Original Article

An engineered cell sheet composed of human islets and human fibroblast, bone marrow-derived mesenchymal stem cells, or adipose-derived mesenchymal stem cells: An *in vitro* comparison study

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Running head: Engineered cell sheets with human islets

Abbreviations:

DM, diabetes mellitus; MSCs, mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; ADSCs, adipose-derived mesenchymal stem cells; VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor beta 1; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; IEQ, islet equivalent

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Authorship:

HI, TA, SO, SE: designed the study. HI: performed the experiments. HI, TA, TA: collected data. HI, TA, YS: analyzed the data. AS, MH, MT, TK, AMJS: contributed reagents/ materials. HI, TA, TK, SE: wrote the manuscript.

Abstract

Background: We previously reported the utility of engineered cell sheets composed of human islets and supporting cells *in vitro* and *in vivo*. It is unclear which type of supporting cell is most suitable for constructing cell sheets with human islets. The present study aimed to compare human fibroblasts, bone marrow-derived mesenchymal stem cells (BM-MSCs), and adipose-derived mesenchymal stem cells (ADSCs) as a supporting source for cell sheets. **Methods:** Engineered cell sheets were fabricated with human islets using human fibroblasts, BM-MSCs, or ADSCs as supporting cells. The islet viability, recovery rate, glucose-stimulated insulin release (determined by the stimulation index), and cytokine secretion (TGF-β1, IL-6, and VEGF) of groups—including an islet-alone group as a control—were compared. **Results:** All three sheet groups consistently exhibited higher viability, recovery rate, and stimulation index values than the islet-alone group. The ADSC group showed the highest viability and recovery rate among the three sheet groups. There were no discernible differences in the stimulation index values of the groups. The fibroblast group exhibited significantly higher TGF-β1 values in comparison to the other groups. The IL-6 level of the ADSC group was more than five times higher than that of the other groups. The ADSC group showed the VEGF level; however, it did not differ from that of the BM-MSC group to a statistically significant extent. **Conclusion:** Engineered cell sheets composed of islets and supporting cells had a cytoprotective effect on islets. These results suggest that individual cell types could be a more attractive source for

crafting engineered cell sheets in comparison to islets alone.

Keywords: Islet transplantation; Fibroblast; Bone marrow-derived mesenchymal stem cell; Adipose-derived mesenchymal stem cell; Tissue engineering; Cell sheet

Introduction

Since the introduction of the Edmonton protocol in 2000, clinical islet transplantation has become an attractive therapy for type 1 diabetes mellitus (DM).[1] However, many hurdles must still be overcome in order to achieve the long-term survival of intraportal islet grafts.[2,3] Various factors contribute to acute and chronic islet loss, including an instant blood-mediated inflammatory reaction,[4,5] insufficient blood supply and immunoreactions associated with intraportal islet transplantation,[6,7] and procedure-related complications, such as portal vein thrombosis and hemorrhage.[8,9] To overcome these issues associated with intraportal islet transplantation, several studies have attempted to transplant islets at extrahepatic sites, including the renal subcapsular space,[10] omental pouch,[11,12] and subcutaneous space.[3]

We previously reported a method of islet transplantation using engineered cell sheets composed of islets and mesenchymal stem cells (MSCs) that overcomes the issue of poor vascularization in subcutaneous sites and succeeded in reversing DM in a rodent model.[13] MSCs are known to improve the engraftment of islets by secreting angiogenic and anti-apoptotic cytokines, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor, interleukin (IL)-6, IL-10, and transforming growth factor beta 1 (TGF-β1).[14-18] We further reported that engineered cell sheets composed of human islets and human fibroblasts provided an ideal substrate for human islets as a source of cytokines and extracellular matrix.[19] Fibroblasts

are easily harvested and are known to play an important role in promoting vascularization through the secretion of VEGF and fibroblast growth factor, and to improve islet viability.[20-24]

In the clinical setting, scaffold-free engineered cell sheet technology has been applied in the treatment of various diseases, including conditions affecting the cornea, esophagus, heart, periodontal ligament, and cartilage.[25] The transplantation of cell sheets composed of islets and supporting cells at extrahepatic sites may be beneficial for the survival and function of islets through both the preservation of endogenous extracellular matrix and adhesive factors and the secretion of several cytokines. Various cells types can be used to create engineered cell sheets, including MSCs and fibroblasts.[14,23,26] However, the type of supporting cell that is most suitable for constructing cell sheets with human islets remains unclear. Furthermore, the effect and function of cell sheets composed of islets and supporting cells must be thoroughly evaluated before introducing this technology to the clinical setting.

We used human fibroblasts, human bone marrow-derived MSCs (BM-MSCs), and human adipose-derived MSCs (ADSCs) to construct cell sheets with human islets. We aimed to compare fibroblasts, BM-MSCs, and ADSCs as a supporting source for cell sheets.

Materials and methods

Human islets, fibroblasts, BM-MSCs, and ADSCs

Human islets were isolated from deceased multiorgan donors at the University of Alberta in accordance with the guidelines of the Ethics Committee at the University of Alberta as previously described[27] and were shipped to the Nagasaki University. Consent for the use of islets in research was obtained from the donors' next-of-kin. Human dermal fibroblasts were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Human BM-MSCs and ADSCs were purchased from Lonza (Walkersville, MD, USA). All experiments were conducted at Nagasaki University in accordance with the guidelines of the Ethics Committee at the Nagasaki University Graduate School of Biomedical Sciences.

Construction of cell sheets

Fibroblasts, BM-MSCs, or ADSCs were seeded onto a 35-mm-diameter temperature-responsive culture dish (UpCell®; CellSeed, Tokyo, Japan) at a density of 0.5×10^6 cells/dish to construct a base cell sheet. After 3 days of culture at 37°C in a humidified air atmosphere with 5% CO_2, islets (500 islet equivalent [IEQ]) were seeded onto each culture dish. After an additional 3 days of co-culture in CMRL 1066 tissue culture media (Mediatech, Manassas, VA, USA), cell sheets composed of islets and fibroblasts, BM-MSCs, or ADSCs were constructed. The cell sheets were detached freely from the dish by reducing the culture temperature from 37°C to 20°C. The cell

sheets were then used in experiments. As a control, islets (500 IEQ) were cultured in CMRL 1066 tissue culture media using a 35-mm dish with an ultra-low-attachment surface (Corning Life Sciences, Corning, NY, USA) for 3 days. Thus, there were four groups: islets-alone without a sheet, and islets with a fibroblast, BM-MSC, or ADSC sheet.

