A method for performing islet transplantation using tissue-engineered sheets of islets and mesenchymal stem cells

Short Title

Tissue engineered sheets of islets and MSCs

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Abstract

Mesenchymal stem cells (MSCs) are known to have a protective effect on islet cells. Cell sheets developed using tissue engineering help maintain the function of the cells themselves. This study describes a tissue engineering approach using islets with MSC sheets to improve the therapeutic effect of islet transplantation. MSCs were obtained from Fischer 344 rats and engineered into cell sheets using temperature-responsive culture dishes. The islets obtained from Fischer 344 rats were seeded onto MSC sheets, and the islets with MSC sheets were harvested by low-temperature treatment after co-culture. The functional activity of the islets with MSC sheets was confirmed by a histological examination, insulin secretion assay, and quantification of the levels of cytokines. The therapeutic effects of the islets with MSC sheets were investigated by transplanting the sheets at subcutaneous sites in severe combined immunodeficiency (SCID) mice with streptozotocin-induced diabetes. Improvement of islet function and viability was shown *in situ* on the MSC sheet, and the histological examination showed that the MSC sheet maintained adhesion factor on the surface. In the recipient mice, normoglycemia was maintained for at least 84 days after transplantation, and neovascularization was observed. These results demonstrated that islet transplantation in a subcutaneous site would be possible by using the MSC sheet as a scaffold for islets.

Introduction

In the field of cell transplantation therapy, mesenchymal stem cells (MSCs) have been shown to have a protective effect on islet cells (1,2). Cell sheets developed using tissue engineering help maintain the function of the cells via a trophic effect (3,4). The protective effect of MSCs engineered into a cell sheet is thus thought to be improved. In the present study, we attempted to create MSC sheets co-cultured with islets as an approach to islet transplantation.

In islet transplantation recipients who have undergone an intraportal injection, the ability to achieve long-term glycemic control remains insufficient (5). During intraportal transplantation, 60%–80% of the islets are lost within 1 h after transplantation due to immediate blood-mediated inflammatory reactions (IBMIRs), activation by direct exposure to foreign immunological cells, and the toxic effects of the immunosuppressive compounds on the transplanted islets (6). Additionally, an insufficient blood supply and immunoreactions associated with intraportal islet transplantation are primary causes of islet loss (6,7).

Several studies described the transplantation of islets at extrahepatic sites, including the omentum (8), spleen (9), testes (10), and renal subcapsular space (11). However, a sufficient long-term control of blood glucose levels has been not shown after implantation at these sites. In 2009, Shimizu et al. (12) reported the creation of islet cell sheets using a tissue engineering method involving subcutaneous transplantation. Saito et al. (13) reported that subcutaneously transplanted islet cell sheets maintain their function over the long term. Tissue engineering methods used for islet

transplantation could generate islets to be transplanted at subcutaneous sites and serve as the foundation for generating a new therapeutic modality.

MSCs are known to differentiate into endothelial cells (14,15) and improve the engraftment of islets by secreting anti-apoptotic and angiogenic cytokines such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and transforming factor beta 1 (TGFβ1) (16–18).

These MSCs ability could solve problems associated with graft loss following various forms of transplantation. Additionally, there are reports that cell sheets created by tissue engineering preserve cellular communication junctions, the endogenous extracellular matrix (ECM), and integrative adhesive agents (3), and further maintain the functions of the cells themselves (4).

The ECM provides the necessary structural and adhesive properties for maintaining cell sheet integrity during transplantation. More recently, our group reported that human hepatic cell sheets could be made rapidly and efficiency by using fibroblasts cells (19). We propose that the protective and engraftment effects of MSCs for transplanted islets could be improved by using MSCs engineered into cell sheets.

In the present study we applied a tissue engineering approach using islets with MSC sheets (islets + MSC sheet) for transplantation at subcutaneous sites. The purpose of this study was to confirm the protective and therapeutic effects of using MSCs engineered into a cell sheet by tissue engineering as a scaffold for islet cell transplantation at an extrahepatic site.

Materials and Methods

Animals

Eight-week-old male Fischer 344 rats, 200–300-g male Fischer 344 rats, and 6-wk-old male SCID mice (Charles River Laboratories Japan, Yokohama, Japan) were used.

This study was carried out in strict accordance with the recommendations in 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 the Nagasaki University.

Isolation and culture of MSCs derived from bone marrow

We isolated bone marrow cells from the femurs of 8-wk-old rats by flushing the femurs with alpha minimum essential medium (αMEM) (Invitrogen GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine saline (FBS) (Invitrogen), 100 IU/mL penicillin (Invitrogen) and 100 μg/mL streptomycin (Invitrogen). These MSCs were used for the experiments at passage two or three.

Characterization of the MSCs

Flow cytometry (FACS) analysis

The MSCs were prepared for use with the following markers: CD29, CD31, CD34, and CD90. First, MSCs were incubated with fluorescence-conjugated primary antibodies for 1 h. Then the cells were washed three times in phosphate-buffered saline (PBS) and incubated with secondary

antibodies for 1 h. After three washing steps, the MSCs were acquired using a FACSCanto II (Becton Dickinson, Lincoln Park, NY) flowcytometer and analyzed with the FACSDiva software program (Becton Dickinson). The following antibodies were employed: PE mouse anti-rat CD29 (Miltenyi Biotec, Auburn, CA), FITC mouse anti-rat CD31 (LSBio, Seattle, WA), PE mouse anti-CD34 (Santa Cruz Bio-Technology, Santa Cruz, CA), and FITC mouse anti-rat Thy-1/CD90 (LSBio).

Differentiation of MSCs in vitro

We induced the differentiation of isolated MSCs into mesenchymal osteogenic and adipogenic lineages according to published protocols (20). Adipocytes were detected by standard Oil Red O staining. Osteocytes were detected by Alizarin Red staining.

Isolation of islets

Fischer 344 rats (200–300 g) were used as donors for islet transplantation. The islets were isolated using collagenase digestion according to published methods (21). The islets were stained with dithizone (140 mmol/L) and counted under a microscope, and the number was converted into standard islet equivalents (IEs).

Preparation of islets co-cultured with the MSC sheets (islets + MSC sheets)

We seeded MSCs at a density of 5×10^5 cells/dish onto 35-mm-dia. temperature-responsive culture dishes (CellSeed, Tokyo). For the cells' culture, αMEM supplemented with 10% FBS was used. Over-confluent MSCs on the temperature-responsive dishes were transferred to another incubator set at 20°C for approx. 30 min, causing the MSC sheet to detach spontaneously. To create islets + MSC sheets, we seeded 400–500 islets onto the MSCs after the MSCs reached 90% confluence. Following an additional 48–72 h in culture, confluent MSCs topped with islets were harvested as an islet + MSC sheet. MSCs were seeded at the density of 5×10^5 cells/dish onto 35-mm-dia. dishes. After 7 days' cultivation, the MSCs were over confluence in the culture dish, and the number of MSCs was 1×10^6 cells/dish. We found that 96–120-h cultivation was required for the cells to reach 90% confluence. Islets + MSC sheets were detached by the same procedure as that used for the MSC sheets. As a control, 400–500 islets were cultivated alone under the same conditions in sterilized, non-coated culture dishes.

Histological and immunohistochemical analysis

The islets + MSC sheets were fixed in 10% formalin and sectioned. Serial sections were then cut from the paraffin-embedded blocks and stained with hematoxylin and eosin (H&E). The presence of cytoplasmic insulin and glucagon in the islets on the MSC sheets was confirmed via immunostaining using pig polyclonal anti-insulin antibodies (LSBio) and mouse polyclonal anti-glucagon antibodies (Sigma Chemical, St. Louis, MO).

Electron microscopy

We used electron microscopy to confirm the presence of ECM on the surface of the MSC sheet and to observe an adhesion between islets and the MSC sheet. The islets + MSC sheets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate-buffered 1% osmium tetroxide. The dehydrated samples were cut into ultrathin section and then examined using an electron microscope (JEM-1200EX, JEOL, Tokyo).

Islet recovery after incubation

Islets were co-cultured with MSCs or an MSC sheet, and we counted the number of islets and calculated the IE after 24 and 72 h of incubation. The IE of the islets present following the incubation/IE of the seeded islets was considered to indicate the recovery rate.

