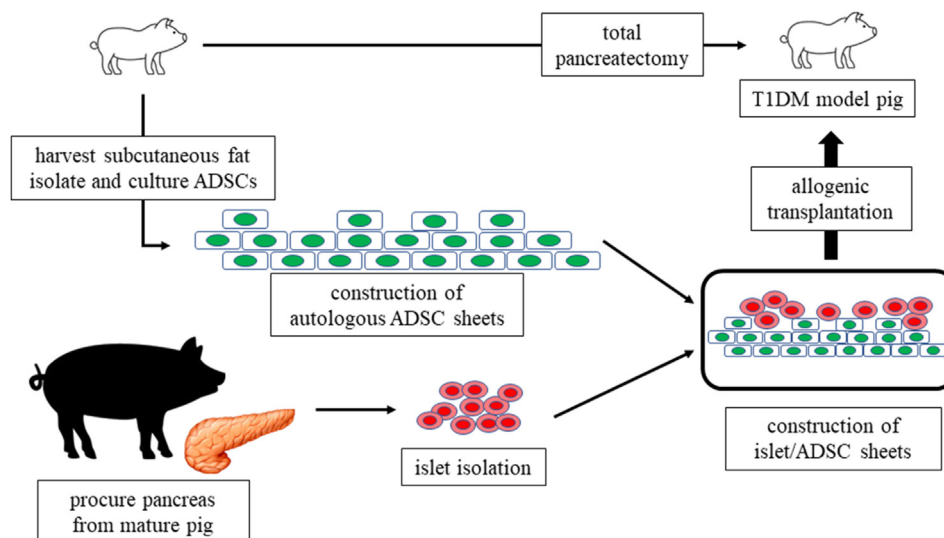
Fig. 1. Experimental design for allogenic transplantation. Subcutaneous fat was harvested from a young pig and isolated ADSCs were used to generate sheets. Pancreatic islets were procured from mature pigs, and islet/ADSC sheets were constructed by seeding isolated islets on the ADSC sheets and incubating them for 24 h before allogenic subcutaneous transplantation into the waist of T1DM model pigs generated by total pancreatectomy.

time PCR system with the TaqMan Gene Expression Assay Kit (Applied Biosystems) following the manufacturer's instructions. In brief, PCR amplification was performed using a reaction mixture containing 1 μL cDNA sample, 0.5 μL TaqMan Gene Expression Assay probe, 5 μL TaqMan Fast Advanced Master Mix (Applied Biosystems), and 13.5 μL nuclease-free water. The cycling conditions were 95 °C for 1 s and 60 °C for 20 s. The comparative cycle threshold method was used to quantify relative gene expression levels normalized to those of pig beta-actin.

2.6. Islet isolation, purification, and culture

Pig pancreases were procured from a local slaughterhouse. We used five-year-old mature pigs weighing approximately 250 kg, as they have large pancreases (about 250 g) that are easy to cannulate. Approximately 10 min after cessation of the heart beating, the operation was started. All pancreases were procured within 10 min of the onset of the operation and were weighed immediately. As soon as each pancreas was removed, we inserted a cannula into the main pancreatic duct and slowly injected the University of Wisconsin (UW) solution (Belzer UW®; Bridge to Life Ltd, Columbia, USA) into it at a concentration of 1 mL/g pancreas. The pancreases were placed into the UW solution at 4 °C for approximately 2 h before islet isolation.

Islet isolation was performed following the standard Ricordi technique, with changes introduced in the Edmonton protocol [21—24]. After decontamination of the pancreas, the pancreatic ducts were perfused with a cold blend of Collagenase I/II Blend Research Grade and Thermolysin Research Grade packed in Liberase T-Flex Research Grade (all Roche Diagnostics Corporation, Indianapolis, IN, USA) through the ductal cannula. Then, approximately 100 g of the pancreas was cut into eight pieces, placed in a Ricordi chamber, and gently shaken. The temperature of the enzyme solution was maintained at 37 °C during digestion. Once islet digestion was confirmed by dithizone staining, collection and washing was performed using cold 25% albumin and Minimum Essential Medium (MEM; Gibco) and preserved in UW solution with gentle stirring for 30 min before purification.

Islet purification was performed using a UW/Ficoll continuous gradient on a COBE 2991 cell processor (COBE; CaridianBCT, Denver, CO, USA). Density gradients of 1.075 and 1.085 g/mL were loaded into the COBE bag using a gradient-making device. The digested tissue suspended in UW solution was added and centrifuged for 5 min at 3000 rpm. Approximately 20 fractions were collected and examined for islet purity and quantity.

Collected islets were cultured in CMRL-1066 medium (Sigma-−Aldrich, St Louis, MO, USA) containing 10% FBS, 100 IU/mL penicillin, and 100 μL/mL streptomycin and incubated at 22 °C, 5% CO_2, and 95% humidity for 24 h.