Immunohistochemistry

Insulin staining was performed to confirm the presence of β cells within cell sheets. After the detachment of cell sheets, they were fixed with 4% paraformaldehyde (PFA; Wako Pure Chemical Industries Ltd., Osaka, Japan). Cell sheets were embedded in paraffin (Paraplast Plus; Leica Biosystems Inc., Richmond, IL, USA). Hematoxylin and eosin (H&E; Muto Pure Chemicals Co. Ltd., Tokyo, Japan) staining was performed according to the standard protocol. For immunohistochemical staining, the sections were deparaffinized with xylene and rehydrated with a graded ethanol series. Consecutive sections were incubated with 10% hydrogen peroxide to quench endogenous peroxidases, blocked with 20% non-specific serum, and incubated with primary antibodies (1/1000 mouse anti-insulin; DAKO Mississauga, Ontario, Canada) for 60 minutes. Sections were washed three times with PBS and incubated with biotinylated goat anti-mouse secondary antibodies (1:200; Vector Laboratories, Burlingame, CA, USA) for 60 minutes followed by activin-biotin enzyme complex with 3,3-diaminobenzidinetetrahydrochloride chromagen and counterstained with H&E. Negative

controls were incubated without primary antibodies and human pancreas sections were used as positive controls. All slides were visualized with an Axioscope II equipped with AxioCam MRC and analyzed with the Axiovision software program (ver. 4.6, Carl Zeiss, Gottingen, Germany).

Islet viability and recovery rate

Calcein-acetoxymethyl and propidium iodide (Cellstain Double Staining Kit; Dojindo, Kumamoto, Japan) were used to assess the viability of the islets. The assay solution was prepared as follows: 10 μl of Solution A (1 mmol/l Calcein-AM/DMSO) and 15 μl of Solution B (1.5mmol/l PI/purified water) were mixed in 5 ml of D-PBS as staining solution. A 35-mm dish was washed with PBS and incubated with staining solution for 15 minutes at 37°C. Islet viability was determined by counting the viable cells (yellowish-green fluorescent staining) and non-viable cells (red fluorescent staining) under a fluorescence microscope (Nikon). Total viability was calculated by dividing the number of viable cells by the total number of cells assessed. Because the islet mass is known to decrease during culturing,[27] we evaluated the islet recovery rate by assessing the islet mass based on the IEQ at baseline and on day 3 after co-culture.

Insulin secretion assay

We conducted a glucose-stimulated insulin secretion test, as previously described.[19] In brief, Krebs solution was prepared by adding 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2•6H_2O$, 0.1% bovine serum albumin, and 2.5 mM $CaCl_2•2H_2O$ (Wako Pure Chemical Industries). Islets alone (without sheets) and cell sheets composed of islets and supporting cells were sequentially incubated for 1 h in Krebs solution containing 2.8 and 28 mM glucose. The supernatant was collected after each incubation period, and the insulin concentration was measured by an enzyme-linked immunosorbent assay (ELISA) using an Abcam ELISA Kit (Cambridge, MA, USA) according to the manufacturer's instructions. The stimulation index was calculated by dividing the amount of insulin secreted under a high glucose concentration by the amount secreted under a low glucose concentration. We performed a glucose-stimulated insulin secretion test on day 3 after co-culture.

Measurement of cytokine secretion

Prior to detachment of the cell sheets, the culture media were collected and frozen at −80°C until use. We used an ELISA Kit (Abcam) to measure the secretion of TGF-β1, IL-6, and VEGF (human cytokines) according to the manufacturer's instructions.

Statistical analysis

All data are expressed as the mean ± standard error of the mean. The results were analyzed by a

one-way analysis of variance and a post hoc Tukey's test. *P* values of <0.05 were considered to indicate statistical significance. The GraphPad PRISM version 5 software program (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses.

Results

Characteristics of the transported human islets

We transported human islets from five donors. It took three days for the human islets to be transported to Nagasaki from Alberta. The details (age, sex, cause of death, cold ischemic time, transported IEQ/viability/purity) are shown in Table 1. The health statuses of the donors prior to death were as follows: Donor Nos. 1 and 3 (no particular conditions); Donor No. 2, splenectomy and cholecystectomy in 1985 due to complication from lupus/hemolytic anemia; Donor No. 4, aneurysm repair in 2016; and Donor No. 5, hypothyroidism since 2002.

Characteristics of the cell sheets

Upon the arrival of the human islets from the University of Alberta, we confirmed the morphological integrity of the islets by dithizone staining (Fig. 1a, 1b, 1c). After 3 days of co-culturing the islets with a base cell sheet, each cell sheet composed of islets and the supporting cells was detached from the dish by reducing the temperature to 20°C. Macroscopically, the islets appeared to be well adhered to the sheets (Fig. 2a). A histological examination revealed the presence of intact islets attached to each cell sheet as well as insulin-positive cells within the islets. Figures 2b and 2c show an example of a cell sheet composed of islets and ADSCs.

The effects of cell sheets on islet viability and the recovery rate

The islet viability of the islet-alone, fibroblast, BM-MSC, and ADSC groups was 22.7%±5.8%, 63.9%±3.2%, 33.8%±3.8%, and 76.1%±4.5%, respectively (Fig. 3). With the exception of the BM-MSC group, all sheet groups exhibited significantly higher viability than the islet-alone group. The ADSC group showed the highest viability among the three sheet groups; however, the viability of the ADSC and fibroblast groups did not differ to a statistically significant extent. Six to nine independent experiments were performed in each group with four separate donors to assess the viability. The islet recovery rates are shown in Figure 4. With the exception of the BM-MSC group, all sheet groups showed significantly higher recovery rates in comparison to the islet-alone group. Although the ADSC group showed the highest recovery rate, the recovery rate did not differ among the three sheets groups to a statistically significant extent. Six to nine independent experiments were performed in each group with three separate donors to assess the recovery rate.

G*lucose-stimulated insulin release*

Unexpectedly, the stimulation index values during the glucose-stimulated insulin release assay of all groups were <1 (Fig. 5). However, the stimulation index values of all cell sheet groups were significantly higher than that of the islet-alone group. No significant differences were observed

in the stimulation index values of the sheets groups. To assess the stimulation indices, 8–15 independent experiments were performed in each group with three separate donors.

