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Mesenchymal Stem Cells Secretions Enhanced ATP Generation on Isolated Islets during Transplantation

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ABSTRACT

The success of islet transplantation in both basic research and clinical settings has proven that cell therapy has the potential to cure diabetes. Islets intended for transplantation are inevitably subjected to damage from a number of sources, including ischemic injury during removal and delivery of the donor pancreas, enzymatic digestion during islet isolation, and reperfusion injury after transplantation in the recipient. Here, we found that protein factors secreted by porcine adipose-tissue mesenchymal stem cells (AT-MSCs) were capable of activating preserved porcine islets. A conditioned medium was prepared from the supernatant obtained by culturing porcine AT-MSCs for 2 days in serum-free medium. Islets were preserved at 4°C in University of Wisconsin solution during transportation and then incubated at 37°C in RPMI-1620 medium with fractions of various molecular weights prepared from the conditioned medium. After treatment with certain fractions of the AT-MSC secretions, the intracellular ATP levels of the activated islets had increased to over 160% of their initial values after 4 days of incubation. Our novel system may be able to restore the condition of isolated islets after transportation or preservation and may help to improve the long-term outcome of islet transplantation.

Abbreviations: AT-MSC, adipose-tissue mesenchymal stem cell; Cas-3, caspase-3; DAPI, 4,6-diamidino-2-phenylindole; DTZ, dithizone; ES cell, embryonic stem cell; FITC, fluorescein isothiocyanate; IEQ, islet equivalent; INS, insulin; iPS cell, induced pluripotent stem cell; Luc-Tg rat, luciferase-transgenic rat; PCNA, proliferating cell nuclear antigen; PDX1, pancreatic and duodenal homeobox protein-1; UW, University of Wisconsin; ZO1, zona occludens 1.

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Introduction

For patients with type 1 diabetes, islet transplantation is a promising therapy due to its therapeutic effect and safety.[1] During islet transplantation, donor islets are infused into the hepatic portal vein, which then engraft to the hepatic parenchyma. In Japan, the islet transplantation program has adopted the immunosuppressive regimen developed by Shapiro et al.[2] which is known as the Edmonton Protocol, with the major adaptation that islets are isolated from donors after cardiac death as dictated by the Japanese national protocol for islet donation, isolation, and transplantation.[3,4]

Clinical islet transplantation can be accomplished in two ways: 1) a cold-preserved brain-dead donor's pancreas is transported to a cell processing center, where it is then transplanted into a recipient; or 2) a donor pancreas is procured and then the islets are isolated, preserved, cultured, and then transplanted into a recipient in the same facility. For islets to be useful in research and clinical applications, they must maintain their function after shipment from one location to another. It has been reported that the system through which surgeons send procured pancreas to remote islet isolation centers and the center sends back isolated islets is effective within 2,500 km.[5–7] According to a recent report, isolated human islets were successfully shipped over 10,000 km internationally, a journey longer than 48 h, with gas-permeable bags being used to maintain clinical grade.[8]

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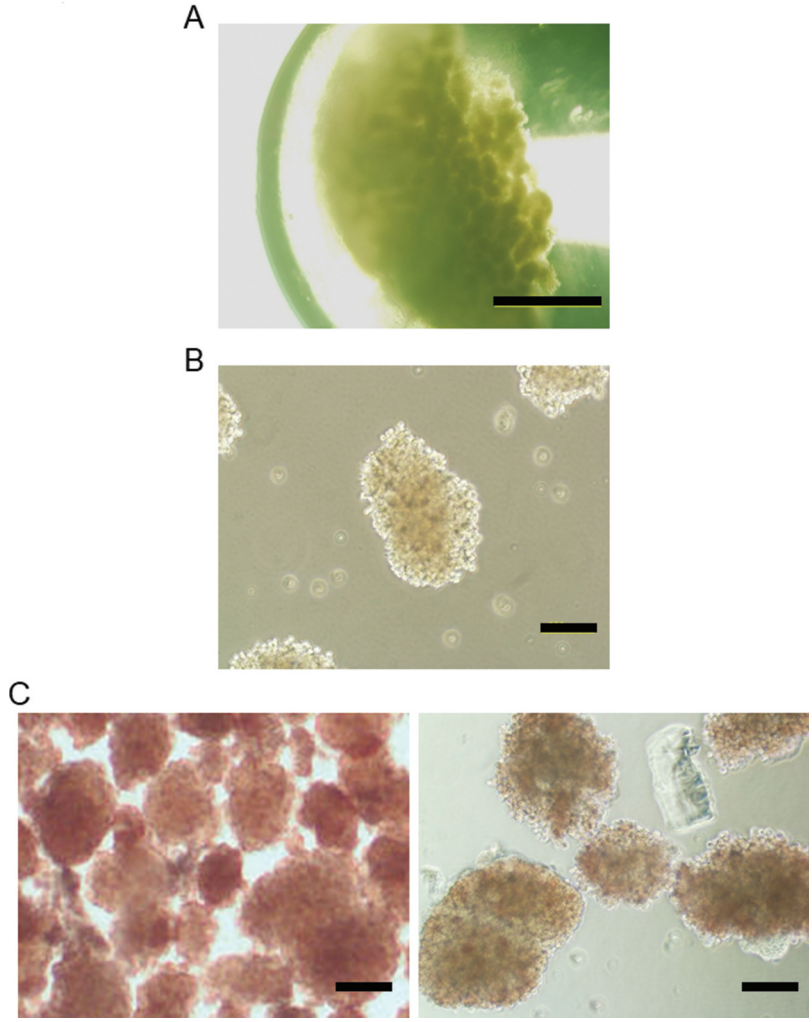