Islet viability after incubation

We assessed the viability of the islets co-cultured with MSCs or an MSC sheet after 72 h of incubation using calcein-AM and propidium iodide (PI) (Cellstain Double Staining Kit; Dojindo, Kumamoto, Japan) staining. The samples were placed on a fluorescent microscope (Eclipse Ti-U; Nikon, Tokyo). Viable cells were stained green and dead cells were stained red. The degree of cell viability was assessed according to published protocols (2). We also evaluated the viability by cell

size (<150 μm, 151−250 μm, 251−500 μm). Ten or more islets were evaluated for each size category.

Insulin secretion assay

For the insulin secretion assay, islets co-cultured with MSCs or an MSC sheet after 72 h of incubation were preincubated for 1 h at 37°C with RPMI-1640 medium containing 3.3 mM glucose. After preincubation step, the culture medium was changed to fresh RPMI-1640 containing 3.3 mM glucose for an additional 1 h. The media were replaced with 20 mM glucose for 1 h. For the final step, the medium was changed to 3.3 mM glucose for 1 h. The culture medium was collected and frozen at −20°C until the analysis. The amount of secreted insulin was measured using an Ultrasensitive Rat Insulin ELISA Kit (Morinaga Institute of Biological Science, Kanagawa, Japan). The stimulation index (SI) was calculated as follows: SI = (insulin content in the 20 mM glucose media)/(insulin content in the initial 3.3 mM glucose media).

Cytokine quantification

We measured the secretion of cytokines in the supernatants using the VEGF Rat ELISA Kit (Abcam, Cambridge, MA), Rat HGF EIA (Institute of Immunology Co., Tokyo), and Rat TGF-beta 1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN).

Induction of diabetes mellitus (DM) and transplantation of the

islet + MSC sheets

Severe combined immunodeficiency (SCID) mice were rendered diabetic via an intraperitoneal injection of 200 mg/kg of streptozotocin (Sigma). SCID mice were categorized in diabetic mice that exhibited a nonfasting blood glucose (NFBG) level of more than 350 mg/dL for 2 consecutive days. The islets + MSC sheets were harvested via low-temperature treatment and placed on glass plates (GPs) for subcutaneous transplantation into the abdomen. To transplant the islets + MSC sheets (500 islets were riding on each MSC sheet), we created an arc-shaped incision in the abdominal skin of the mouse. After the attachment of the islets + MSC sheet to the subcutaneous site, the GP was immediately and carefully removed. Another islets + MSC sheet was transplanted on the initial sheet. Two or four islets + MSC sheets were transplanted in the subcutaneous site.

Two thousands islets were transplanted to SCID mice within 24 h after isolation without or with 4×10^6 MSCs, and four MSC sheets were also transplanted in the subcutaneous site. The ratio of the number of MSCs in an MSC sheet and the number of islets was 1×10^6 cells:500 islets, and the number of MSCs and the number of islets was 4×10^6 cells:2,000 islets. An investigation was also performed in sham-operated diabetic SCID mice (DM sham). Diabetic sham-operated (DM-Sham) mice (n=5), and recipient mice were used: 2,000 islets alone (n=5), MSC sheet alone (n=5), 2,000 islets with MSCs (n=5), two islets + MSC sheets (1,000 islets) (n=5), and four islets + MSC sheets (2,000 islets) (n=6). For an investigation of the long-term efficacy of the islets + MSC sheets, another six mice were transplanted with four islets + MSC sheets (2,000 islets).

Validation of the therapeutic effects of the islets + MSC sheets

The level of NFBG was measured twice weekly. On day 28, we obtained serum samples to measure the rat-nonspecific insulin levels using ELISA kits (Morinaga Institute of Biological Science). In other experiments, to confirm the long-term therapeutic effects (-day 84), five diabetic SCID mice underwent transplantation of four islets + MSC sheets, and the transplanted islets + MSC sheets were removed by abdominal wall resection on day 84.

Intraperitoneal glucose tolerance test

We evaluated the functionality of the islets + MSC sheets *in vivo* by conducting intraperitoneal glucose tolerance tests (IPGTTs) on day 56 in the mice transplanted with four islets + MSC sheets. The mice received an intraperitoneal inoculation of glucose solution (2 g/kg body weight) after 18 h of fasting.

Immunohistochemical and immunofluorescence examinations

On day 28, specimens of subcutaneous tissue were fixed in 10% buffered formalin and sectioned (5-μm-thick sections) and stained with H&E and Azan Mallory. To evaluate the degree of vascularization, we immunostained the specimens using anti-von Willebrand factor (vWF) polyclonal antibodies (1:50; Chemicon-Millipore, Billerica, MA), anti-insulin polyclonal antibodies

(1:50; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-pancreas duodenum homeobox (PDX)-1 antibodies (1:100; Upstate, Charlottesville, VA). The number of vessels was determined by counting the vessels randomly in five different subcutaneous areas.

Statistical analysis

Data are presented as the mean ± standard error (SEM). Statistical analyses were performed using GraphPad Prism software (version 6:00; GraphPad, San Diego, CA) for numerical variables, using a repeated-measures analysis of variance (ANOVA) when comparing more than two groups, Student's *t*-test when comparing two groups, and the Mann-Whitney U-test. P-values < 0.05 were considered significant.

Result

Characteristics of MSCs isolated from rat bone marrow

The isolated cells were positive for the mesenchymal markers CD29 and CD90, and negative for the hematopoietic markers CD31 and CD34 (Fig. 1A). These findings indicated that the characteristic immunophenotype of rat bone marrow-derived MSCs was exhibited in the isolated cells (22–24). MSCs have differentiation ability into osteogenic and adipogenic lineages (22, 23). The cells described herein are able to differentiate into osteocytes and adipocytes (Fig. 1B).

Harvest of the islet + MSC sheets

The islets seeded on the MSC sheet immediately sank to the bottom of the culture dishes and came into contact with the MSCs (Fig. 2A, B). After a 72-h culture, the islets adhered to the MSCs. The MSCs detached from the culture dish while shrinking slowly as a cell sheet following low-temperature treatment (Fig. 2C, D). The islets were confirmed on the sheet using dithizone staining (Fig. 2E).

Histological assessment of the adherent islets *in vitro*

H&E staining showed that the islets adhered to the sheet while maintaining a spherical shape in their physiological form. In addition (Fig. 2F), the MSC sheets firmly adhered to the islets, which demonstrated cytoplasmic immunostaining for both insulin and glucagon (Fig. 2G,H).

Transmission electron microscopy

The islets adhered firmly to the MSC sheets and partially to the ECM (Fig. 2I, J), forming tight junctions (Fig. 2K). The MSC sheets contained multiple cell layers (Fig. 2L) that established cell-to-cell connections via the formation of tight and gap junctions (Fig. 2M).

Recovery rate of islets

After 24 h, there were no differences in the recovery rate among the islets co-cultured with

the MSC sheet and the groups of MSCs and islets cultured alone. However, the recovery rate significantly improved in the co-cultured groups compared to the group of islets cultured alone. There were no significant differences between the MSCs group and the MSC sheet group at 72-h culture (Fig. 3).

Viability of the islets

We assessed the viability of the islets using calcein-AM /PI staining. Viable cells were stained in green, and dead cells were stained in red (Fig. 4A). The MSCs were almost 100% viable in the range observed. The 72-h viability of the co-cultured islets groups was significantly improved compared to the islets cultured-alone group (Fig. 4B). There were no significant differences between the MSCs group and the MSC sheet group.

Insulin secretion assay of the islet function

In the co-culture with MSCs and MSC sheet groups, the SI values were significantly higher than in the cultured-alone group. There were no significant differences between the MSCs group and the MSC sheet group (Fig. 5).

Cytokine secretion

The VEGF, HGF, and TGFβ1 levels were significantly higher in the co-culture groups

compared to the cultured-alone islets group. The TGFβ1 level was significantly higher in the co-culture with MSC sheet group than in the co-culture with MSCs group (Fig. 5).