Secretion of cytokines during co-culture

The cytokine secretion results are shown in Figures 6–8. As expected, the cytokine secretion levels (TGF-β1, IL-6, and VEGF) in the islet-alone group were the lowest among the four groups and were always significantly lower than those of at least two sheet groups. The TGF-β1 levels of the islet-alone, fibroblast, BM-MSC, and ADSC groups were 0.61±0.01, 2.17±0.05, 1.66±0.10, and 1.63±0.10 ng/ml, respectively (Fig. 6). The secretion of TGF-β1 in the fibroblast group was significantly higher than that in the other sheets groups (p<0.05). The IL-6 levels of the islet-alone, fibroblast, BM-MSC, and ADSC groups were 2.73±0.3, 20.9±2.1, 46.3±4.2, and 228.0±19.4 ng/ml, respectively (Fig. 7). The IL-6 level of ADSC group was significantly higher than that in the other sheet groups, and was five times higher than that in the BM-MSC group. The VEGF levels of the islet-alone, fibroblast, BM-MSC, and ADSC groups were 1.15±0.15, 2.2±0.09, 5.4±0.31, and 6.6±0.75 ng/ml, respectively (Fig. 8). The ADSC group showed the highest VEGF secretion level of the three sheet groups; however, the value did not differ from that in the BM-MSC group to a statistically significant extent. Nine to 12 independent experiments with three separate donors were performed to assess the cytokine secretion levels of each group.

Discussion

In the present *in vitro* study, we successfully fabricated cell sheets with human islets using three types of human-derived supporting cells. We also demonstrated that, in comparison to islets alone, all three cell sheets exerted cytoprotective effects on human islets, likely through the secretion of several cytokines or growth factors. Although individual cell types may be a more attractive source for crafting engineered cell sheets than islets alone, the favorable results observed in the ADSC group, including islet viability and cytokine secretion, suggest that using ADSCs to create tissue-engineered cell sheets with human islets is suitable in the clinical setting. To our knowledge, this is the first report to investigate the ideal cell source for cell sheets composed of islets and supporting cells by comparing individual human-derived cell types.

Cell sheets composed of islets and supporting cells have been shown to have beneficial effects on the survival and function of human islets in previous reports, as they maintain the natural structure of the islets and preserve the extracellular matrix components.[13,19] A temperature-responsive culture dish is coated with poly (N-isopropylacrylamide), which changes from hydrophobic to hydrophilic at temperatures of <32°C. Cells cultured on such a dish can thus be harvested as monolayer cell sheets without trypsinization by simply decreasing the culture temperature. In the present study, we fabricated cell sheets with human islets using three types of human-derived supporting cells, and all types of cell sheets composed of islets and supporting cells exerted cytoprotective effects on human islets. These results suggest that human

islet transplantation with cell sheets may have beneficial effects in comparison to the transplantation of islets alone. Cell sheets can be fabricated simply and efficiently with the use of commercially available temperature-responsive culture dishes. In addition, cell sheets composed of islets and supporting cells are easy to manipulate in culture dishes. We believe that these cell sheets will be applied in extrahepatic islet transplantation.

Unexpectedly, the stimulation indices during the glucose-stimulated insulin release assay of all groups were <1. However, the stimulation index values of all cell sheet groups were significantly higher than that of the islet-alone group. Park et al. reported that MSC monolayers improved the survival and function of islets.[28,29] However, several authors conversely reported that direct co-culturing did not improve the islet function.[30,31] In the present study, cell sheets composed of islets and supporting cell seemed to contribute to the improvement of the islet function, regardless of the type of cell. This benefit might be derived from the maintenance of the natural structure of islets by cell sheets. The cell-to-cell junctions have been reported to play an important role in the secretion of insulin.[32]

The three cytokines investigated in the present study (TGF-β1, IL-6, and VEGF) were selected because they have been reported to have beneficial effects on islets.[14,28,29] TGF-β1 stimulates the production of heat shock protein 32 and X-linked inhibitor of apoptosis protein.[33] Heat shock protein 32 has a protective effect on islets and suppresses inflammatory reactions and oxidative stress.[34,35] X-linked inhibitor of apoptosis protein is known to have an anti-apoptotic

effect on β cells.[36,37] IL-6 is a multifunctional cytokine produced by both lymphoid and non-lymphoid cells, including macrophages, fibroblasts, and endothelial cells, and is involved in antigen-specific immune responses and inflammatory reactions.[38,39] IL-6 has been shown to prevent the functional impairment of IFN-γ-, TNF-α-, and IL-1β-treated mouse islets and is considered to have an anti-apoptotic effect on islets.[14,40] With regard to the protective mechanism, it is assumed that IL-6 induces the expression of Bcl-2 and Bcl-xL,[14,41] which have been reported as anti-apoptotic signaling molecules in β cells.[42,43] Thus, even though the expression of Bcl-2 and Bcl-xL was not investigated in the present study, IL-6 secretion may be related to decreased islet cell death and the maintenance of the recovery rate. Of note, the IL-6 secretion in the ADSC group was more than five times higher than in the other sheet groups.

Regarding extrahepatic sites, the subcutaneous space seems to be an attractive transplantation site for several reasons. It provides an adequate space to accommodate a large volume of islets and prevents acute islet loss due to instant blood-mediated inflammatory reactions. In addition, subcutaneous transplantation does not require general anesthesia, and the transplanted islets can be safely removed or biopsied if needed. However, subcutaneous transplantation has been suggested to be associated with a lack of graft viability, as the superficial tissue oxygen tension is relatively low in comparison to other vascularized organs.[44] One of the most impressive findings of the present study was that the secretion of VEGF in the ADSC and BM-MSC groups was significantly higher in comparison to the other groups. VEGF

has been shown to induce revascularization, angiogenesis, and the proliferation of endothelial cells in islet grafts after transplantation. These results suggest that islet engraftment would be promoted under hypoxic conditions, such as in the subcutaneous space, when ADSC or BM-MSC sheets are utilized. One possible advantage of ADSCs over BM-MSCs may be their high affinity for the subcutaneous space, as ADSCs are originally isolated from subcutaneous tissues.

The characteristics of the MSC donors, including their age, sex, and basal disease status, influence the therapeutic potential of MSCs.[45] Hayward et al. reported that MSCs from patients with psoriatic arthritis (an autoimmune disease in which the joints are targeted for immune attack and inflammation) had a detrimental effect on the ability of neonatal pig islets to reverse DM.[46] The authors suggested that these MSCs had functional impairments, such as reduced VEGF expression levels or reduced anti-inflammatory effects. In the present study, we used MSCs that had been obtained from a healthy donor by a company. To improve the outcomes of islet transplantation, it will be necessary to examine and optimize the conditions of cell sheets composed of islets with supporting cells, including the number of islets, the duration for which the cells are cultured, and the donor characteristics. The characteristics of the MSC donor should be considered in both research and the clinical setting.

The present study is associated with a limitation that warrants mention. The human islets were transported for a long distance, as it is difficult to obtain human islets at present of Japan.