2.7. Addition of islets to ADSC sheets

After 24 h of ADSC sheet culture on temperature-responsive dishes, the islets were isolated and preserved in CMRL-1066 medium. After an additional 24 h, 10,000 islet equivalents (IEQs) were gently seeded on each ADSC sheet and stirred evenly in all directions. The procedures of seeding and stirring were gently and carefully carried out on a heating board at 37 °C so as to not detach the ADSC sheets. Islet/ADSC sheets were cultured for 24 h in 5% CO_2 at 37 °C before transplantation. Sheets were confirmed by immunohistochemistry 24 h after preparation, and their function was evaluated by comparing the islet viability, IL8 and IL16 secretion, and glucose-stimulated insulin secretion in sheets containing equal amounts of islets.

2.8. Immunohistochemistry

Islet/ADSC sheets were detached, fixed with 4% paraformaldehyde (Wako Pure Chemical Industries Ltd., Osaka, Japan), and embedded in paraffin (Paraplast Plus; Leica Biosystems Inc., Richmond, IL, USA). Then, serial sections were generated and stained with hematoxylin and eosin (H&E; Muto Pure Chemicals Co. Ltd., Tokyo, Japan). The presence of β cells was confirmed by immunostaining with guinea pig anti-pig insulin antibodies (Bio-Rad, Hercules, CA, USA).

2.9. Islet viability assays

The Cellstain Double Staining Kit (Dojindo, Kumamoto, Japan), consisting of calcein acetoxymethyl and propidium iodide, was used to analyze islet viability according to the manufacturer's instructions. Samples were observed on an Eclipse Ti−U fluorescent microscope (Nikon, Tokyo, Japan). Islet viability was determined by counting viable (yellow-green) and non-viable (red) cells. The degree of cell viability was assessed as previously described [25]. Total viability was calculated by dividing the number of viable cells by the total number of cells assessed.

2.10. Cytokine and insulin secretion assays of islet/ADSC

IL8 and IL16 secretions were measured to evaluate the islet-protective effect of the ADSC sheet. IL8 is a potent inducer of angiogenesis, and it is reported for the islets [26]. On the other hand, IL16 production is reported to augment the severity of insulitis during the onset of T1DM, and neutralization of IL16 is a strategy to prevent T1DM [27]. The concentrations of pig IL8 and IL16 were measured in the medium 24 h after islet/ADSC sheet preparation using enzyme-linked immunosorbent assay (ELISA) kits (IL8; Abcam, Cambridge, MA, USA: IL16; Toronto Research Chemicals, North York, ON, Canada) according to the manufacturer's instructions. DMEM was used as a control sample.

To measure the reaction of the islet/ADSC sheet to blood sugar, glucose-stimulated insulin secretion tests were conducted 24 h after preparing islet/ADSC sheets as previously described [19] In brief, Krebs solution was prepared by combining 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid, 115 mM NaCl, 24 mM NaHCO$_3$, 5 mM KCl, 1 mM MgCl$_2$·6H$_2$O, 0.1% bovine serum albumin, and 2.5 mM CaCl$_2$·2H$_2$O (Wako Pure Chemical Industries). Islet/ADSC sheets and equal amounts of islets alone were sequentially incubated for 1 h each in Krebs solutions containing 2.8 and 28 mM of glucose. After each incubation period, the supernatant was collected, and the insulin concentration was measured using an ELISA kit (Mercodia AB, Uppsala, Sweden) according to the manufacturer's instructions. The insulin stimulation index was calculated by dividing the amount of insulin secreted at 28 mM glucose by the amount secreted at 2.8 mM glucose.

2.11. Preparation of T1DM model pigs

To generate T1DM model pigs ($n = 5$), total pancreatectomy was performed with general anesthesia and tracheal intubation as previously described [28]. A central venous line was then inserted into the right jugular vein for administering medication and painless regular blood sampling. Furthermore, a continuous glucose

monitor was attached (CGM; Abbott Japan Co., Ltd, Tokyo, Japan) to the anterior chest, allowing blood glucose measurements at an interval of 15 min.

2.12. Biochemical examination

Blood samples were obtained from each pig through the central venous line. The blood insulin concentration was measured using a chemiluminescent enzyme immunoassay and the ketone body level was measured using the enzymatic method (SRL, Inc. Okayama, Japan). When necessary, the blood glucose levels were measured using a One Touch Ultra View Blood Glucose Monitor (Johnson & Johnson Co., Ltd, Tokyo, Japan).

2.13. Subcutaneous transplantation of islet/ADSC sheets into pigs

After 24 h of culture of the islet/ADSC sheets on temperature-responsive culture dishes, they were transferred to an operating room at 20 °C and detached spontaneously through low-temperature treatment. Sheets were subcutaneously transplanted into the waists and buttocks of T1DM pigs under general anesthesia. In this allogenic large animal experiment, observations were made without the use of immunosuppressants. The glucose levels and insulin secretion by CGM and blood sampling were monitored. Diet was unchanged before and after total pancreatectomy and subcutaneous transplantation. For the first week after transplantation, saline and glucose were injected to stabilize general conditions, including the blood glucose level. Intravenous glucose tolerance tests (IVGTTs) were performed 10 d after transplantation. Graftectomy, including the removal of subcutaneous fat and fascia from the transplant site, was performed 14 d after transplantation based on the survival of T1DM model pigs, and monitoring was continued. Extracted grafts were examined by immunohistochemistry as described above.