Therapeutic effects of the engrafted islets + MSC sheets

Islets + MSC sheet transplantation was performed as described in the Materials and Methods section (Fig. 6A). In the recipient SCID mice treated with two islets + MSC sheet transplantation, three of the five recipient mice had maintained normoglycemia 2 wks after transplantation, two of the five recipient mice had maintained normoglycemia at 3 wks after transplantation, and all mice became hyperglycemic more than 3 wks after transplantation. Therefore, four islets + MSC sheet transplantation was performed. All recipient SCID mice returned to a normoglycemic state within 1 wk, whereas all sham-operated mice remained hyperglycemic. The transplantation of 2,000 islets alone and that of the same numbers of islets with MSCs was performed within 24 h after islet isolation. All recipient mice exhibited a minimal decrease in the NFBG level and remained hyperglycemic. In the diabetic SCID mice transplanted with MSC sheets only, all mice remained hyperglycemic (Fig. 6B). The body weight as a clinical condition of recipient mice in the four islets + MSC sheet transplantation group improved (Fig. 6C). All recipient SCID mice treated with a four islets + MSC sheet remained normoglycemic for 84 days, and the NFBG levels rose rapidly after graft removal (Fig. 6D). An IPGTT was performed in the recipient mice treated with four islets + MSC sheets and control (nondiabetic naive) SCID mice. The blood glucose levels returned to

normal levels after elevations at 15 and 30 min. (Fig. 6E).

Assessment of the engrafted islets and serum insulin levels

The appearance of connective tissue was observed at the subcutaneous sites of transplantation, and collagen-rich connective tissue was noted on Azan Mallory staining. In addition, on immunofluorescence, islets with the expression of insulin and Pdx1 were observed in the new tissue. Islets maintaining their physiological shape were also detected (Fig. 7A), although no islets were apparent at the subcutaneous sites in the specimens in the islet transplantation alone group (data not shown). A significant amount of insulin was seen in the four islets + MSC sheet transplantation group compared to that observed in the DM-sham mice group. There was no significant difference among the 2,000 islets, 2,000 islets with MSCs, two islets + MSC sheet transplantation, and the DM sham-operated group (Fig. 7B).

Angiogenesis associated with the transplantation of islets + MSC sheets

The numbers of detectable vessels in the MSC sheet and islets + MSC sheet groups were significantly higher than in the sham-operated and islets alone groups. There was no significant difference in the vessel numbers among DM sham-operated, islets alone, and islets with MSCs groups (Fig. 8A, B).

Discussion

Previous studies have examined MSCs' abilities to preserve the inflammatory response (25,26), modulate the immune reaction (27,28), inhibit apoptosis (29), and promote vascularization (30,31). Such effects may solve problems associated with graft loss following various forms of transplantation. In islet transplantation, MSCs secrete a variety of cytokines that modulate intracellular signaling related to the cell function and increased survival of islets both *in vitro* and *in vivo* (1,2). The results of the present study suggest that the use of the islets + MSC sheet improves graft survival compared to islets with MSCs in transplantation into diabetic mice.

VEGF, HGF, and TGFβ1 are the major cytokines secreted by MSCs that exhibit effects in graft protection. TGFβ1 stimulates the production of heat shock protein HSP32 and X-linked inhibitor of apoptosis protein (XIAP) (32). HSP32 has a protective effect on islets and suppresses inflammatory reactions and oxidative stress (33,34). XIAP is known to be anti-apoptoticmolecules in β cells (35,36). And, previous studies indicated the effects of MSC neovascularization due to secretion of angiogenic growth factors, such as VEGF and HGF (16,37), and differentiation themselves into endothelial cells and create new vessels (38,39). Moreover, Spees et al. (40) reported that MSCs provide mitochondria to and inhibit the apoptosis of cells damaged by ischemia-reperfusion. Therefore, providing mitochondria to islets by MSCs may inhibit the apoptosis of islets. In the present study, we did not clarify particular elements that improve the function and survival of islets; however, we observed improvement in the function and survival of islets *in situ* on

the MSCs and MSCs sheets.

Several studies have described the usefulness of islet transplantation using MSCs (1,2), but islet transplantation using MSC sheets is thought to be more useful regarding the therapeutic effect. Although the MSCs were sheeted using tissue engineering techniques, the MSCs maintained their function, including the production of cytokines and their protective effects on islets. Our present findings verified that the secretion of angiogenic factors is not deteriorated even when MSCs are modified into a cell sheet.

It is also interesting that TGFβ1 was significantly increased by the modification into MSC sheets. Electron microscopy showed the attachment between the islet cells and MSCs to be sufficient for harvesting sheets as islets + MSC sheets, and the attachment withstood the transplantation procedures. ECM components such as adhesion factor were also observed on the surface of the MSC sheets. Previous studies reported that the use of tissue-engineered cell sheets resulted in a greater degree of engraftment at the transplantation sites compared to cell transplantation (41,42) because the cell sheets preserve adhesion factors when harvested without trypsin. We also found that although the protective effect of MSCs was comparable to that of MSC sheets *in vitro*, the therapeutic effects of the islets + MSC sheet transplantation were significantly higher than those of islet transplantation with MSCs.

These findings suggest that MSC sheets improve the engraftment rate of islets at subcutaneous sites depending on the presence of adhesion factors in the MSC sheets. The MSC sheet

improved the efficiency of islets transplantation more than the same number of MSCs did. Moreover, the creation of the MSC sheets does not require a scaffold, such as a laminin coating or Matrigel, and only donor cells will be used. The main advances shown by our study were the fabrication of the islets + MSC sheets and the evaluation of the efficacy of islets + MSC sheet transplantation in a subcutaneous site. Therefore, we used an immunodeficiency animal model in the experiments, and the immune-activity of the MSC sheets was not clarified in this study. The immunomodulatory ability of the MSCs should be evaluated in a future study using an immunocompetent animal model.

The successful improvement of the blood glucose levels in diabetic mice was reported in a study using approx. 400–500 islets for portal vein islet transplantation (43). A marginal amount of islets was detected following the transplantation of 1,000–2,000 islets using the islet + MSC sheet procedure. The major reasons underlying these findings are thought to be: [1] the engrafted islets were damaged following culture for over 48 h to establish islet-MSC co-cultured sheets, and [2] the effects of vascularization in the MSC sheets were insufficient in the early phase of islet engraftment at the subcutaneous sites, although the MSC sheets exerted a stimulating effect on angiogenesis. Fumimoto et al. (44) reported that in their study, the engraftment and function of islets was remarkably increased following transplantation at subcutaneous sites treated pre-vascularization with MSCs. When performing transplantation into subcutaneous tissue lacking an adequate blood flow, vascularization is an important factor enabling the therapeutic effects of islets + MSC sheet transplantation. In order to improve the efficiency of transplantation, obtaining efficient early

angiogenesis and/or using other sites with a sufficient blood flow for engraftment is required. In addition, in the present study angiogenesis was significantly increased in the islets + MSC sheet group compared to the MSC sheet alone group. We suspect that the islets exposed to hypoxia induced some signals to the MSC sheet for the angiogenesis effect.

Hasegawa et al. (45) reported that MSCs can differentiate into β cells, however another study reported low levels of regeneration (46). In the present study, no insulin-positive and Pdx1-positive cells were detected by immunofluorescence examinations around the islets in the specimens of the recipient mice treated with islets + MSC sheet transplantation. These results indicate that MSC sheets are unable to differentiate into β cells around islets at subcutaneous sites under hyperglycemic conditions. Conversely, connective tissue was observed at the subcutaneous sites of islets + MSC sheet transplantation, although the outgrowth of connective tissue was not detected in the specimens of the recipient mice transplanted with islets only and the sham-operated mice. These findings thus indicate that the outgrowth of connective tissue is due to the presence of transplanted MSC sheets.

In addition, the connective tissue containing a rich vascular bed that appeared following transplantation provided ideal conditions for islet engraftment. In the present study, the differentiation of transplanted MSCs was insufficiently clarified. However, no abnormal proliferation was detected in the recipient mice by the macroscopic or microscopic examinations. A confirmation of the differentiation of MSCs transplanted into humans is absolutely necessary.