Transported human islets have been valuable and have contributed to the continued progress of islet-based basic research. Yamashita et al. reported that dispersed islet cells obtained from long-distance-transported human islets were valuable for basic medical research, despite the vast distance over which they had been transported.[47] In contrast, although the conditions of transportation or handling of human islets would have been similar, the islets used in the present study might not have had typical characteristics. Thus, our results might not be generalizable to healthier islets. We strongly recognize that it is ideal to use human islets as soon as possible after isolation. Future studies should therefore attempt to avoid using long-distance-transported human islets.

In conclusion, we showed that engineered cell sheets composed of islets and supporting cells had favorable effects on the outcome. Their cytoprotective effects and their effects on the secretion of several cytokines and growth factors were superior in comparison to the islet-alone group. These results suggest that individual cell types could be a more attractive source for crafting engineered cell sheets than islets alone. Cell sheets composed of islets and supporting cells may prove useful for extrahepatic islet transplantation.

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References

1. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med. 2000;343:230-8. PMID: 10911004

2. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, Lakey JR, Shapiro AM. Five-year follow-up after clinical islet transplantation. Diabetes. 2005;54:2060-9. PMID: 15983207

3. Bruni A, Gala-Lopez B, Pepper AR, Abualhassan NS, Shapiro AJ. Islet cell transplantation for the treatment of type 1 diabetes: Recent advances and future challenges. Diabetes Metab Syndr Obes. 2014;7:211-23. PMID: 25018643

4. Bennet W, Groth CG, Larsson R, Nilsson B, Korsgren O. Isolated human islets trigger an instant blood mediated inflammatory reaction: Implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. Ups J Med Sci. 2000;105:125-33. PMID: 11095109

5. Eich T, Eriksson O, Lundgren T, Nordic Network for Clinical Islet T. Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. N Engl J Med. 2007;356:2754-5. PMID: 17596618

6. Ryan EA, Lakey JR, Rajotte RV, Korbutt GS, Kin T, Imes S, Rabinovitch A, Elliott JF,

Bigam D, Kneteman NM, et al. Clinical outcomes and insulin secretion after islet transplantation with the edmonton protocol. Diabetes. 2001;50:710-9. PMID: 11289033

7. Korsgren O, Lundgren T, Felldin M, Foss A, Isaksson B, Permert J, Persson NH, Rafael E, Ryden M, Salmela K, et al. Optimising islet engraftment is critical for successful clinical islet transplantation. Diabetologia. 2008;51:227-32. PMID: 18040664

8. Low G, Hussein N, Owen RJ, Toso C, Patel VH, Bhargava R, Shapiro AM. Role of imaging in clinical islet transplantation. Radiographics. 2010;30:353-66. PMID: 20228322

9. Kawahara T, Kin T, Kashkoush S, Gala-Lopez B, Bigam DL, Kneteman NM, Koh A, Senior PA, Shapiro AM. Portal vein thrombosis is a potentially preventable complication in clinical islet transplantation. Am J Transplant. 2011;11:2700-7. PMID: 21883914

10. Jindal RM, Sidner RA, McDaniel HB, Johnson MS, Fineberg SE. Intraportal vs kidney subcapsular site for human pancreatic islet transplantation. Transplant Proc. 1998;30:398-9. PMID: 9532100

11. Kin T, Korbutt GS, Rajotte RV. Survival and metabolic function of syngeneic rat islet grafts transplanted in the omental pouch. Am J Transplant. 2003;3:281-5. PMID: 12614282

12. Berman DM, Molano RD, Fotino C, Ulissi U, Gimeno J, Mendez AJ, Kenyon NM, Kenyon NS, Andrews DM, Ricordi C, et al. Bioengineering the endocrine pancreas:

Intraomental islet transplantation within a biologic resorbable scaffold. Diabetes. 2016;65:1350-61. PMID: 26916086

13. Hirabaru M, Kuroki T, Adachi T, Kitasato A, Ono S, Tanaka T, Matsushima H, Sakai Y, Soyama A, Hidaka M, et al. A method for performing islet transplantation using tissue-engineered sheets of islets and mesenchymal stem cells. Tissue Eng Part C Methods. 2015;21:1205-15. PMID: 26066973

14. Choi SE, Choi KM, Yoon IH, Shin JY, Kim JS, Park WY, Han DJ, Kim SC, Ahn C, Kim JY, et al. IL-6 protects pancreatic islet beta cells from pro-inflammatory cytokines-induced cell death and functional impairment in vitro and in vivo. Transpl Immunol. 2004;13:43-53. PMID: 15203128

15. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation. 2004;109:1292-8. PMID: 14993122

16. Aksu AE, Horibe E, Sacks J, Ikeguchi R, Breitinger J, Scozio M, Unadkat J, Feili-Hariri M. Co-infusion of donor bone marrow with host mesenchymal stem cells treats GVHD and promotes vascularized skin allograft survival in rats. Clin Immunol. 2008;127:348-58. PMID: 18387852

17. Boumaza I, Srinivasan S, Witt WT, Feghali-Bostwick C, Dai Y, Garcia-Ocana A, Feili-Hariri M. Autologous bone marrow-derived rat mesenchymal stem cells promote

PDX-1 and insulin expression in the islets, alter T cell cytokine pattern and preserve regulatory t cells in the periphery and induce sustained normoglycemia. J Autoimmun. 2009;32:33-42. PMID: 19062254

18. Yeung TY, Seeberger KL, Kin T, Adesida A, Jomha N, Shapiro AM, Korbutt GS. Human mesenchymal stem cells protect human islets from pro-inflammatory cytokines. PLoS One. 2012;7:e38189. PMID: 22666480

19. Matsushima H, Kuroki T, Adachi T, Kitasato A, Ono S, Tanaka T, Hirabaru M, Kuroshima N, Hirayama T, Sakai Y, et al. Human fibroblast sheet promotes human pancreatic islet survival and function in vitro. Cell Transplant. 2016;25:1525-37. PMID: 26877090

20. Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JC, Trent JM, Staudt LM, Hudson J Jr., Boguski MS, et al. The transcriptional program in the response of human fibroblasts to serum. Science. 1999;283:83-7. PMID: 9872747

21. Pinney E, Liu K, Sheeman B, Mansbridge J. Human three-dimensional fibroblast cultures express angiogenic activity. J Cell Physiol. 2000;183:74-82. PMID: 10699968

22. Jalili RB, Moeen Rezakhanlou A, Hosseini-Tabatabaei A, Ao Z, Warnock GL, Ghahary A. Fibroblast populated collagen matrix promotes islet survival and reduces the number of islets required for diabetes reversal. J Cell Physiol. 2011;226:1813-9. PMID: 21506112

23. Liu D, Xiao H, Du C, Luo S, Li D, Pan L. The effect of fibroblast activation on vascularization in transplanted pancreatic islets. J Surg Res. 2013;183:450-6. PMID:

23369362

24. Perez-Basterrechea M, Obaya AJ, Meana A, Otero J, Esteban MM. Cooperation by fibroblasts and bone marrow-mesenchymal stem cells to improve pancreatic rat-to-mouse islet xenotransplantation. PLoS One. 2013;8:e73526. PMID: 24009755

25. Matsuura K, Utoh R, Nagase K, Okano T. Cell sheet approach for tissue engineering and regenerative medicine. J Control Release. 2014;190:228-39. PMID: 24858800

26. Yamada S, Shimada M, Utsunomiya T, Ikemoto T, Saito Y, Morine Y, Imura S, Mori H, Arakawa Y, Kanamoto M, et al. Trophic effect of adipose tissue-derived stem cells on porcine islet cells. J Surg Res. 2014;187:667-72. PMID: 24238974

27. Kin T, Senior P, O'Gorman D, Richer B, Salam A, Shapiro AM. Risk factors for islet loss during culture prior to transplantation. Transpl Int. 2008;21:1029-35. PMID: 18564983

28. Park KS, Kim YS, Kim JH, Choi BK, Kim SH, Oh SH, Ahn YR, Lee MS, Lee MK, Park JB, et al. Influence of human allogenic bone marrow and cord blood-derived mesenchymal stem cell secreting trophic factors on ATP (adenosine-5'-triphosphate)/ADP (adenosine-5'-diphosphate) ratio and insulin secretory function of isolated human islets from cadaveric donor. Transplant Proc. 2009;41:3813-8. PMID: 19917393

29. Park KS, Kim YS, Kim JH, Choi B, Kim SH, Tan AH, Lee MS, Lee MK, Kwon CH, Joh JW, et al. Trophic molecules derived from human mesenchymal stem cells enhance survival, function, and angiogenesis of isolated islets after transplantation.

Transplantation. 2010;89:509-17. PMID: 20125064

30. Johansson U, Rasmusson I, Niclou SP, Forslund N, Gustavsson L, Nilsson B, Korsgren O, Magnusson PU. Formation of composite endothelial cell-mesenchymal stem cell islets: A novel approach to promote islet revascularization. Diabetes. 2008;57:2393-401. PMID: 18519803

31. Duprez IR, Johansson U, Nilsson B, Korsgren O, Magnusson PU. Preparatory studies of composite mesenchymal stem cell islets for application in intraportal islet transplantation. Ups J Med Sci. 2011;116:8-17. PMID: 21050099

32. Santos-Silva JC, Carvalho CP, de Oliveira RB, Boschero AC, Collares-Buzato CB. Cell-to-cell contact dependence and junctional protein content are correlated with in vivo maturation of pancreatic beta cells. Can J Physiol Pharmacol. 2012;90:837-50. PMID: 22712703

33. Kutty RK, Nagineni CN, Kutty G, Hooks JJ, Chader GJ, Wiggert B. Increased expression of heme oxygenase-1 in human retinal pigment epithelial cells by transforming growth factor-beta. J Cell Physiol. 1994;159:371-8. PMID: 8163576

34. Pileggi A, Molano RD, Berney T, Cattan P, Vizzardelli C, Oliver R, Fraker C, Ricordi C, Pastori RL, Bach FH, et al. Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. Diabetes. 2001;50:1983-91. PMID: 11522663

35. Lee DY, Lee S, Nam JH, Byun Y. Minimization of immunosuppressive therapy after islet transplantation: Combined action of heme oxygenase-1 and PEGylation to islet. Am J Transplant. 2006;6:1820-8. PMID: 16780547

36. Emamaullee JA, Rajotte RV, Liston P, Korneluk RG, Lakey JR, Shapiro AM, Elliott JF. XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes. Diabetes. 2005;54:2541-8. PMID: 16123341

37. Plesner A, Liston P, Tan R, Korneluk RG, Verchere CB. The X-linked inhibitor of apoptosis protein enhances survival of murine islet allografts. Diabetes. 2005;54:2533-40. PMID: 16123340

38. Matsuda T, Suematsu S, Kawano M, Yoshizaki K, Tang B, Tanabe O, Nakajima T, Akira S, Hirano T, Kishimoto T. IL-6/BSF2 in normal and abnormal regulation of immune responses. Ann N Y Acad Sci. 1989;557:466-76. PMID: 2786703

39. Kamimura D, Ishihara K, Hirano T. IL-6 signal transduction and its physiological roles: The signal orchestration model. Rev Physiol Biochem Pharmacol. 2003;149:1-38. PMID: 12687404

40. Karaoz E, Genc ZS, Demircan PC, Aksoy A, Duruksu G. Protection of rat pancreatic islet function and viability by coculture with rat bone marrow-derived mesenchymal stem cells. Cell Death Dis. 2010;1:e36. PMID: 21364643

41. Ogawa M, Nishiura T, Oritani K, Yoshida H, Yoshimura M, Okajima Y, Ishikawa J,

Hashimoto K, Matsumura I, Tomiyama Y, et al. Cytokines prevent dexamethasone-induced apoptosis via the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways in a new multiple myeloma cell line. Cancer Res. 2000;60:4262-9. PMID: 10945640

42. Dupraz P, Rinsch C, Pralong WF, Rolland E, Zufferey R, Trono D, Thorens B. Lentivirus-mediated Bcl-2 expression in betaTC-tet cells improves resistance to hypoxia and cytokine-induced apoptosis while preserving in vitro and in vivo control of insulin secretion. Gene Ther. 1999;6:1160-9. PMID: 10455420

43. Klein D, Ribeiro MM, Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, Molano RD, Inverardi L, Ricordi C, Pastori RL. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun. 2004;323:473-8. PMID: 15369775

44. Sakata N, Aoki T, Yoshimatsu G, Tsuchiya H, Hata T, Katayose Y, Egawa S, Unno M. Strategy for clinical setting in intramuscular and subcutaneous islet transplantation. Diabetes Metab Res Rev. 2014;30:1-10. PMID: 24000195

45. Wang J, Liao L, Wang S, Tan J. Cell therapy with autologous mesenchymal stem cells-how the disease process impacts clinical considerations. Cytotherapy. 2013;15:893-904. PMID: 23751203

46. Hayward JA, Ellis CE, Seeberger K, Lee T, Salama B, Mulet-Sierra A, Kuppan P, Adesida

A, Korbutt GS. Cotransplantation of mesenchymal stem cells with neonatal porcine islets improve graft function in diabetic mice. Diabetes. 2017;66:1312-21. PMID: 28246290

47. Yamashita S, Ohashi K, Utoh R, Kin T, Shapiro AM, Yamamoto M, Gotoh M, Okano T. Quality of air-transported human islets for single islet cell preparations. Cell Med. 2013;6:33-8. PMID: 26858878

Figure legends

Figure 1. (a) The morphological integrity of the human islets. (b) Human islets stained with dithizone for characterization. Scale bar: 100 μm. (c) A whole image of shipped human islets with dithizone. Scale bar: 500 μm.