2.14. IVGTT

IVGTT was conducted as previously reported [29], 10 d after transplantation. A bolus injection of 50% glucose was administered at 0.5 g/kg body weight. Blood was collected through the central line at 0, 1, 3, 5, 7, 10, 20, 30, 45, and 60 min to measure blood glucose levels and insulin concentrations.

2.15. Statistical analysis

Statistical analyses were performed using Stat Mate V (ATMS, Tokyo, Japan). Data are expressed as means ± standard deviation (SD). The Mann−Whitney U test was used for inter-group
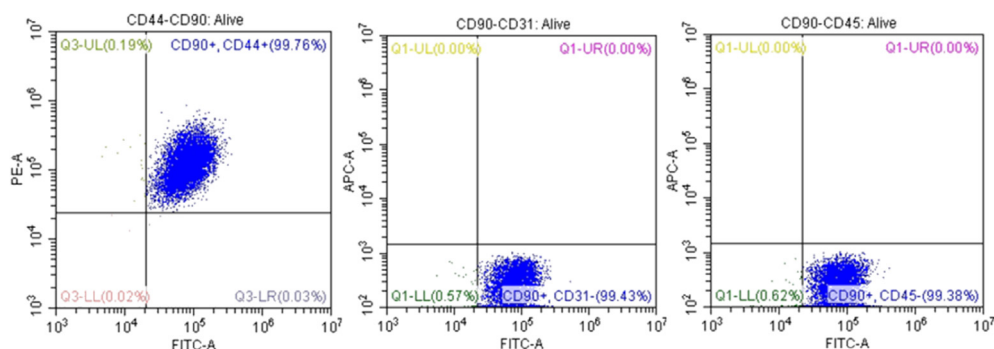

Fig. 2. Flow cytometric analysis of cells isolated from inguinal subcutaneous fat. Cells were assayed for expression of the mesenchymal markers CD44 and CD90, the vascular endothelial cell marker CD31, and the hematopoietic stem cell marker CD45.

comparisons. Differences were considered significant when P values were <0.05.

3. Results

3.1. ADSC characteristics

Cells isolated from the inguinal subcutaneous fat were positive for the mesenchymal markers CD44 and CD90 but negative for the vascular endothelial cell marker CD31 and the hematopoietic stem cell marker CD45 (Fig. 2). These results indicate that the isolated cells exhibited the characteristic immunophenotype of ADSCs.

3.2. Evaluation of ADSC sheets

Next, the optimal cell density for preparing ADSC sheets was determined. ADSC sheet strength was measured by electronic microscope ($n = 5$). ADSC sheets with 2.0×10^6 cells/sheet were significantly thicker than sheets with 1.0×10^6 cells/sheet (32.0 ± 2.7 vs 25.7 ± 5.2 nm, $p < 0.05$, Fig. 3A). Vascularization and oxygenation were unaffected by the cell concentration ($n = 6$; Fig. 3B and C). To examine the proportion of anaerobic metabolism, the ratio of lactate production to glucose consumed was measured by ELISA ($n = 6$). The proportion of anaerobic metabolism was lower in the sheets containing 2.0×10^6 cells (0.036 ± 0.002 vs 0.047 ± 0.003, $p < 0.05$). Based on these results, ADSC sheets were prepared with 2.0×10^6 cells/sheet. Although the higher density resulted in thicker sheets, they displayed fewer ischemic changes.

3.3. Evaluation of islets on ADSC sheets

H&E and insulin staining revealed a strong ADSC scaffold that enabled the adherence of most of the seeded islets. Islet/ADSC sheets shrunk during their removal from temperature-responsive cell culture dishes; therefore, some islets appeared to be incorporated into the ADSC sheets (Fig. 4A). The function of islet/ADSC sheets was evaluated compared to equal amounts of isolated islets. Islet viability was higher in islet/ADSC sheets than in islets alone (Fig. 4B and 918 ± 4.3 vs $81.7 \pm 4.1\%$, $p < 0.05$). Islet/ADSC sheets displayed significantly higher secretion of vascularization cytokine IL8 (381.6 ± 249.5 vs 0 ± 0 pg/mL, $p < 0.05$) than islets alone, and the secretion of IL16, an indicator of T1DM onset and exacerbation, was significantly lower in the islet/ADSC sheets (1875.3 ± 135.3 vs 2179.2 ± 264.0 pg/mL, $p < 0.05$) than in islets alone (Fig. 4C). Glucose-stimulated insulin release showed that islet/ADSC sheets were more sensitive to glucose (2.8 ± 2.0 vs 0.8 ± 0.3, $p < 0.05$) than islets alone (Fig. 4C). These results confirm that islet/ADSC sheets were superior to isolated islets in terms of islet protection.