Conclusions

We successfully fabricated islets + MSC sheets by using tissue engineering. Our findings indicate that the MSC sheets exerted protective effects on the viability and function of islets and improved the engraftment of islets at subcutaneous sites. The islets engrafted with MSC sheets at subcutaneous sites had a therapeutic effect on hyperglycemia compared to that observed following islet transplantation without MSC sheets. In the future, more detailed elucidation of the mechanisms in MSCs' activity and cell sheet potentiality would help to expand the clinical applications of islet transplantation therapy.

Acknowledgement

We thank Dr. Tatsuya Kin (University of Alberta) for providing technical advice regarding the isolation of islets.

Author Disclosure Statement

Teruo Okano is an investor in CellSeed, Inc., and is an inventor/developer designated on the patent for temperature-responsive culture surfaces. The other authors declare that there is no duality of interest associated with this manuscript.

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Figure Legends

Fig. 1. (A) A flow cytometric analysis. CD29 and CD90 such as mesenchymal markers were positive, and CD31 and CD34such as hematopoietic markers were negative. (B) To confirm a capability of MSC differentiation into osteogenesis and adipogenesis, Alizarin Red S staining and Oil Red O staining were performed.

Fig. 2. (A, B) The islets were seeded at a density of 50 islets/cm^2 in 35-mm temperature-responsive dishes. The islets were riding on the confluent MSCs in the temperature-responsive dish. (C) The islets + MSC sheets were harvested by low-temperature treatment after 72 h co-culture. (D) The islet + MSC sheets were harvested while shrinking during low-temperature treatment. (E) The islets were stained with dithizone. (F) H&E staining showed that the sheets adhered to the islets in the shape of spheres. (G, H) Rat insulin and glucagon immunostaining of islets co-cultured with MSC sheets. Ultrastructures of the islets + MSC sheets were observed by electron microscopy. (I, J) ECM was partially detected between the islets and MSC sheets. (K) The islets and MSC sheets were connected via the formation of tight junctions. (L) The MSC sheets consisted of multiple layers. (M) Cell-to-cell connections were observed in the MSC sheets due to the formation of tight and gap junctions. ECM: extracellular matrix; N: nucleus; TJ: tight junctions (arrow); GJ: gap junctions (arrowhead).

Fig. 3. The recovery rate was calculated after 24 and 72 h of incubation. n=5 each; ***P*<0.01 compared to the group of islets cultured alone.

Fig. 4. (A) The viability of the islets was assessed using calcin-AM and propidium iodide (PI). Viable cells were stained green and dead cells were stained red. Almost all MSCs and MSC sheets were viable. (B) Viability of the islets cultured alone and co-cultured with MSCs and MSC sheets. n=5 each; **P*<0.05, ***P*<0.01.

Fig. 5. The insulin levels changed along with the change in the glucose concentration. The SI was calculated in the cultured-alone group and co-cultured with MSCs and MSC sheet groups. n=5 each. The secretions of VEGF, HGF, and TGFβ1 in the supernatants obtained from the islets alone, islets co-cultured with MSCs, and MSC sheets groups. n=5 each. **P*<0.05, ***P*<0.01.

Fig. 6. (A) The islets + MSC sheets adhered to glass plates (GP). The islets + MSC sheets were attached to the surrounding tissue. The implanted islets + MSC sheets are indicated by a dashed line. (B) The blood glucose levels of diabetic sham-operated (DM-Sham) mice (n=5) and those of the recipient mice: 2,000 islets alone (n=5), MSC sheet alone (n=5), 2,000 islets with MSCs (n=5), two islets + MSC sheets (1,000 islets) (n=5), and four islets + MSC sheets (2,000 islets) (n=6). **P*<0.05, ***P*<0.01 compared to the DM-Sham group. (C) Body weight changes in the recipient mice treated

with four islets + MSC sheets (2,000 islets; black circles, n=6) and the DM-Sham mice (white triangles, n=5). **P*<0.05, ***P*<0.01 compared to the DM-Sham group. (D) The recipient mice were transplanted with four islets + MSC sheets (2,000 islets; black circles, n=6). The graft tissue was surgically removed on day 84. †: graft removal. (E) The IPGTT was performed in the control mice (white squares, n=9) and recipient mice treated with four islets + MSC sheets (2,000 islets; black circles, n=6) on day 56.

Fig. 7. (A) Histological, immunohistochemical, and immunofluorescence analyses on day 28 after the subcutaneous transplantation of the islets + MSC sheets. The expression of insulin and PDx1 on islets was observed in the connective tissue. (B) Serum insulin level was investigated in the DM-Sham (n=5), recipient SCID mice [2,000 islets (n=5), 2,000 islets with MSCs (n=5), two islets + MSC sheets (1,000 islets) (n=5), four islets + MSC sheets (2,000 islets) (n=6)], and normal SCID mice (n=7). ***P*<0.01 compared to the DM-Sham group.

Fig. 8. (A) The expression of vWF was observed in the subcutaneous site. (B) The degree of vascularization was evaluated according to the number of vessels for anti-vWF immunostaining. In the implanted subcutaneous tissue, the number of vessels per square millimeter was counted. At 28 days after treatment, specimens were obtained from DM-Sham mice (n=5), recipient SCID mice treated with islets alone (n=5), four MSC sheets alone (n=5), 2,000 islets with MSCs (n=5), and four

islets + MSC sheet (n=6). *P<0.05, **P<0.01.