Figure 1. Shipping of porcine islets and establishment of porcine adipose-tissue mesenchymal stem cells (AT-MSCs). (a) Phase-contrast image of cold-preserved porcine islets in a 1.5-mL tube. (b) Morphology of porcine islets after 2 h under culture conditions. (c) Dithizone staining of fresh-porcine islets (left) and (b)(Right). (d) Morphology of Kusabira–Orange transgenic porcine-derived AT-MSCs at passage 6. (e) The differentiation potential of porcine AT-MSCs (passage 8) into adipocytes and osteocytes were evaluated using differentiation-induction media purchased from Lonza Walkersville, Inc. (http://www.lonza.com) according the manufacturer's protocols. (F) Analysis of porcine MSCs marker genes by RT-PCR. Scale bar (a): 1 mm (b), (c), (d), (e): 500 µm.

The success of islet transplantation greatly depends on the number of islets transplanted to the recipient (usually > 13,000 islet equivalent [IEQ]/recipient kg), and insulin independence is generally only achieved after transplantation from more than one donor preparation per recipient.[9–11] After intraliver islet transplantation, most recipients achieve insulin independence, but this condition is not permanent.[12] Although transplantation of sufficient islets makes it unnecessary for most patients to require insulin administration, the rate of independence decreases over time, with less than 10% of transplant recipients remaining insulin independent at five years.[13] It is worthwhile noting that although the liver has been extremely well studied and characterized both in animal models and humans, it is widely recognized that it may not provide the ideal microenvironment for islets due to the immunologic, anatomic, and physiologic factors that contribute to loss of islet mass soon after infusion.[14–19]

Mesenchymal stem cells (MSCs) are thought to be pluripotent cells that can differentiate into a variety of cells and can be an ideal resource for transplantation therapy.[20,21] Additionally, MSCs have been confirmed to secrete a variety of cytokines.[22]

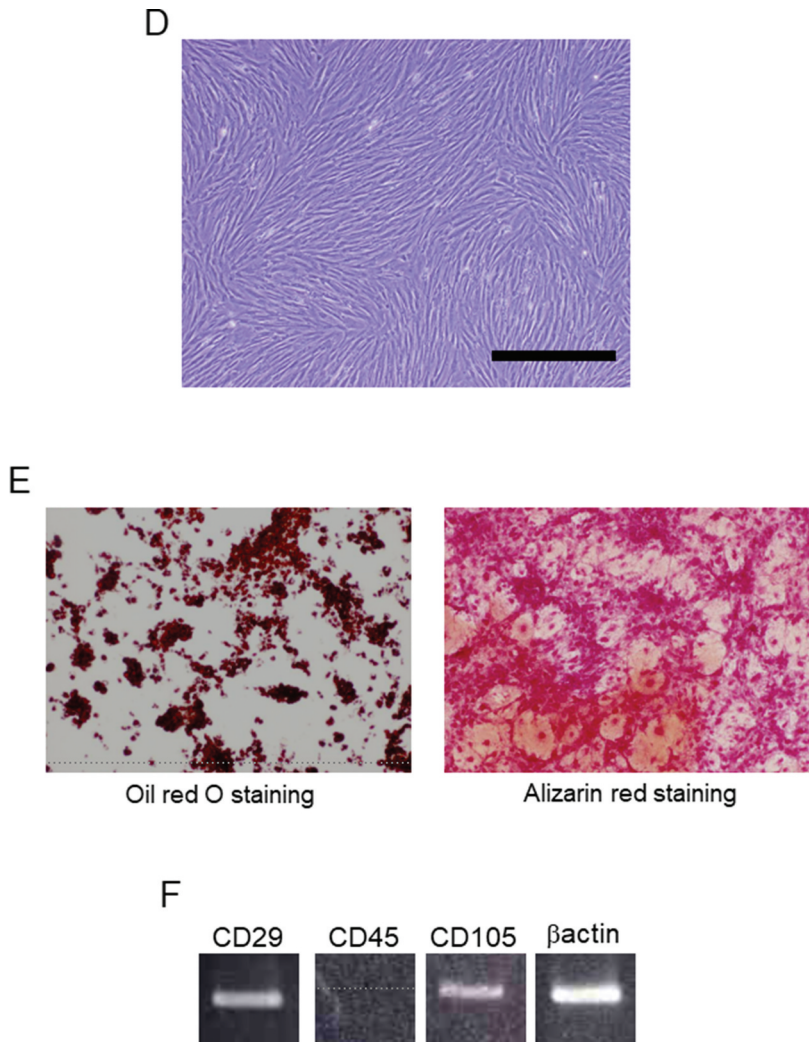

Figure 1. Continued.