3.4. T1DM model pigs

To confirm the blood glucose levels of the diabetic model pigs in these experiments, a total pancreatectomy was performed on five pigs and their glucose levels were monitored. Also, the insulin secretion and total ketone bodies were monitored in the blood of three of them. The blood glucose levels were elevated immediately after surgery (Fig. 5A) and were higher than the normoglycemic range (145 ± 33 mg/dL) [28]. Blood glucose level was measured until the death of the animals, and median survival was 11 d (9–15 d) without interventional treatment. Fig. 5B shows the insulin and total ketone body levels of pigs 3 and 7 d after pancreatectomy. The insulin secretion was lower than the detectable levels (0.3 μIU/mL) at both time points and significantly lower than in normal pigs used as controls (0.877 ± 0.722 μIU/mL, $p < 0.05$). Moreover, T1DM pigs produced significantly more total ketone

bodies 3 d (222.7 ± 149.0 μmol/L, $p < 0.05$) and 7 d (2065 ± 2991 μmol/L, $p < 0.05$) after pancreatectomy than control pigs owing to insulin deficiency.

3.5. Islet/ADSC sheet transplantation into diabetic pigs

Transplantation of subcutaneous islet/ADSC sheet was performed in two diabetic pigs following total pancreatectomy (Fig. 6A). The number of islets transplanted was defined by the amount yielded from islet isolation, as shown in Table 1. Warm ischemic time is defined as the time that elapsed between the cessation of heartbeat and the placement of the pancreas into the preservation solution. Cold ischemic time is defined as the time that elapsed between the placement of the pancreas into the preservation solution and the start of islet isolation. Thus, normoglycemia was confirmed within 2 weeks, and graftectomy was performed on day 14 as planned.

Both cases displayed hypoglycemia in the first week after transplantation that was sufficient to require glucose injection and later displayed normoglycemia not requiring any glucose injection until graftectomy. After graftectomy, the animals died at 7 and 1 d, respectively (Fig. 6B). Both cases revealed rapid increases in insulin the day after transplantation, which then normalized and remained consistent until graftectomy. After graftectomy, insulin secretion was undetectable, as in untreated T1DM pigs (Fig. 6C). IVGTT was conducted on case 1, 10 days after transplantation. The blood glucose level rose immediately after injection of 50% glucose and the insulin concentration also increased gradually in response. After 10 min, the blood glucose level was within the normal range (<200 mg/dL; Fig. 6D). The existence of islets in the extracted graft of case 1 was confirmed by insulin immunohistochemistry; however, the sheet structure could not be detected, and the placement of the islets was not sheet-like (Fig. 6E).

4. Discussion

This study describes an effective technique to normalize the blood glucose levels of T1DM pigs in 2 weeks, using subcutaneously transplanted islet/ADSC sheets. Engraftment was demonstrated by normalized blood glucose levels and the ability to secrete insulin, as well as the pathological findings of the graft. Importantly, this is the first report describing normoglycemia upon subcutaneous islet transplantation in a large animal.

To promote islet protection, the ADSC sheet secretes major cytokines such as transforming growth factor (TGF)β1, VEGFA, hepatocyte growth factor (HGF), and IL6. TGFβ1 stimulates the production of heat shock protein 32 (HSP32) and X-linked inhibitor of apoptosis protein (XIAP) [30]. HSP32 has a protective effect on islets and suppresses inflammatory reactions and oxidative stress [31,32], whereas XIAP has an anti-apoptotic effect on β cells [33,34]. IL6 can prevent functional impairment in interferon γ-, tumor necrosis factor α-, and IL1β-treated mouse islets, with anti-apoptotic effects [25,35]. In particular, ADSCs secrete more IL6 than fibroblasts and bone marrow MSCs [20]. Previous studies have indicated that ADSCs promote vascularization not only by secreting angiogenic growth factors such as VEGFA and HGF [36,37] but also by differentiating into endothelial cells [38,39]. These features could have also a protective effect on islet/ADSC sheets.