Figure 1

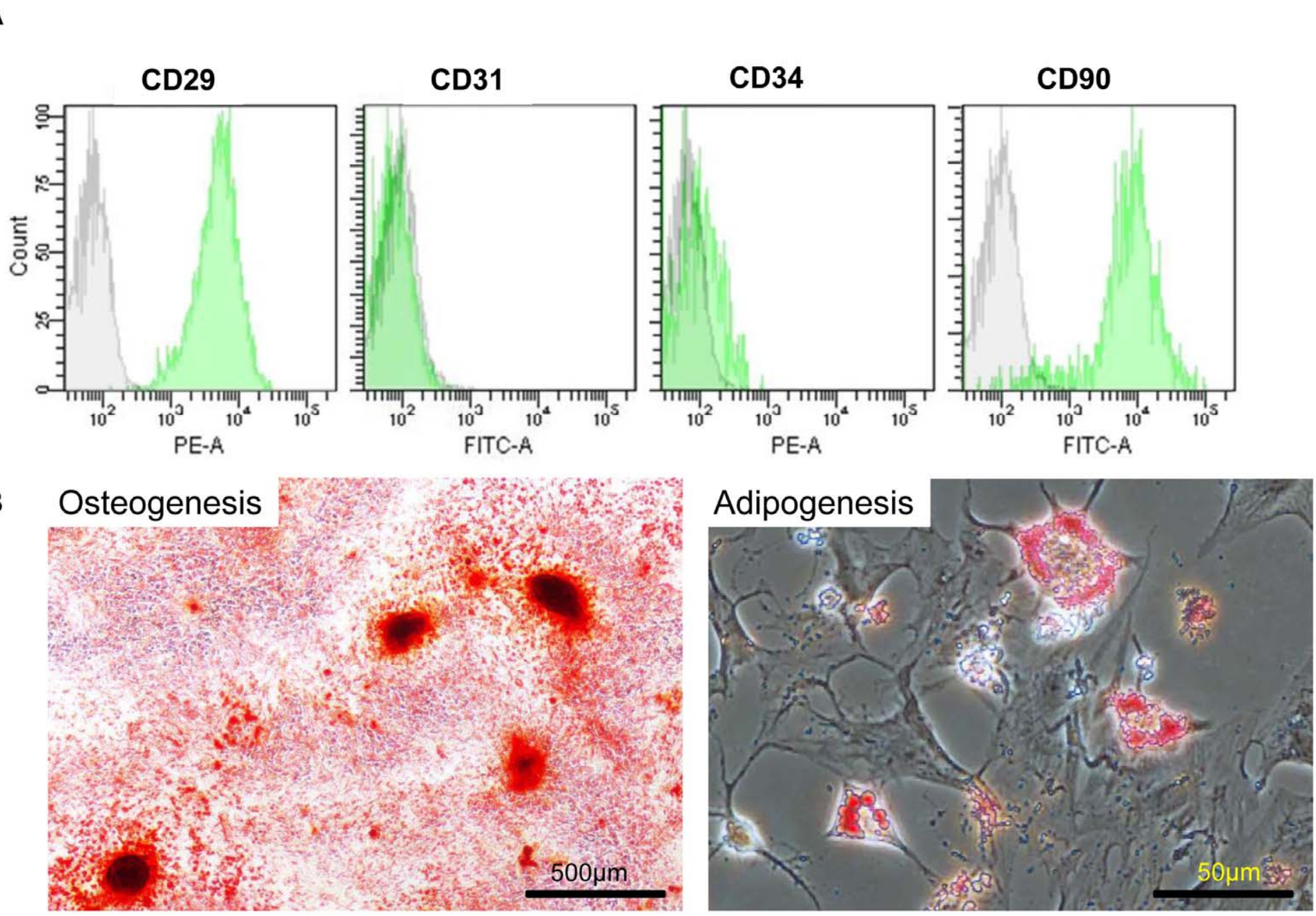
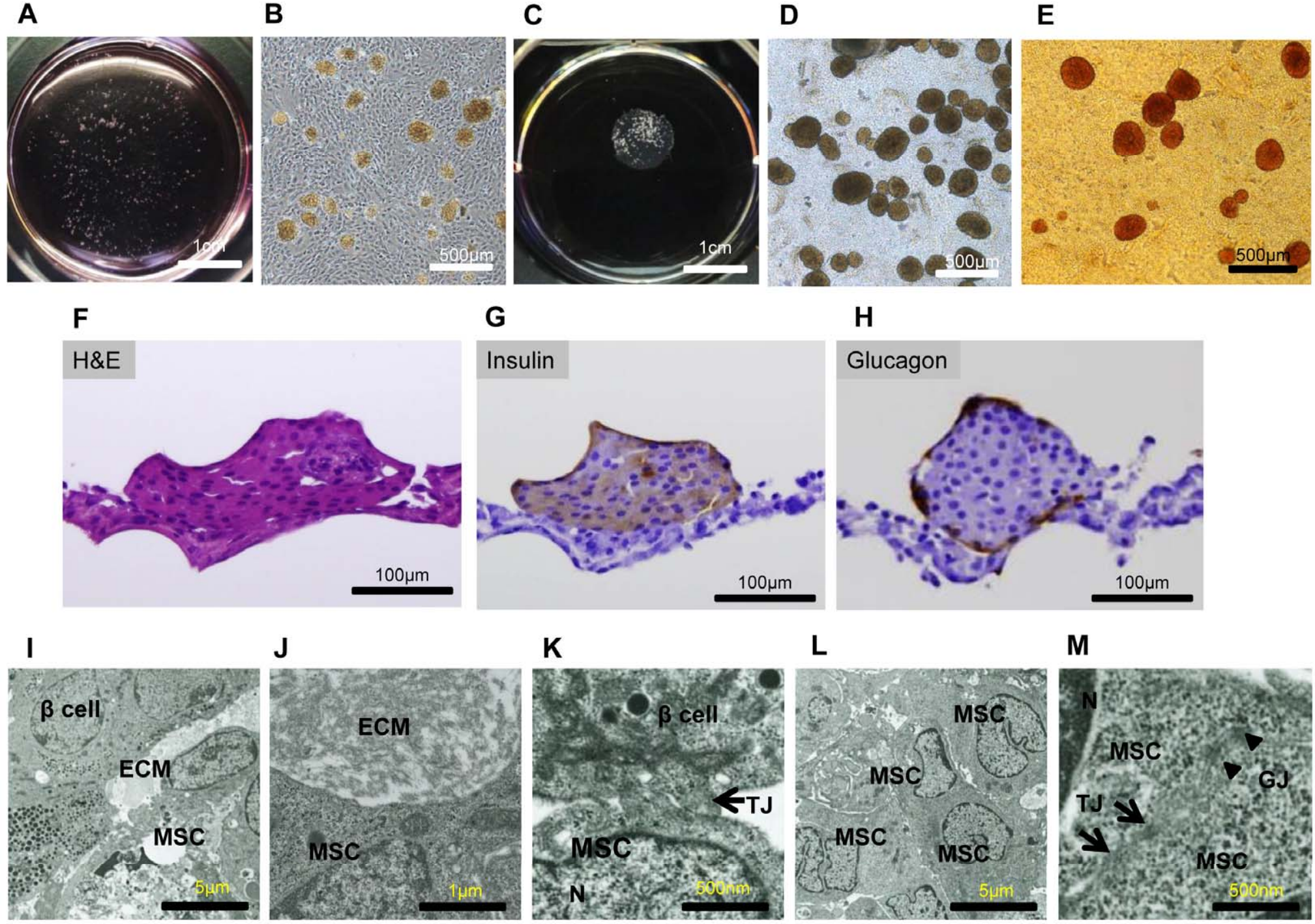

A
CD29
CD31
CD34
CD90
Count
PE-A
FITC-A
PE-A
FITC-A
B
Osteogenesis
500μm
Adipogenesis
50μm

Figure 2


A
B
C
D
E
1cm
500μm
1cm
500μm
500μm
F
G
H
H&E
Insulin
Glucagon
100μm
100μm
100μm
I
J
K
L
M
β cell
ECM
MSC
5μm
ECM
MSC
1μm
β cell
TJ
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5μm
N
MSC
GJ
TJ
MSC
500nm

Figure 3

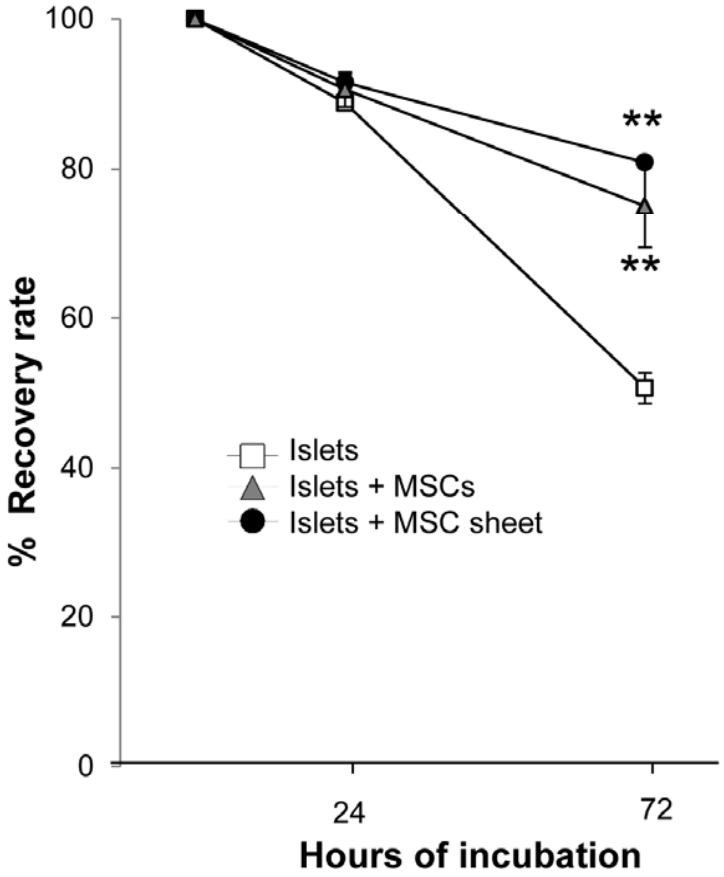
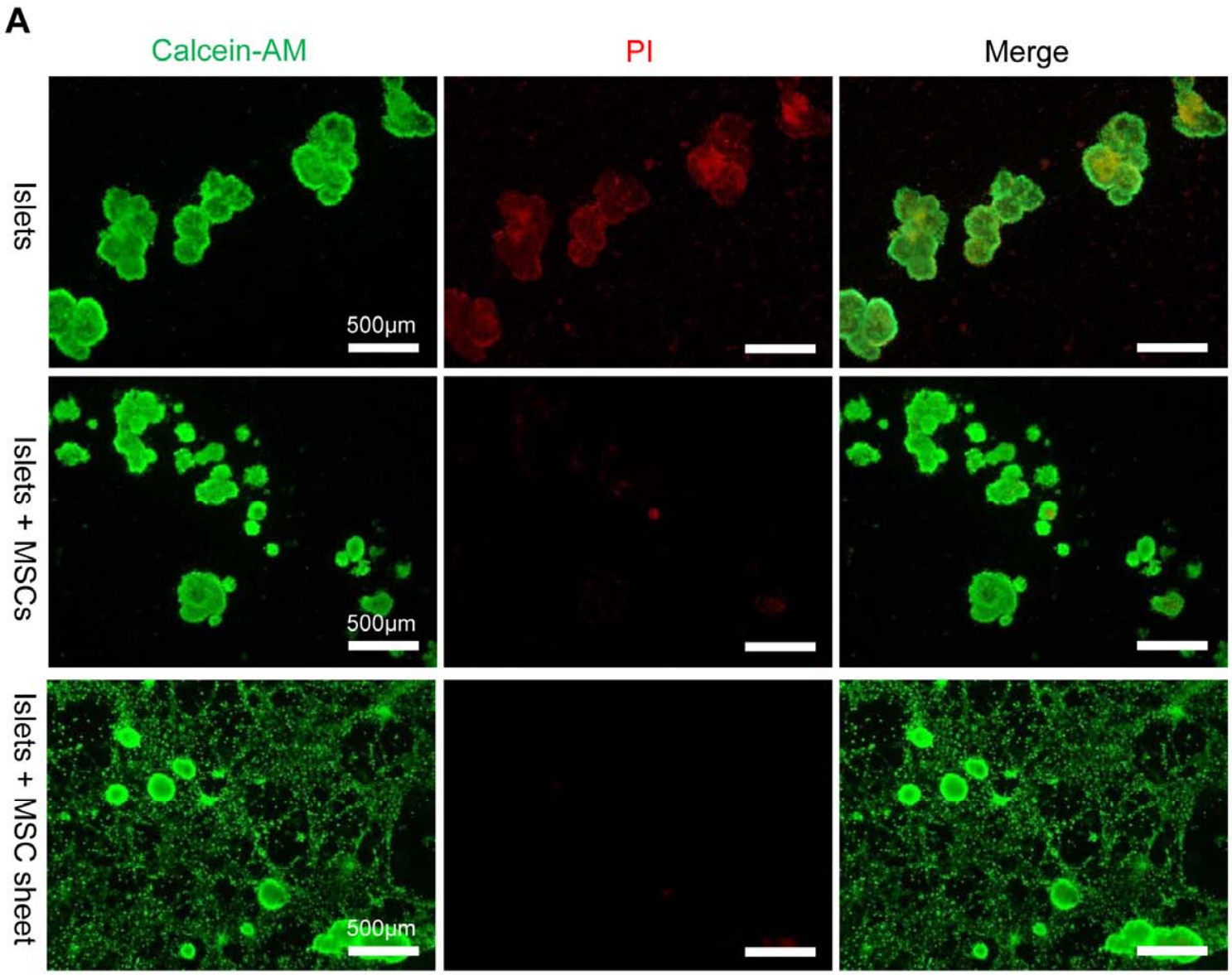