Figure 2. (a) After 3 days of co-culturing islets with a base cell sheet, cell sheets composed of islets and supporting cells were detached from the dish by reducing the temperature to 20°C. Scale bar: 10 mm. (b) H&E staining of a detached cell sheet composed of human islets (*black arrow*) and ADSCs (*white arrow*). Scale bar: 100 μm. (c) Insulin staining was performed to confirm the presence of β cells within cell sheets. Insulin-positive cells within the islets were detected on cell sheets composed of human islets (*black arrow*) and ADSCs (*white arrow*). Scale bar: 100 μm.

Figure 3. The islet viability. (a)The ADSC group showed the highest viability (76.1%±4.5%); however, the value did not differ from that of the fibroblast group to a statistically significant extent (63.9%±3.2%). *p<0.05. (b) Representative merged images of the viability staining by calcein-AM and PI. Viable cells were stained green and dead cells were stained red. (c) The majority of cells located in the central area of the islets were dead.

Figure 4. The islet recovery rate. The islet recovery rates of the ADSC (81.2±4.1%) and fibroblast sheet (78.5±3.9%) groups were similar and higher than in the islet-alone group at 3 days after cultivation. *p<0.05.

Figure 5. Glucose-stimulated insulin release. The stimulation index values of all cell sheet groups were significantly higher than that of the islet-alone group. No significant differences were observed in the stimulation index values of the sheet groups. The stimulation index values of the islet-alone, fibroblast, BM-MSC, and ADSC groups were 0.11±0.04, 0.44±0.08, 0.64±0.09, and 0.44±0.07, respectively. *p<0.05.

Figure 6. The TGF-β1 levels. The fibroblast group showed significantly higher TGF-β1 levels (2.17±0.05 ng/ml) than the other groups (p<0.05). The TGF-β1 levels of the islet-alone, fibroblast, BM-MSC, and ADSC groups were 0.61±0.01, 2.17±0.05, 1.66±0.10, and 1.63±0.10 ng/ml, respectively. *p<0.05.

Figure 7. The IL-6 levels. The ADSC group showed significantly higher IL-6 levels (228.0±19.4 ng/ml) than the other groups. The IL-6 levels of the islet-alone, fibroblast, BM-MSC, and ADSC groups were 2.73±0.3, 20.9±2.1, 46.3±4.2, and 228.0±19.4 ng/ml, respectively. *p<0.05.

Figure 8. The VEGF levels. The ADSC group showed the highest VEGF secretion level (6.6±0.75 ng/ml); however, the level was not significantly different from that in the BM-MSC group (5.4±0.31 ng/ml). The VEGF levels of the islet-alone, fibroblast, BM-MSC, and ADSC groups were 1.15±0.15, 2.2±0.09, 5.4±0.31, and 6.6±0.75 ng/ml, respectively. *p<0.05.

Table 1. Characteristics of the transported human islets

No.	Age	Sex	Cause of death	Cold ischemic time (hours)	Transported islet equivalent (IEQ)	Viability(%)	Purity(%)
1	32	M	Anoxia (Donation after cardiac death)	6.5	13546	85	30
2	66	F	Cerebrovascular accident	11.5	15517	95.5	40
3	25	M	Anoxia	13	12524	84.5	30
4	56	F	Cerebrovascular accident	14.25	14549	98	40
5	36	F	Anoxia	10.75	18577	78.5	30

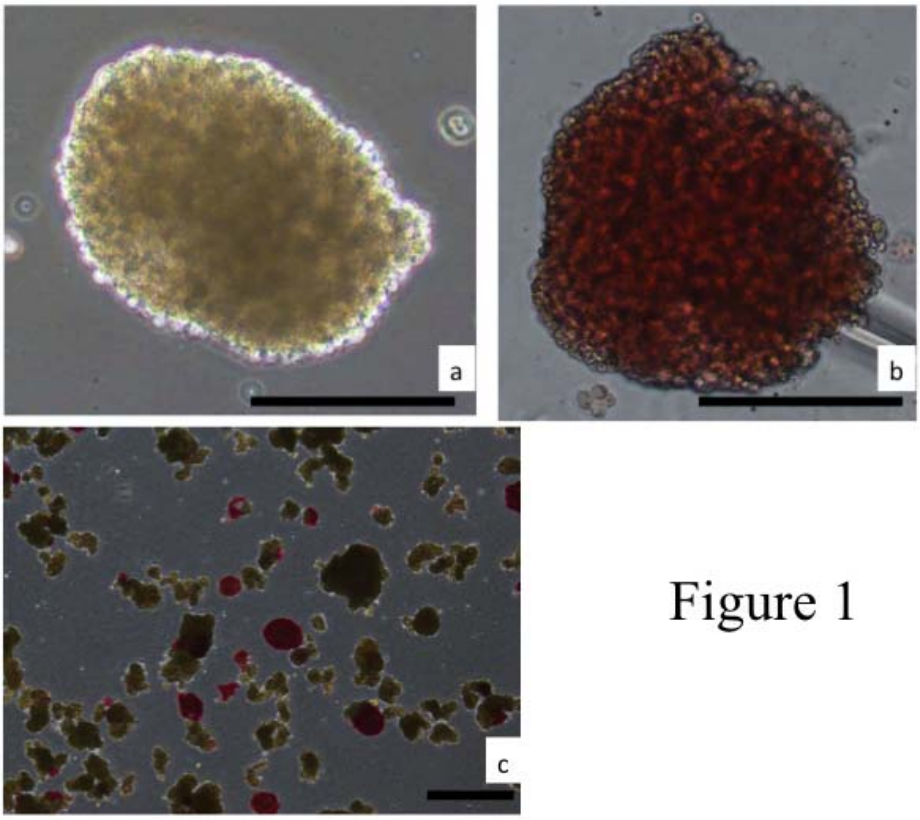


Figure 1

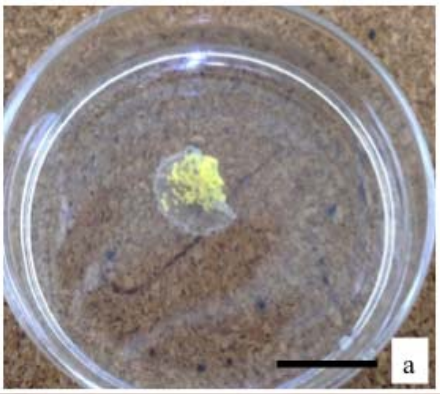
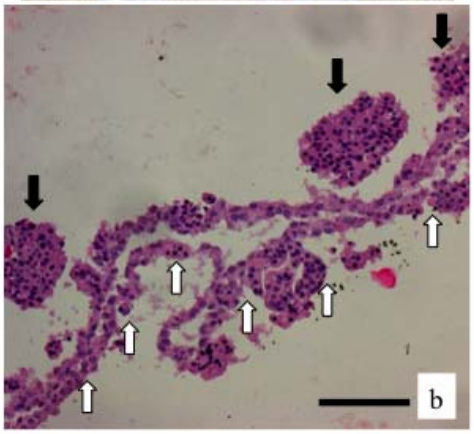