Intraportal islet transplantation is considered a common, safe, and minimally invasive procedure. However, transplantation on the liver surface can minimize IBMIR by avoiding direct infusion of transplanted islets or islet cells by the blood [40]. Moreover, using cell sheet technology enables the direct and stable transplantation of a cellular construct on the liver surface, with the help of an extracellular matrix layer that accumulates on the surface of the
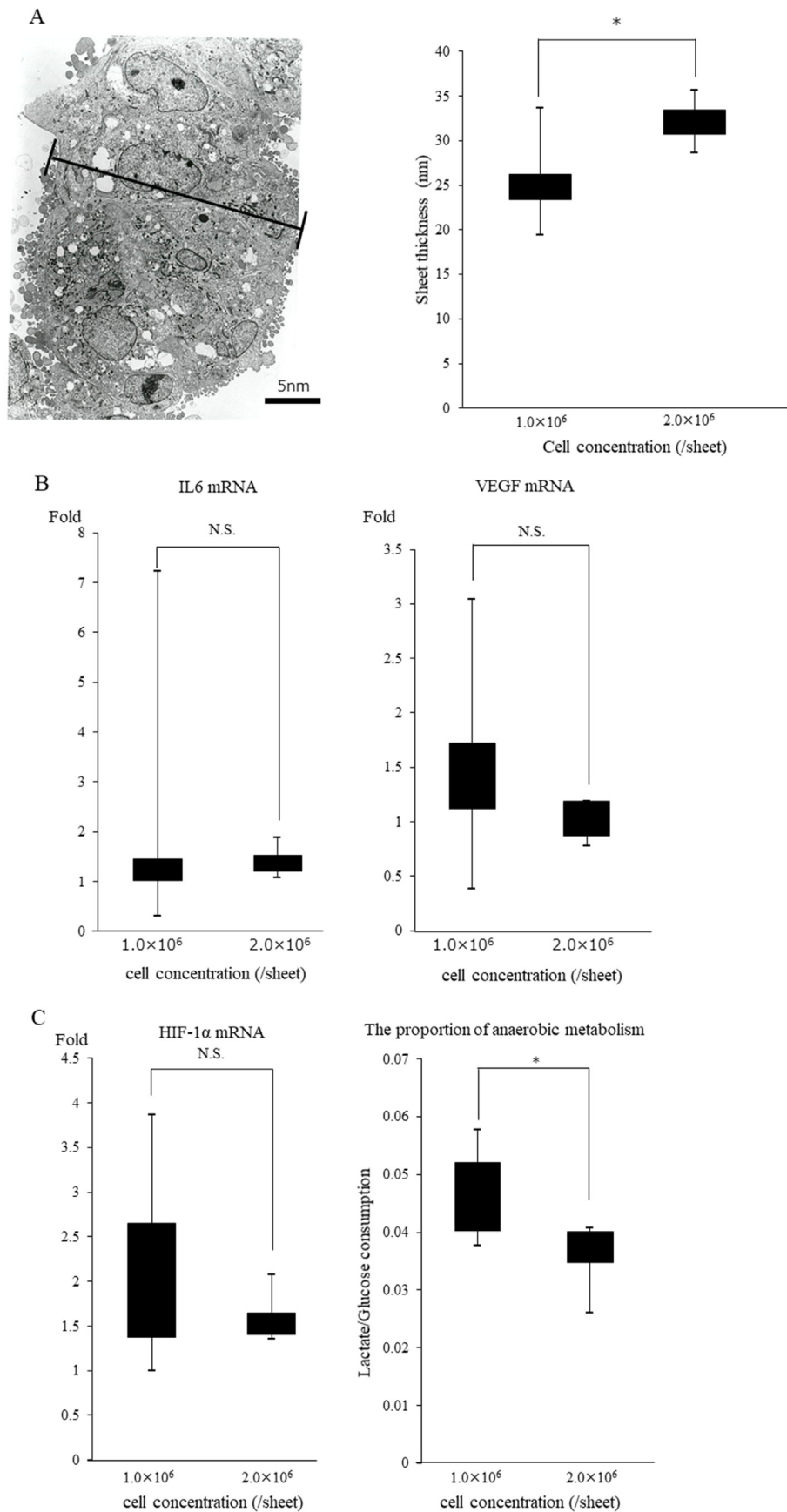
Fig. 3. Effects of cell concentration on ADSC sheet construction. (A) ADSC sheet strength was measured by electronic microscope. *$p < 0.05$; $n = 5$. (B) ADSC sheet vascularization was measured by assaying IL6 and VEGF levels by qRT-PCR. *$p < 0.05$; $n = 6$. (C) ADSC sheet oxygenation was measured by assaying HIF1α levels by qRT-PCR and the proportion of anaerobic metabolism by ELISA. *$p < 0.05$; $n = 6$.

cell sheets [41]. The angiogenic and islet-protective nature of the cell sheets enables engraftment to subcutaneous sites, which have few blood vessels. Therefore, subcutaneous transplantation can prevent acute islet loss owing to IBMIR by avoiding direct infusion by the blood flow. As the skin is the largest organ, the vast subcutaneous space can accommodate a large volume of islets. Also, subcutaneously transplanted islets can be safely and easily biopsied or removed with minimal invasiveness if problems arise with the transplanted graft. As subcutaneous tissue lacks adequate blood flow, vascularization is important to enable the therapeutic effects of islet/ADSC sheet transplantation. By improving engraftment and islet function, ADSC sheets enable efficient transplantation with early angiogenesis. In a previous study, angiogenesis was

significantly increased in islet/MSC sheets compared to that in MSC sheets alone. We suspect that islets exposed to hypoxia may produce signals that induce angiogenesis in the ADSC sheet [15]. Therefore, the subcutaneous islets using ADSC were considered suitable for islet sheet transplantation.