Previously reported, it was suggested that islet co-culture with MSCs are effective in improving the efficiency of clinical islet transplantation.[23] From these reports, we considered that there is the islet-suppressing effect that deteriorates during transport and the islet-activating effect before transplantation.

We previously examined the efficacy of several commonly used organ preservation solutions on the viability of isolated islets from luciferase-transgenic (Luc-Tg) rats and found that proteins secreted by rat adipose-tissue mesenchymal stem cells (AT-MSCs) activated preserved Luc-Tg rat islets.[24–26]

Here, we apply the findings from our rodent model experiments to the preservation and activation of porcine islets in a preclinical study in a large animal. We identified factors secreted from porcine AT-MSCs that markedly activated preserved porcine islets. These data will be helpful for elucidating the precise molecular mechanisms of pancreatic commitment and could be useful in the development of diabetes therapy through the transplantation of preserved islets.

Results

Shipping of porcine islets and establishment of porcine AT-MSCs

Porcine islets were shipped in University of Wisconsin (UW) preservation solution at 4–10°C in a 1.5-mL tube (about 2,000 IEQ/tube; Figure 1(a)), and the median time required for transportation was 20.3 ± 5.43 h (n = 7). Isolated fresh porcine islets the stimulation index in the

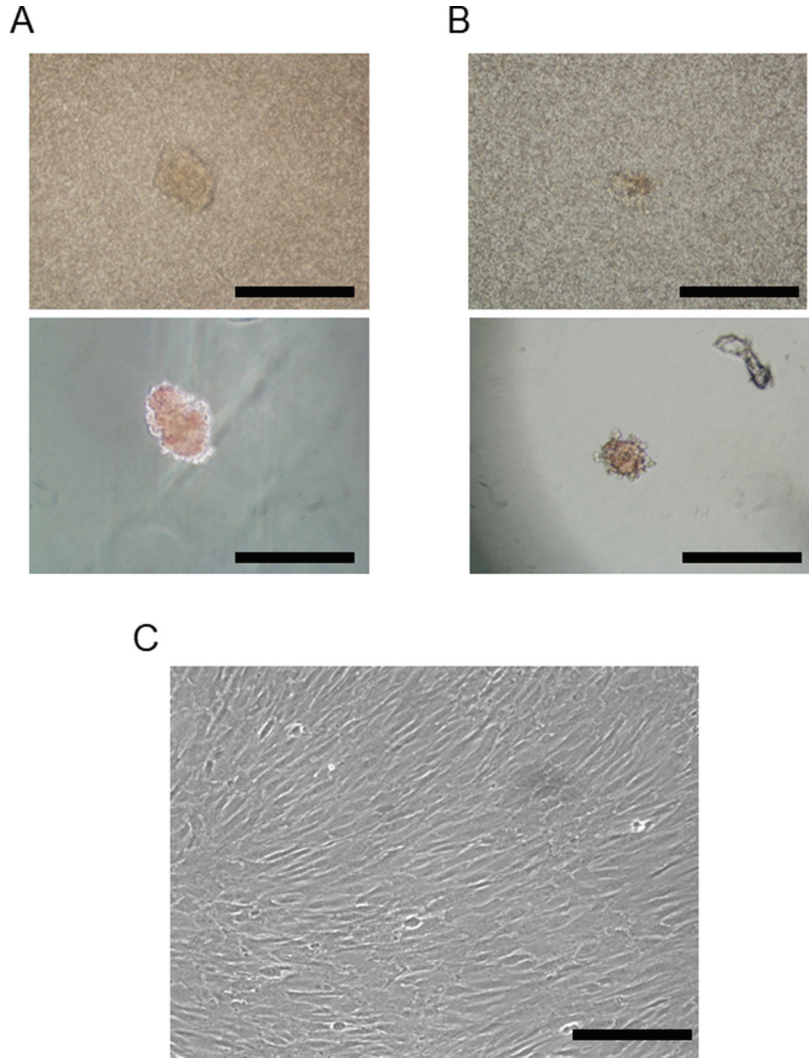

Figure 2. Analysis of degraded porcine islets and ATP content in a co-culture system. (a) Co-culture of porcine adipose-tissue mesenchymal stem cells (AT-MSCs) and porcine islets. (b) Islets only. Upper panels are phase-contrast images and bottom panels are images of dithizone staining. (c) The morphology of the co-cultured MSCs. Scale bar (a), (b), (c): 500 μM (d) Intracellular ATP content of co-cultured islets. White bar represents day 0. The black bar on the left represents the islets-only control group on day 4, whereas that on the right represents islets co-cultured with AT-MSCs on day 4. *There are significant differences between the islets-only control group and the islets co-cultured group ($P < .05$). (e) Blood glucose levels after islet transplantation into the kidney capsule of STZ-induced-diabetic mice are shown. Straight line is UW group. Dashed line is non-transplanted group. Dotted line is UW and AT-MSC secretions group. Data are representative of three independent experiments.

static glucose stimulation test (3.482 ± 1.433) and ADP/ATP ratio (0.0518 ± 0.0354) were normally. The islets regained their three-dimensional morphology post-transportation after culturing in RPMI-1620 medium for 2 h (Figure 1(b)), and the survival rate was 66.15% ± 7.08% (n = 7) by trypan blue staining. We found dithizone (DTZ)-negative islets in the samples (Figure 1(c)), suggesting that porcine islets are damaged during shipping by the long preservation time and low temperature.