Figure 2



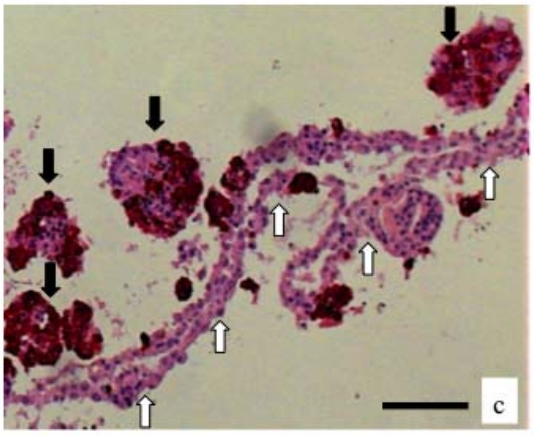
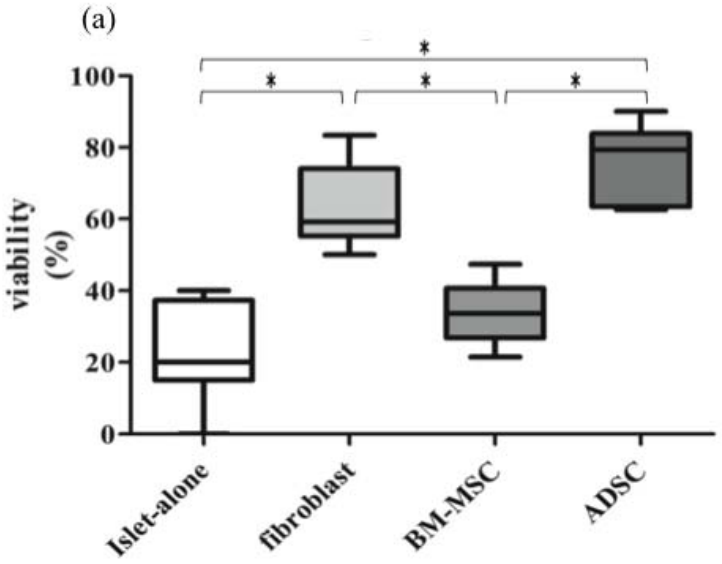
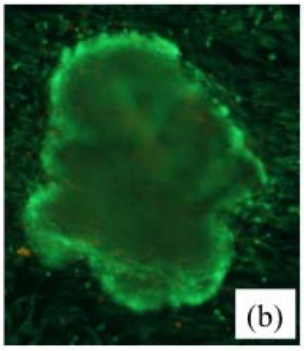


Figure 3


(a)
viability (%)
100
80
60
40
20
0
*
*
*
*
Islet-alone
fibroblast
BM-MSC
ADSC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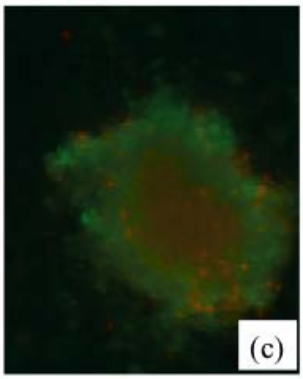
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Figure 4

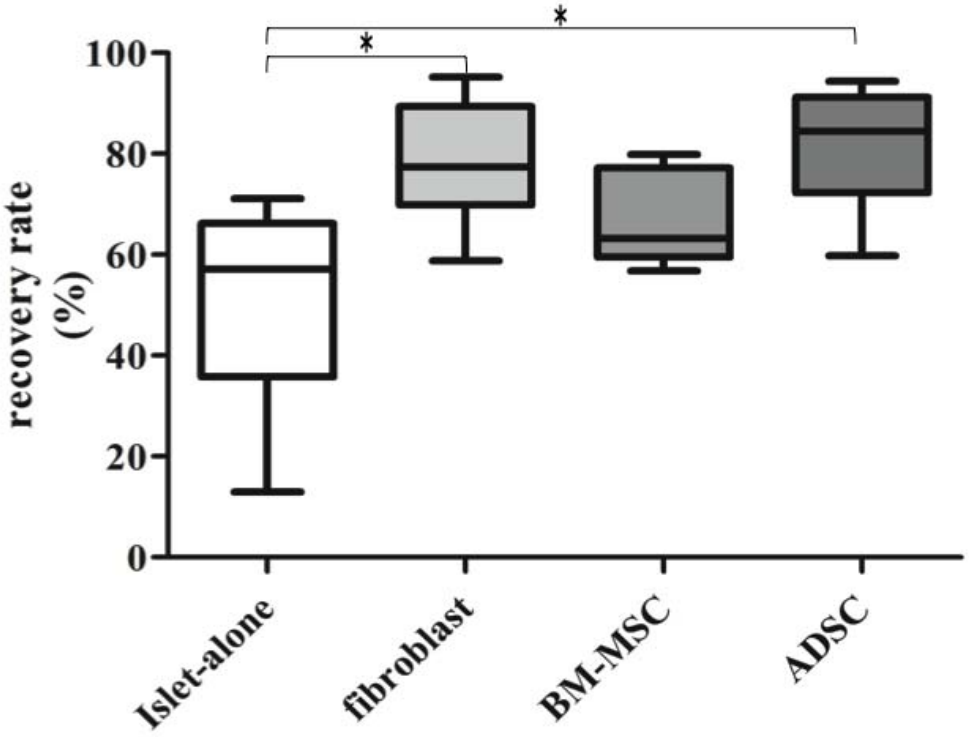
*
*
100
80
60
40
20
0
recovery rate (%)
Islet-alone
fibroblast
BM-MSC
ADSC