In this study, transplantation cases received intravenous glucose injections to counteract hypoglycemia. They exhibited hypoglycemia for approximately 7 d after transplantation, which may have been caused by excessive insulin secretion, and were treated with glucose accordingly. During this period, islet destruction likely occurred. However, at later stages treatment was not required, as the islet conditions and food intake were stable. Moreover, IVGTT demonstrated rapid insulin secretion and glucose normalization in
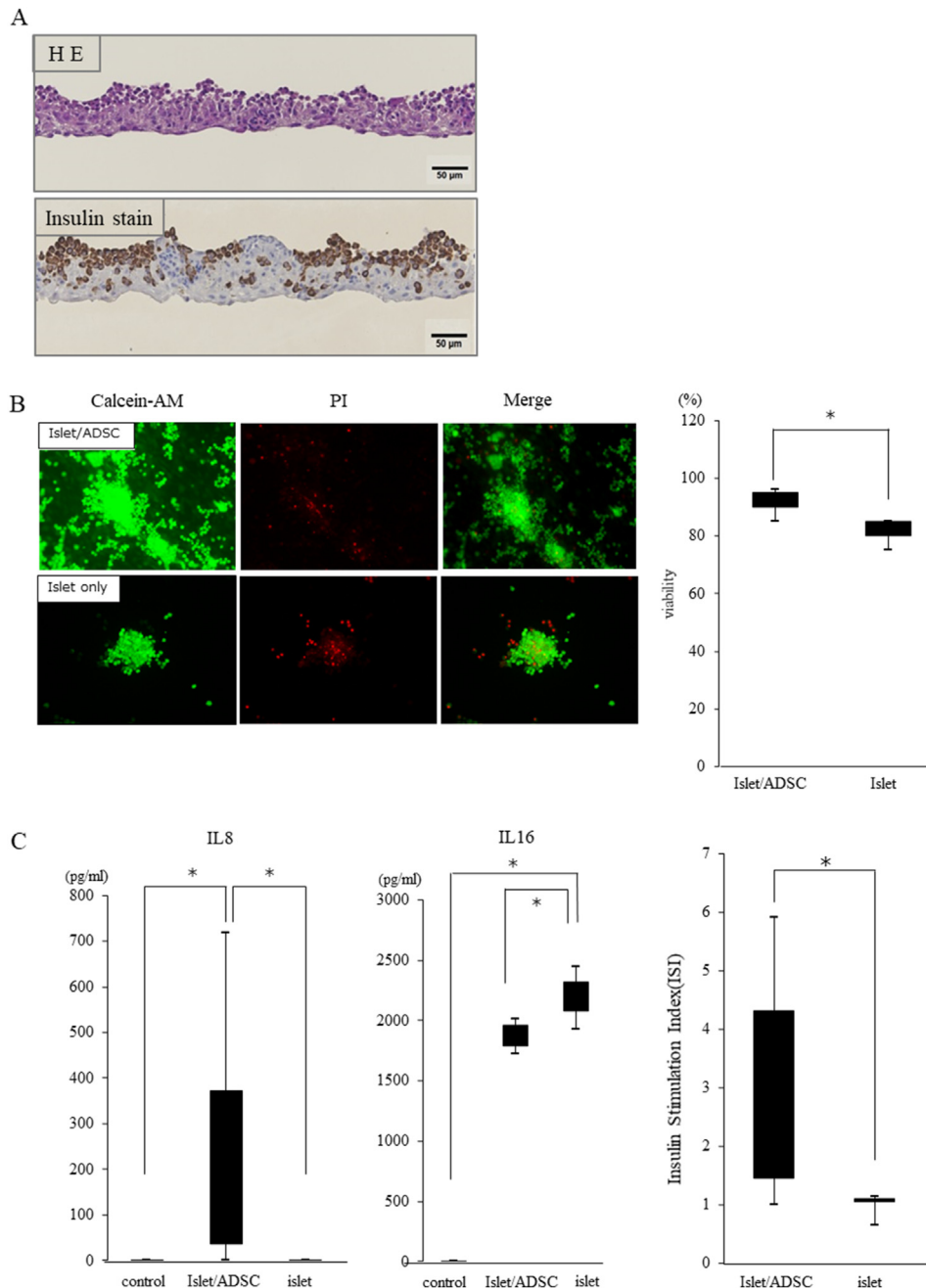