Next, we isolated porcine AT-MSCs from the fat tissue of Kusabira-Orange transgenic porcines (Figure 1(d)). Established porcine AT-MSCs were induced to differentiate into adipocytes and osteoblasts (Figure 1(e)), and expression of MSC-marker genes was detected by reverse transcription–polymerase chain reaction (CD29+, CD45-, and CD105+), such as a porcine BM-MSCs (Figure 1(f)).[27] Thus, our porcine AT-MSCs expressed similar characteristics to those of other animal species, such as rat and human.

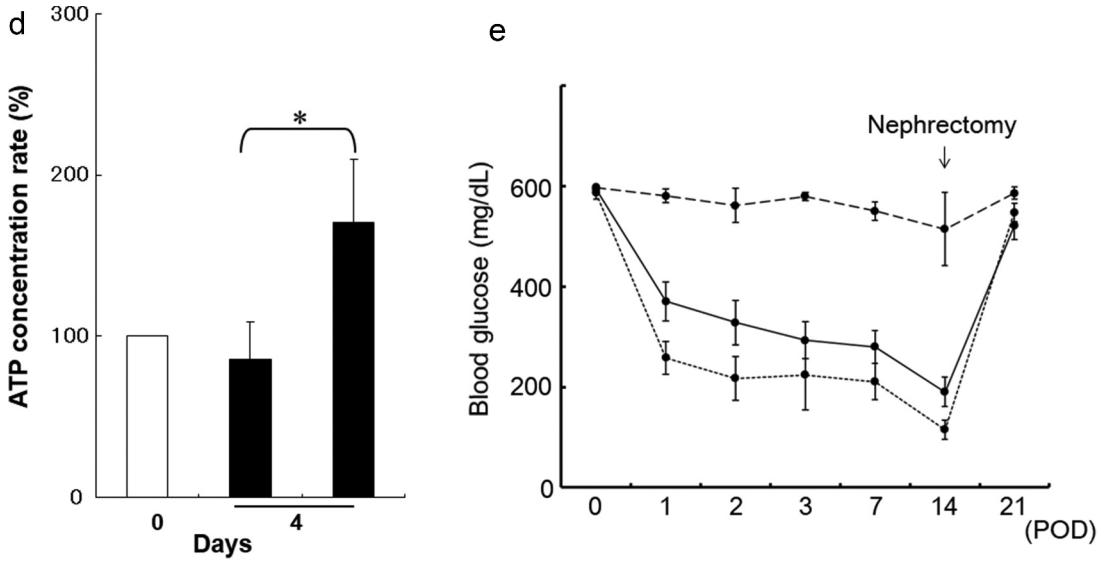

Figure 2. Continued.

Evaluation of co-culture of degraded porcine islets with porcine AT-MSCs

We used a Boyden chamber to examine the activatory effect of porcine AT-MSCs on degraded porcine islets in co-culture. At 4 days, the co-cultured islets had regained their morphology and strong DTZ positivities (Figure 2(a)); however, the islet-only control group had further degraded (Figure 2(b)). The morphology of the co-cultured MSCs has changed due to the use of serum-free medium (Figure 2(c)). The concentration of intracellular ATP had also recovered in the co-cultured islets (Figure 2(d)), whereas it had not in the islet-only control group (170.7% ± 39.1% vs. 85.4% ± 23.6%). This suggests an important islet activatory role for factors secreted by AT-MSCs.

Functionality of the preserved islet in STZ-induced diabetic mice

Approximately 200 IEQs were transplanted into the left kidney capsule of newly diabetic NOD-scid mice. Mice receiving islets preserved in AT-MSC secretion-conditioned medium in UW or fresh showed better glycemic control than those that received UW only preserved porcine islet (Figure 2(e)). Additionally, a recurrence of hyperglycemia was evident in nephrectomized mice, which suggests that the diabetic condition was reversed upon porcine islet transplantation and reappeared when the graft was removed. Thus, the UW and AT-MSC secretion-conditioned medium preserved islets functioned therapeutically *in vivo* and their transplantation ameliorated the effects of STZ-induced diabetes in mice.