100
80
60
40
20
0
% Recovery rate
**
**
Islets
Islets + MSCs
Islets + MSC sheet
24
72
Hours of incubation

Figure 4


A
Calcein-AM
PI
Merge
Islets
Islets + MSCs
Islets + MSC sheet
500μm
500μm
500μm

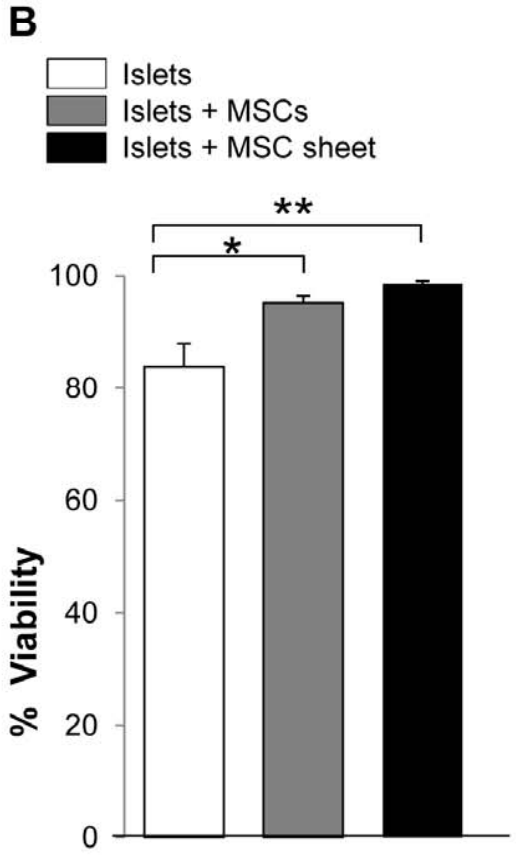

B
Islets
Islets + MSCs
Islets + MSC sheet
**
*
100
80
60
40
20
0
% Viability

Figure 5

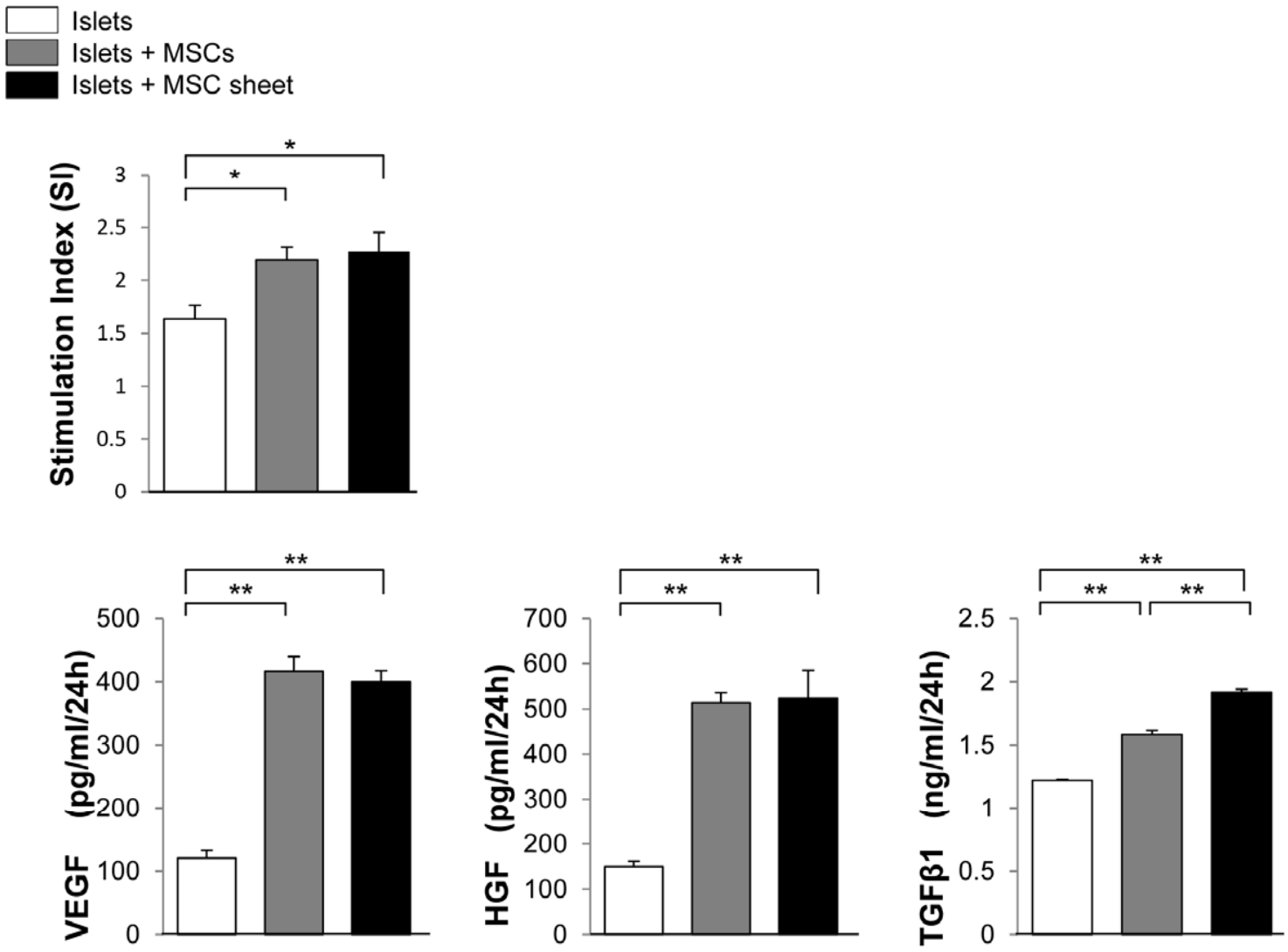

Islets
Islets + MSCs
Islets + MSC sheet
Stimulation Index (SI)
VEGF (pg/ml/24h)
HGF (pg/ml/24h)
TGFβ1 (ng/ml/24h)
*
**