Fig. 4. **Construction of islet/ADSC sheets.** (A) Islet/ADSC sheet structures visualized by H&E and insulin staining. (B) Viability of islets on islet/ADSC sheets and islet only. *$p < 0.05$; $n = 6$. (C) IL8 and IL16 secretion and insulin stimulation in islet/ADSC sheets compared with equal amounts of isolated islets. *$p < 0.05$; $n = 6$.
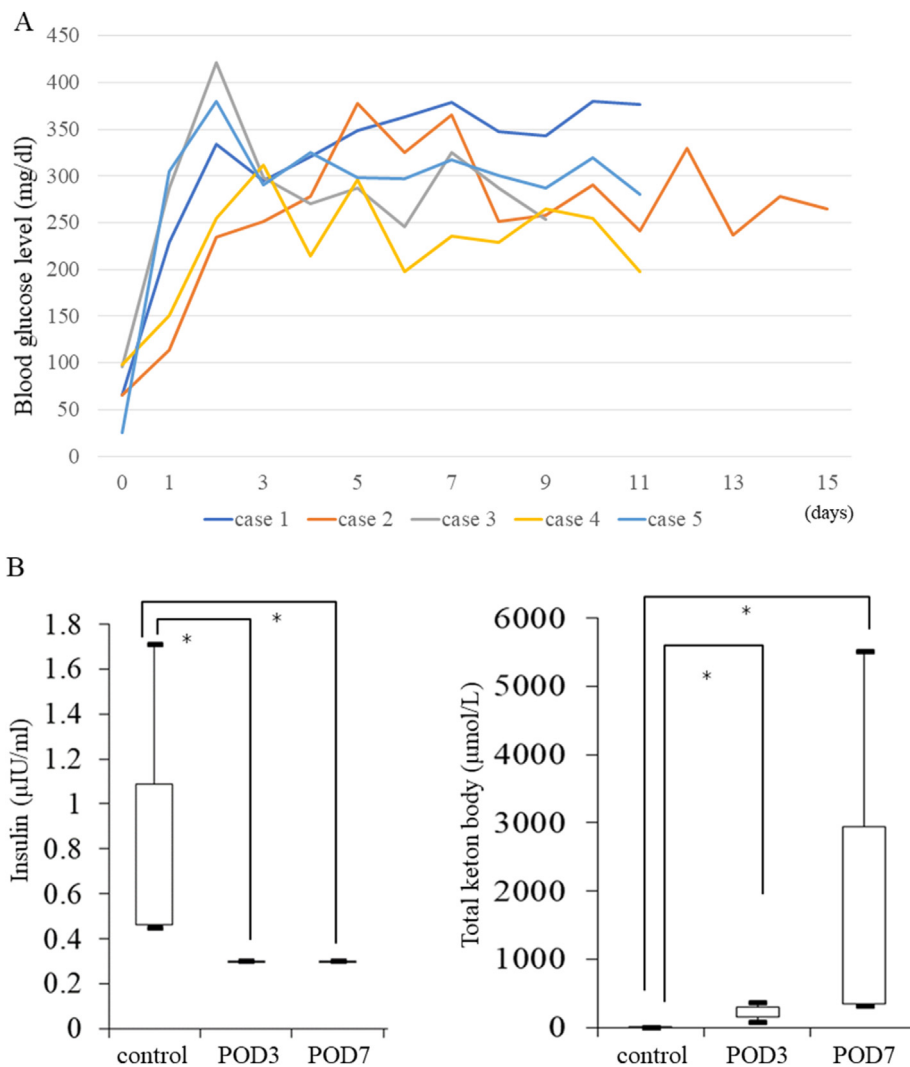
Fig. 5. Preparation of T1DM model pigs by total pancreatectomy. (A) Blood glucose levels after total pancreatectomy were measured by CGM. (B) Insulin secretion and total ketone body production 3 and 7 d after total pancreatectomy. *$p < 0.05$; $n = 3$.

response to glucose stimulation, indicating stable islet engraftment. Additionally, the continued existence of islets was confirmed by insulin immunohistochemical staining of the extracted graft and by the surge in blood glucose level after graftectomy.

Regarding the amount of islets that enables normoglycemia, our previous study showed that 10,000 islets/kg on the MSC sheet transplant to the T1DM rat subcutaneous site enabled normalization of blood glucose level [15]. In the present study, the number of islets was not clearly defined; however, 7000 IEQ/kg was needed to achieve normoglycemia. For clinical application, it is necessary to determine the required amounts of islets using these as an index.

A limitation of this study is that the procedure involves several steps, including ADSC isolation and culture, islet isolation and culture, generation of islet/ADSC sheets, and generation of T1DM pigs. In particular, the total pancreatectomy in pigs often results in difficulties in perioperative management, including intratracheal intubation and extended surgical incision [28]. In a previous intraportal islet auto transplantation on total pancreatectomized pigs, one out of five pigs was able to achieve normoglycemia for three months, but the remaining four pigs died in 12.2 ± 7.2 d with unstable blood glucose [42]. The use of pigs as a large experimental

animal requires anesthesia and surgical equipment comparable to human clinical practice, in addition to veterinary knowledge and clinical expertise. Nevertheless, this study holds clinical relevance as it provides experimental evidence from a large animal as a pre-stage of clinical practice in humans. Experiments involving three pigs or more are considered ideal to confirm reproducibility; however, considering the number of steps involved in transplantation, we used two pigs and obtained data on short-term

Table 1
Transplantation and islet/ADSC sheet characteristics.