Figure 5

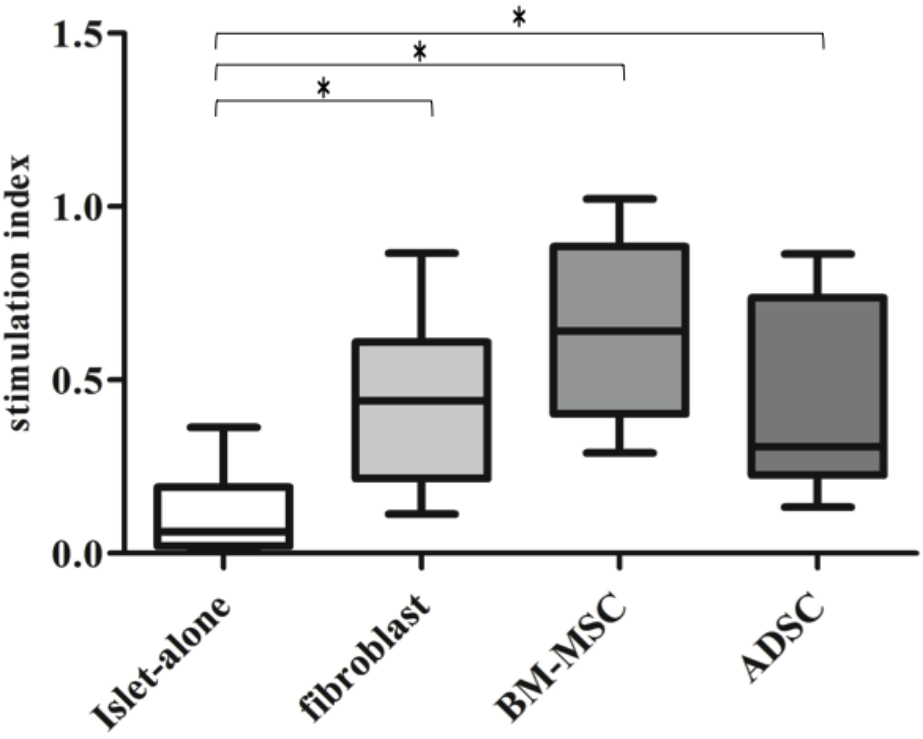
stimulation index
1.5
1.0
0.5
0.0
*
*
*
Islet-alone
fibroblast
BM-MSC
ADSC

Figure 6

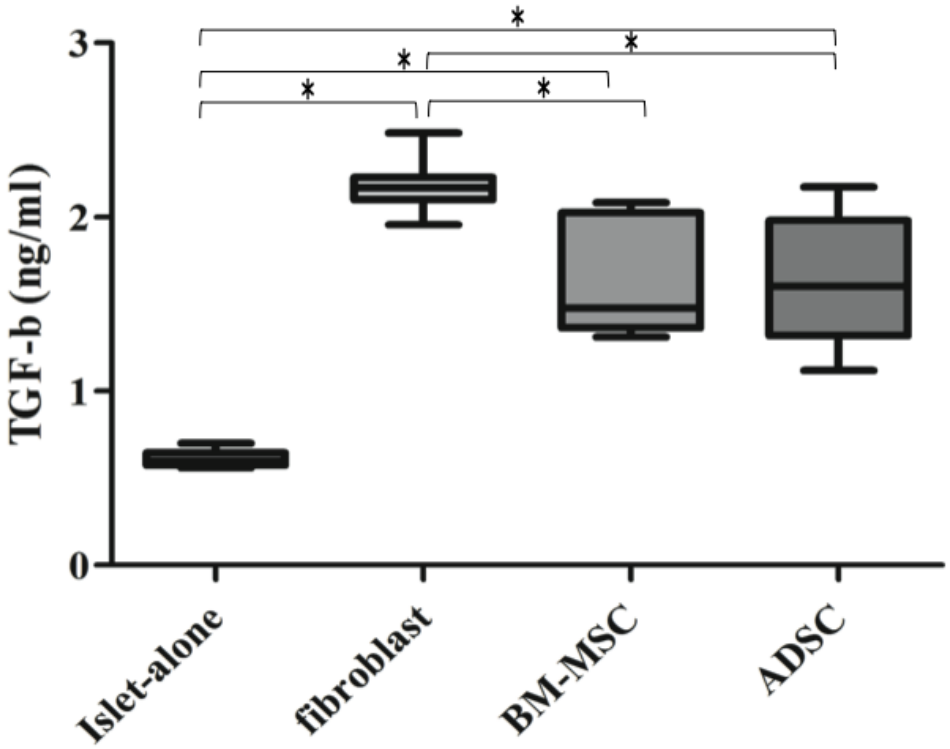

Figure 7

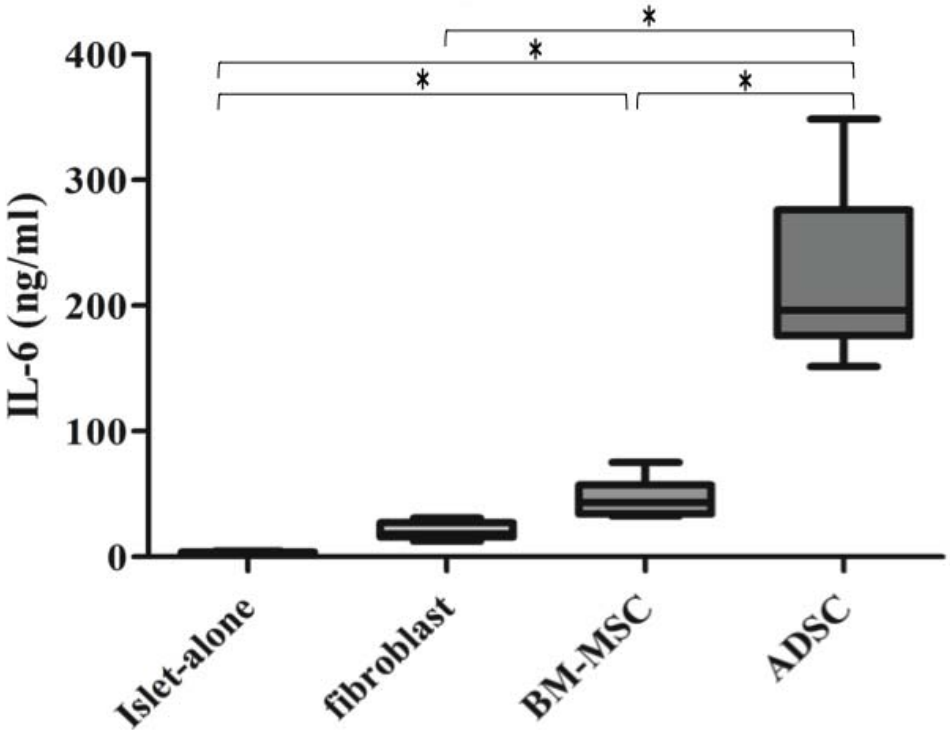

Figure 8