Activation of degraded porcine islets by porcine AT-MSC secretions

We fractionated the AT-MSC secretion-conditioned medium into five fractions by molecular size and treated islet samples with the individual fractions by adding them to the islet culture medium. Islet condition deteriorated when treated with the 3–10 kDa or 30–50 kDa fractions compared with controls (Figure 3(a)). However, when treated with the 10–30 kDa fraction or the fractions above 50 kDa, the islets retained their structure at 4 days. The intracellular ATP content of the cultured islets also recovered in those treated with the 10–30 kDa fraction or the fractions above 50 kDa. However, intracellular ATP content in the groups administered the 3–10 kDa or 30–50 kDa fractions decreased compared with the control group (Figure 3(b)). Thus, we found that factors capable of activating degraded islets were present in the fractions containing secretions with molecular weights between 10 and 30 kDa and above 50 kDa.

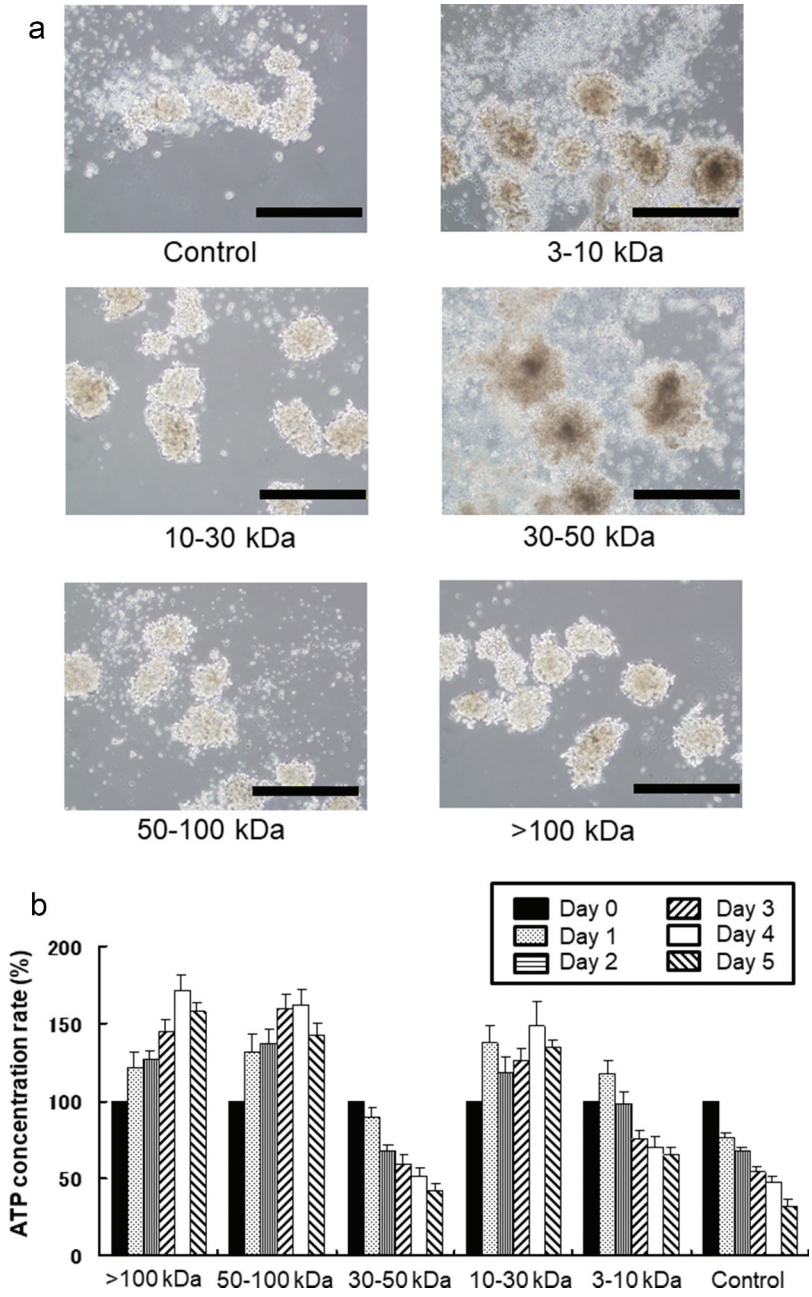

Figure 3. Comparison of porcine islet condition after treatment with various fractions of porcine adipose-tissue mesenchymal stem cell (AT-MSC) secretions. (a) Microscopic morphology of isolated islets after treatment with various fractions of AT-MSC secretions. (b) Intracellular ATP content of each sample of cultured islets after treatment with various fractions of AT-MSC secretions. Intracellular ATP content in the groups administered the 3–10 kDa or 30–50 kDa fractions decreased compared with the control group. Scale bar: 500 μm.