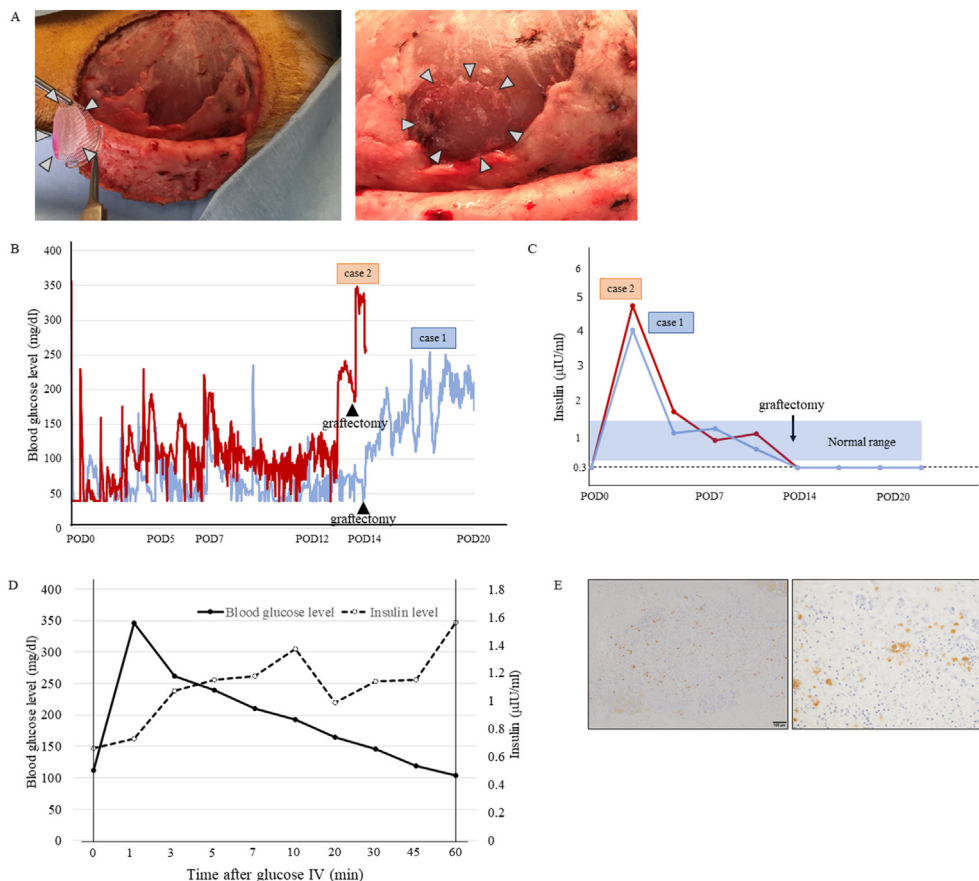|	Case 1	Case 2
Pancreas size of donor (g)	270	220
Warm ischemic time (min)	26	28
Cold ischemic time (min)	187	163
Isolated islet (IEQ)	105,108	150,082
Weight of recipient pig (kg)	15.0	15.0
Transplanted islet (IEQ/kg)	7007	10,005
Number of transplanted sheets	10	15

IEQ, islet equivalent.

Fig. 6. Subcutaneous islet/ADSC sheet transplantation, blood glucose levels, and insulin secretion. (A) Waist and buttock subcutaneous sites were exposed and carefully transplanted under general anesthesia. (B) Blood glucose levels after transplantation. (C) Insulin secretion after transplantation and after graftectomy. (D) Islet/ADSC sheet IVGTT 10 days after transplantation (case 1) (E) Islets were detected in the extracted graft of case 1 by insulin immunohistochemistry.

outcomes. In future studies, it is necessary to confirm long-term normoglycemia.

We did not use immunosuppressive drugs because of the risk of surgical infection, and the immune activity of the ADSC sheets was not determined in this study. We expected that the transplanted islets would be removed by the recipient's immune system; therefore, graftectomy was scheduled for 14 d after transplantation based on the survival of T1DM model pigs. Although the effects of drug-induced immunosuppression in pig pancreas transplantation were not examined owing to the selection of minor and class I antigen-mismatched donor/recipient pairs [43], there have been several reports on the use of immunosuppression in pig organ and cell transplantation [44–47]. Therefore, this study has demonstrated several positive effects of the ADSC sheet and also laid the prospect of evaluating its immunomodulatory ability using an immunosuppressant in future. This will be crucial for the clinical application of this technique.

5. Conclusion

In this study, we successfully normalized the blood glucose levels in T1DM pigs through the subcutaneous transplantation of islet/ADSC sheets. Future experiments should aim to further explore the islet/ADSC function. The findings of the current study might contribute to the development of novel cellular therapies for patients with T1DM.

Ethical approval

This study was approved by the Committee on the Ethics of Animal Experiments of Nagasaki University.

Statement of human and animal rights

All procedures in this study were conducted in accordance with the Committee on the Ethics of Animal Experiments of Nagasaki University's (1604111298) approved protocols.

Statement of informed consent

There are no human subjects in this article and informed consent is not applicable.

Authors contributions

Mampei Yamashita, Toshiyuki Adachi, Tamotsu Kuroki, and Susumu Eguchi: study design. Mampei Yamashita, Toshiyuki Adachi, and Tomohiko Adachi: manuscript writing. Mampei Yamashita, Toshiyuki Adachi, Tomohiko Adachi, Shinichiro Ono, Naomi

Matsumura, Kyoichiro Maekawa, Yusuke Sakai, Masaaki Hidaka, and Kengo Kanetaka: data collection and assembly.Susumu Eguchi: manuscript editing.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgments

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