Figure 6

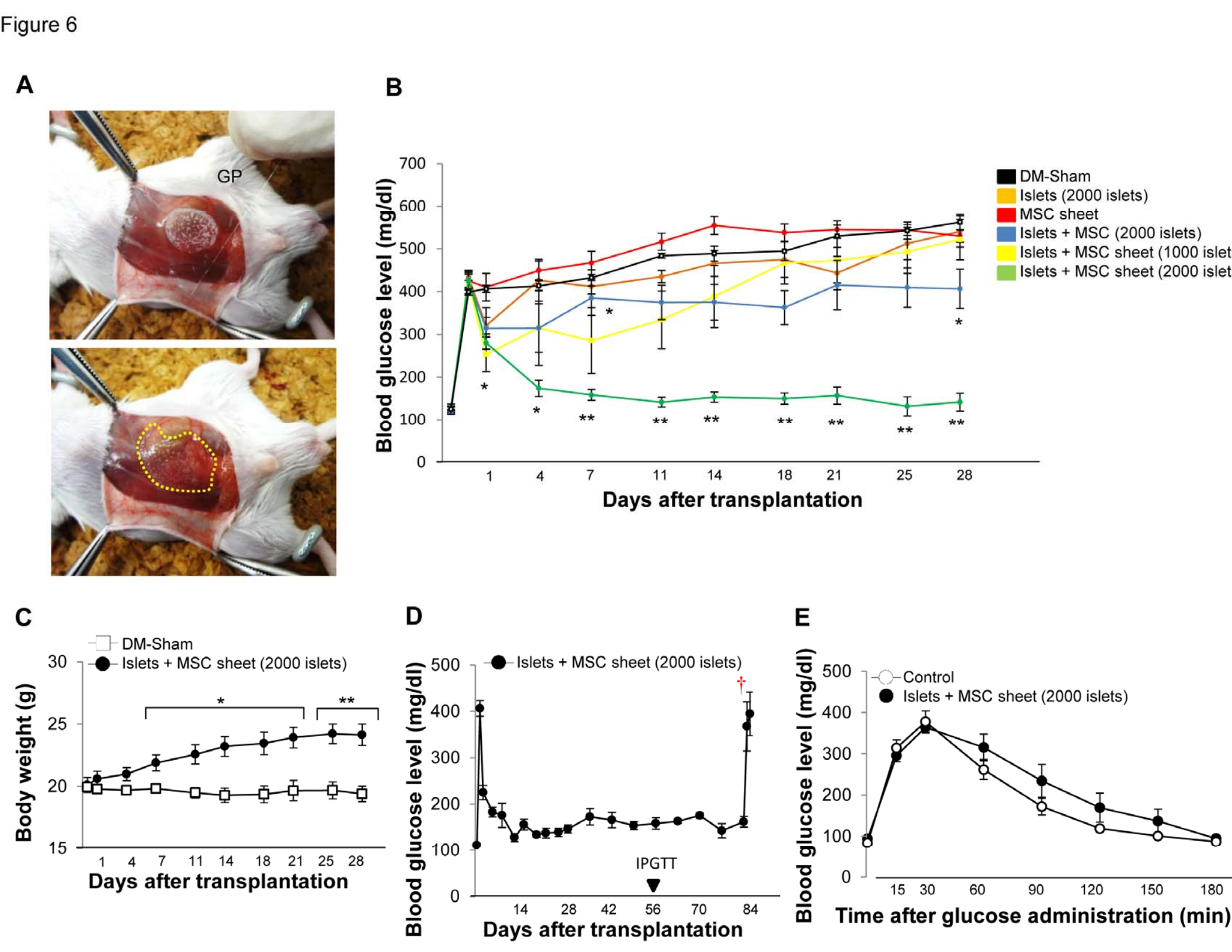

A
GP
B
Blood glucose level (mg/dl)
Days after transplantation
DM-Sham
Islets (2000 islets)
MSC sheet
Islets + MSC (2000 islets)
Islets + MSC sheet (1000 islet
Islets + MSC sheet (2000 islet
C
DM-Sham
Islets + MSC sheet (2000 islets)
Body weight (g)
Days after transplantation
D
Islets + MSC sheet (2000 islets)
Blood glucose level (mg/dl)
IPGTT
Days after transplantation
E
Control
Islets + MSC sheet (2000 islets)
Blood glucose level (mg/dl)
Time after glucose administration (min)