Histological analysis

We analyzed the activation of porcine islets by the factors secreted by AT-MSCs with immunohistochemistry. First, activated porcine islets were stained with markers for functionality (INS and PDX1) and proliferation cells (pancreatic-like stem cells) (Ki67 and PCNA) and then examined under a fluorescence microscope (Figure 4(a)). In the group treated with the 10–30 kDa fraction, we observed a lot of cells strongly positive for INS and PDX1; we also observed some INS- and PDX1-positive cells in the groups treated with the fractions above 50 kDa. In contrast, the numbers of INS- and PDX1-positive cells in the groups treated with the 3–10 kDa and 30–50 kDa fractions were decreased compared with the control group. Ki67- and PCNA-positive cells

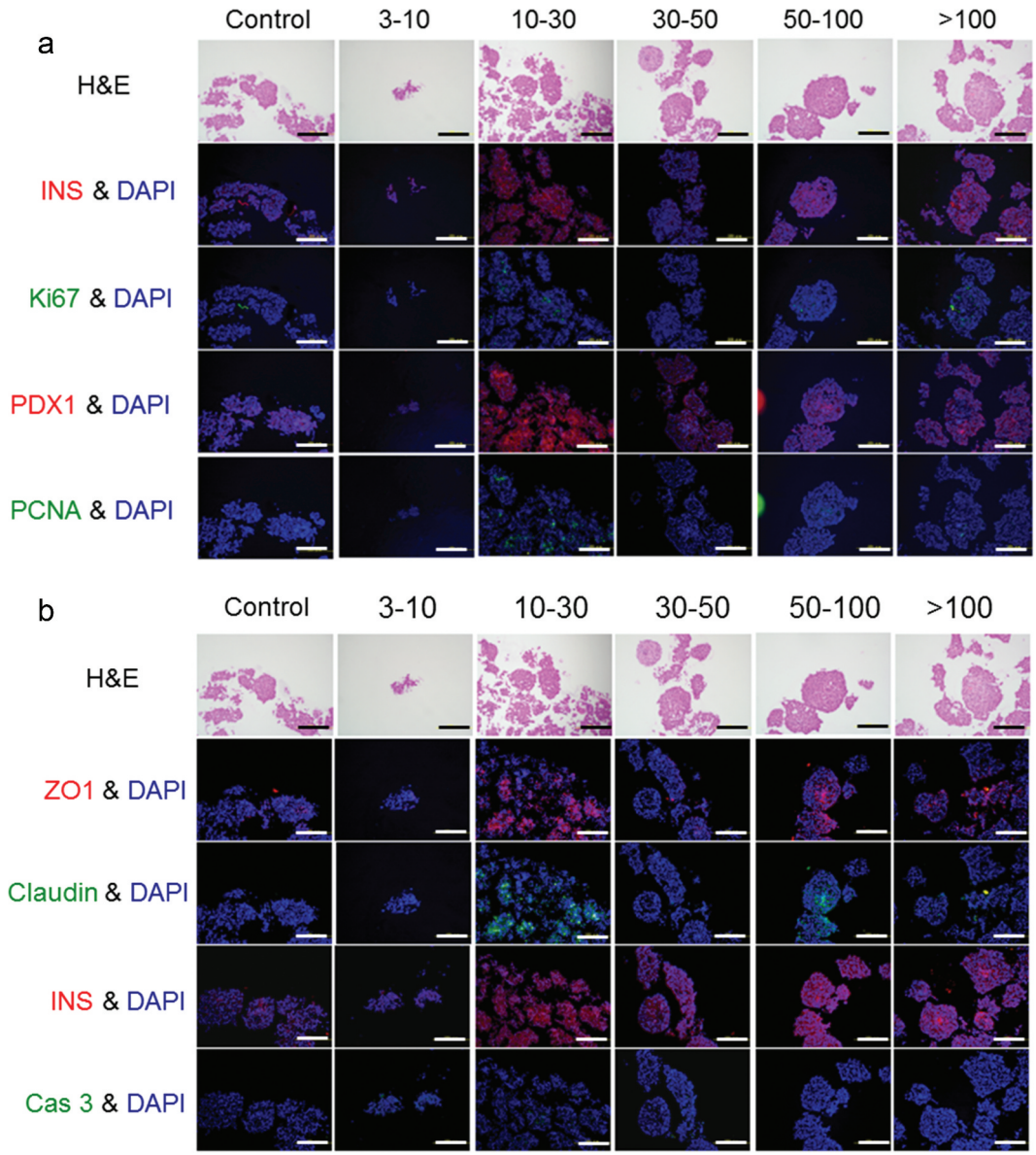

Figure 4. Immunostaining of porcine islets activated by various fractions of porcine adipose-tissue mesenchymal stem cells secretions. (a) Evaluation of markers of function and pancreatic stem cells. (b) Evaluation of tight-junction proteins and apoptosis. These samples are serial sections.

were observed in the central areas of islets treated with the 10–30 kDa fraction or the fractions above 50 kDa. These positive cells were more abundant in the group treated with the 10–30 kDa fraction. Thus, we found that treatment with fractions containing secretions with molecular weights between 10 and 30 kDa and above kDa increased the expression of markers related to islet function and proliferation.