Figure 7

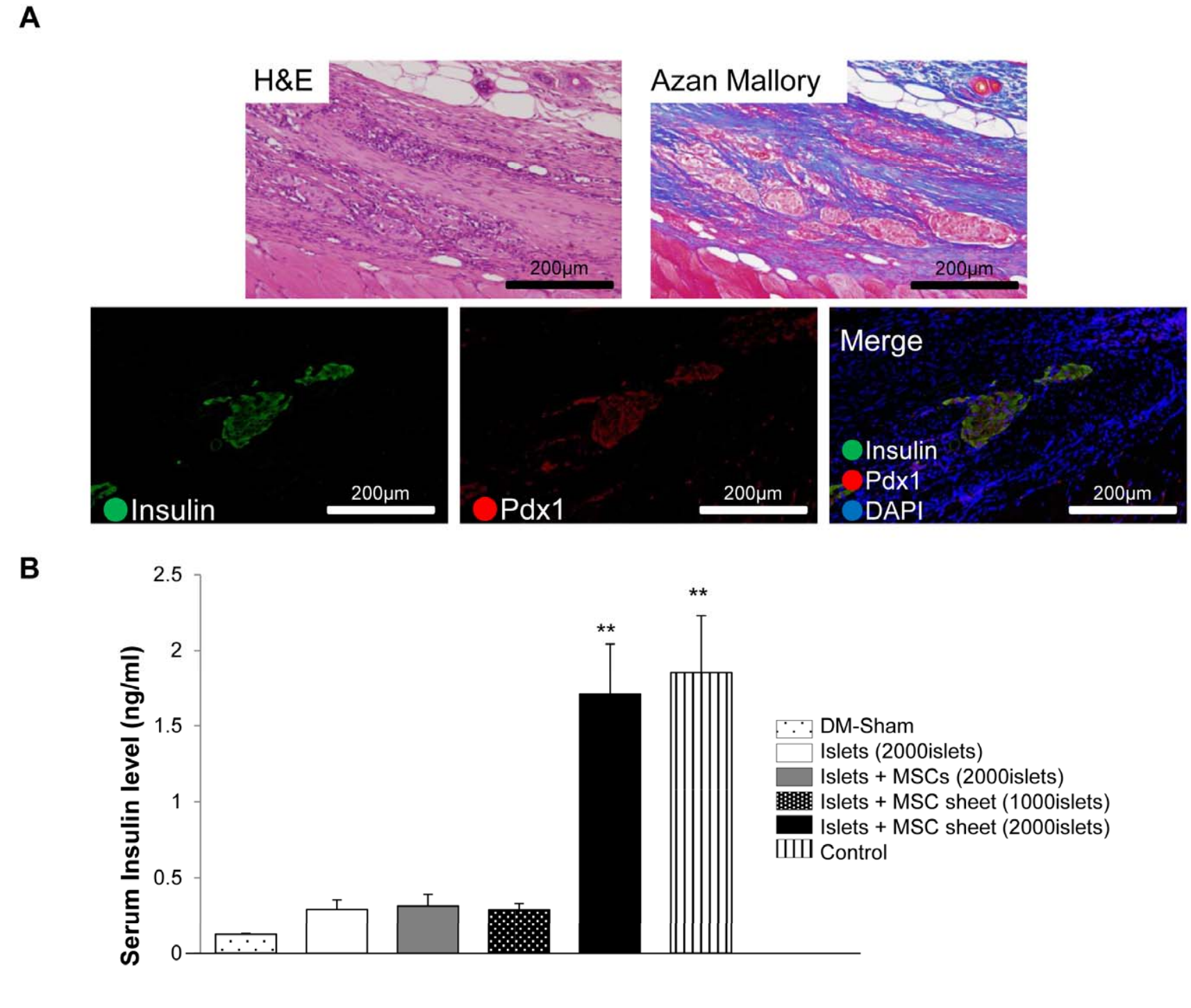
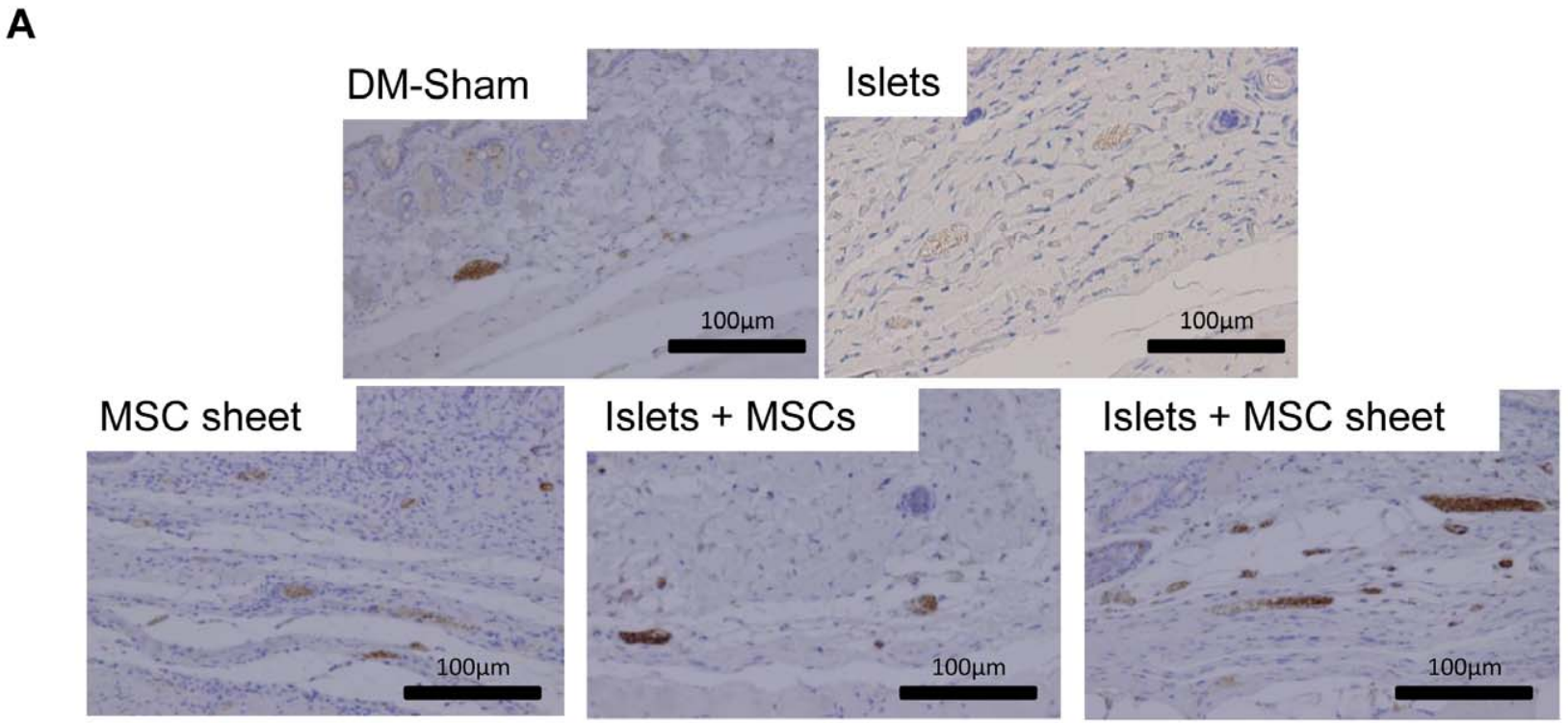
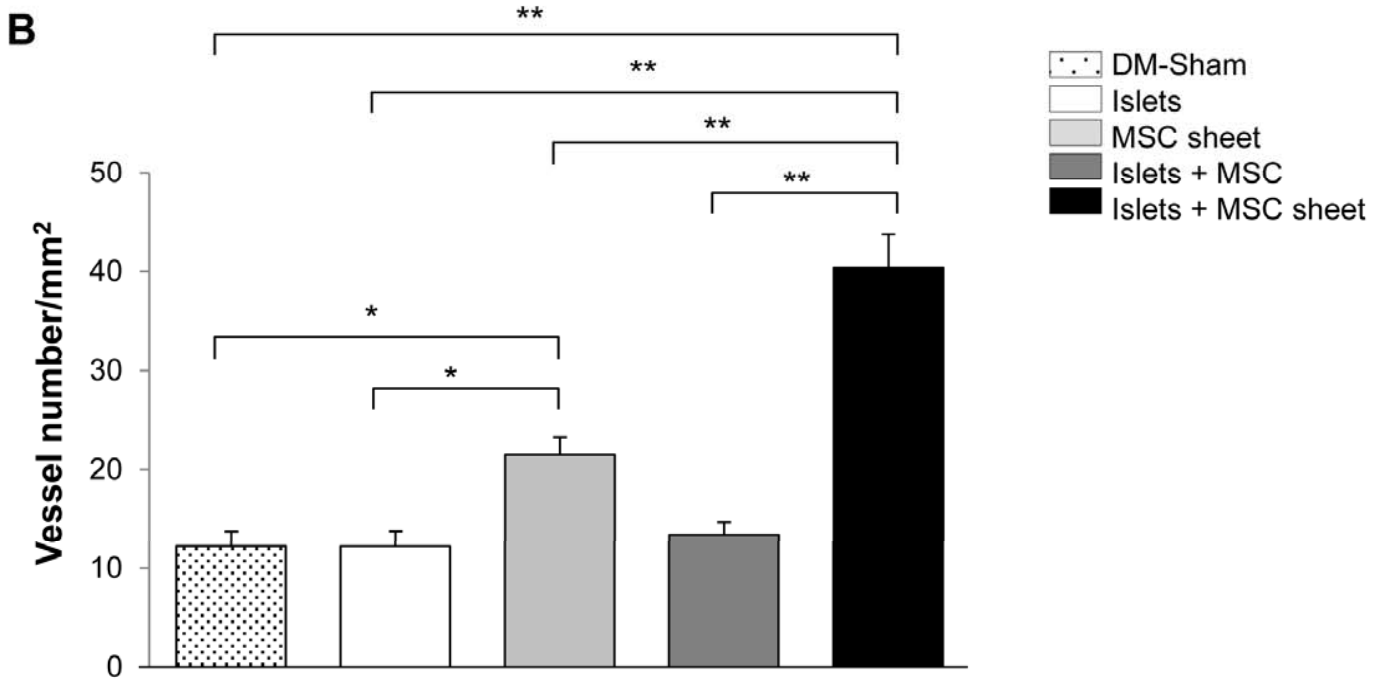

A
H&E
200μm
Azan Mallory
200μm
Insulin
200μm
Pdx1
200μm
Merge
Insulin
Pdx1
DAPI
200μm
B
Serum Insulin level (ng/ml)
2.5
2
1.5
1
0.5
0
**
**
DM-Sham
Islets (2000islets)
Islets + MSCs (2000islets)
Islets + MSC sheet (1000islets)
Islets + MSC sheet (2000islets)
Control

Figure 8


A
DM-Sham
Islets
MSC sheet
Islets + MSCs
Islets + MSC sheet
100μm
100μm
100μm
100μm
100μm


B
Vessel number/mm²
0
10
20
30
40
50
*
*
**
**
**
**
DM-Sham
Islets
MSC sheet
Islets + MSC
Islets + MSC sheet