Tight junction proteins play an important role in the maintenance of islet structure, so we stained activated porcine islets with anti-ZO1 and anti-Claudin-3 antibodies (Figure 4(b)). In the groups treated with the 10–30 kDa fraction or the fractions above 50 kDa, a stable islet structure was observed together with ZO1 and Claudin-3 expression. However, in the groups treated with the other fractions, ZO1 and Claudin-3 expression was low. In addition, the apoptosis marker Cas-3 was expressed in the groups treated with the 3–10 kDa or 30–50 kDa fraction (Figure 4(b)). Previously reported that PDX-1 negative islet cells have been shown to undergo apoptosis.[28]

Discussion

Recent reports have focused on the induction of insulin-secreting cells comparable to βαcells from human-derived embryonic stem (ES) cells and induced pluripotent stem (iPS) cells *in vitro*.[29–32] ES cells and iPS cells have enormous potential; however, limitations, such as teratoma formation followed by tumor genesis and immunogenicity, as well as a range of ethical issues, are preventing them from being applied clinically. Somatic stem cells such as MSCs have also been used to induce insulin-secreting cells *in vitro*.[33,34] MSC-derived insulin-secreting cells present a low risk for tumor genesis and do not raise any ethical issues; however, the clinical application of stem cell-derived insulin-secreting cells is still a long way off. Therefore, patients with severe diabetes are currently treated by through the transplantation of pancreatic tissue or islets from brain-dead donors.

For the quality and quantity of islets from a single donor to be sufficient to cure one recipient in terms of therapeutic effect, the purification rate of isolated islets, and the processes of islet recovery after isolation and maintenance of islet viability must all be improved. To resolve these issues, many researchers have investigated and reported on the use of a range of materials and protocols.[35–42] Our method here is little influenced by the condition of the donor pancreas because we use factors secreted by MSCs that restore islets injured during shipping and/or culturing before transplantation. In preliminary experiments (data not shown), we shipped a sample of human islets (n = 1) kept in common preservation solution from the United States to Japan. We then cultured the islets in standard medium containing factors secreted by human MSCs, and the result was similar to that of the present study regarding porcine islets. In Japan, CMRL solution is not so common and only available for clinical shipping. Furthermore, for the islets shipping solution, have been made reports still different from several research facility, it does not have reached the consensus. In our preliminary verification, the UW solution was not inferior to the islets-culture medium (CMRL or equivalent) in storage effectiveness. Additionally, several studies have been reported that experimentally verified the benefits of using UW solution not only for organs but also for islet preservation.[43]

Cell-based therapy is now viewed as an important tool in regenerative medicine.[37,38] Previous reports have revealed that transplantation of MSCs in mice and rats has functional benefits, in part because of the ability of these cells to produce a large amount of bioactive factors.[20,21] MSCs display self-renewal capacity and multilineage potential that is they have the potential to differentiate into bone, fat, or cartilage cells[44,45] and MSC-like cells have been found in isolated human islets.[46,47] Among the numerous molecules that have been proposed to induce β-cell expansion, hepatocyte growth factor has received much attention. There is increasing evidence suggesting that hepatocyte growth factor (about 83 kDa) and insulin-like growth factors (about 29 kDa) play an important role in the proliferation and survival of pancreatic β-cells both *in vitro* and *in vivo*.[48,49] We think that candidate effectors for activating degraded islets are hepatocyte growth factor, insulin-like growth factor, and the transforming growth factor because previous reports have suggested that these cytokines are factors for the activation of pancreas and/or β cells.[50–52]

In conclusion, we found that certain fractions of the factors secreted by MSCs were able to activate preserved islets. By using these factors, it should be possible to restore islets to the condition they were in prior to isolation and transportation. This is important for the shipping of islets for research purposes and is even more important for entire islet clinical preparations.

Materials and methods

Animals and islet isolation

Retired breeder porcines weighing approximately 200 kg each were used as donors for all experiments as previously described.[53] All animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.[54] Isolation of porcine islets was performed at Tohoku University as previously described,[55–57] with minimal modifications. Purified islet fractions were pooled and cultured in CMRL 1066 medium (Biochrom, Berlin, Germany) supplemented with 20% porcine serum, 2 mM N-acetyl-L-alanyl-L-glutamine, 10 mM HEPES, 100

IU/mL penicillin (GIBCO, Tokyo, Japan), 100 μg/mL streptomycin (Biochrom), and 20 μg/mL ciprofloxacin (Bayer, Leverkusen, Germany) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was subsequently changed to UW solution and the islets translocated to a 1.5-mL Eppndorf tube, which was then put into a Styrofoam container and transported by refrigerated truck to Jichi Medical University, Tochigi, Japan. The porcine islets take about 20 hours to refrigerate and transport the isolated to the Jichi Medical University.

Adipose tissue-derived MSC preparation and culture

Kusabira-Orange transgenic porcine-derived adipose tissue was minced with scissors and scalpels into pieces less than 1 mm in diameter. After gentle shaking of the minced tissue with an equal volume of phosphate-buffered saline (PBS[−]), the mixture was separated into two phases. The upper phase (the phase containing stem cells, adipocytes, and blood) was enzymatically dissociated with 0.125% collagenase (type I) in PBS[−] for 1.5 h at 37°C with gentle shaking. The dissociated tissue was mixed with an equal volume of MEMα (GIBCO, Tokyo, Japan) supplemented with 10% fetal bovine serum (GIBCO, Tokyo, Japan), and incubated for 10 min at room temperature. The solution was left to separate into two phases in a few minutes. The lower phase was centrifuged at 1,200 rpm for 5 min at 20°C to isolate the AT-MSCs. The AT-MSCs were then seeded into 100-mm tissue culture dishes (Thermo Scientific, Tokyo) and cultured in MEMα supplemented with 10% fetal bovine serum. When the cells were 70% to 80% confluent, they were harvested with 0.05% trypsin-EDTA (Invitrogen, Tokyo), replated at 2.0×10^4 cells/cm^2, and cultured in MEMα supplemented with 10% fetal bovine serum at 37°C for 5 days. AT-MSCs between the fifth and eighth passages were used for the experiments.

Table 1. Primer list.

Primer Name	Accession No		Sequence
CD29 Forward	NM213968	5	-ACAGTGAAGACATGGACGCT-3′
CD29 Reveres		5	-CAGGTCTGACACATCTCACA-3′
CD45 Forward	AY444866	5	-TCCAGAATGCGTCACTCTGA-3′
CD45 Reveres		5	-TTGAATGTGAGGCAGACTCC-3′
CD105 Forward	NM214031	5	-CTTTGTGCAGGTGAGCATGT-3′
CD105 Reveres		5	-TGCAGTCTTGTGGACATCCA-3′
βActin Forward	NM007393	5	-AGAGCAAGAGAGGTATCCTG-3′
βActin Reveres		5	-GCAGAAGCCTAGTTGGATCA-3′

Characterization of porcine AM-MSCs

Analysis of stem cell markers genes and differentiation ability in porcine AT-MSCs. Total RNA (0.5 g) was reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Tokyo, Japan) according to the manufacturer's guidelines. PCR analyses were performed using the AmpliTaq Gold kit (Applied Biosystems, Tokyo, Japan). The PCR primer sequences are listed in Table 1. The differentiation potential of porcine AT-MSCs (passage 8) into adipocytes and osteocytes was evaluated using differentiation-induction media purchased from Lonza Walkersville, Inc. (http://www.lonza.com) according the manufacturer's protocols.

Production of conditioned medium

To analyze the factors secreted by AT-MSCs, we prepared a conditioned medium. AT-MSCs were plated into thirty 100-mm tissue culture dishes. Once they had reached confluence, the cells were washed with PBS[−] and incubated in serum-free MEMααmedium (GIBCO). After 2 days, the supernatant was collected and then centrifuged, filtered, and concentrated at 12,000 rpm using Amicon Ultra centrifugal filters (Millipore, Tokyo, Japan; molecular weights 3, 10, 30, 50, and 100 kDa).

Immunohistochemical analysis of preserved islets

Islet samples were cultured for 4 days at 37°C in an atmosphere of 5% CO_2, before being fixed in 10% formalin and embedded in paraffin. Histological analysis was conducted by serial tissue section followed by staining with hematoxylin and eosin for conventional morphological evaluation or with anti-INS-1 (sc-7839, Dilute 150 times), anti-PDX-1 (sc-14662, Dilute 150 times), anti-Ki67 (E1870, Dilute 200 times), anti-PCNA (sc-7970, Dilute 200 times), anti-ZO1 (HP9044, Dilute 100 times), or anti-Claudin-3 (sc-17662, Dilute 100 times) antibodies. Rhodamine- or FITC-conjugated secondary antibodies were applied for 30 min (Santa Cruz Biotechnology, Dilute 2000 times). Nuclei were stained with DAPI.

Measurement of ATP

ATP was measured by means of an ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics, Tokyo, Japan), as per the manufacturer's instructions. Luminescence was measured with a Mithras LB940 multimode microplate reader (Berthold, Tokyo, Japan). The total amount of ATP was normalized for total protein level using a Pierce BCA Protein Assay Kit (TaKaRa, Kyoto, Japan).

Streptozotocin-induced diabetic mice

STZ (Sigma, Tokyo, Japan) was prepared in citrate buffer (pH4.5) and delivered by intraperitoneal injection (50 mg/kg) for 5 consecutive days before transplantation. Mice with blood glucose levels >400 mg/dl were considered as diabetic.

Analysis of preserved porcine islets for diabetic mice

The porcine islets were cultured in preservation solution (UW contacting AT-MSC secretion-conditioned medium or UW only) at 4°C. After 24 h, preserved islets were injected under the kidney capsule. An incision was made in the renal capsule and advanced in the subcapsular space, to the kidney. Isolated porcine islets were slowly injected and allowed to spread at the pole. The blood glucose level was checked at days 0, 1, 2, 3, 7, 14, and 21. After 14 days, the prince islet containing transplanted kidney was resected, and monitoring of blood glucose continued 7 days.

Statistical analysis

Data are presented as means ± SEM. Results were analyzed by using a two-tailed Student's *t*-test. A *P* value of less than 0.05 was considered significant.

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Disclosure statement

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Duality of interest

The authors state that they have no duality of interest.

Author contributions

Conceived and designed the experiments: TT and YF
Performed the experiments: TT, NK, MG
Analyzed the data: TT, NK, YS
Contributed reagents/materials/analysis tools: TT, MG, NK, AM, NS, JK.
Wrote the paper: TT

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