

MESENCHYMAL STEM CELLS PROMOTE ISLET SURVIVAL IN VITRO AND FUNCTION IN VIVO

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ABSTRACT

Background: Transplanted islets may undergo primary graft non-function for various reasons. The aim of this study was to evaluate whether mesenchymal stem cells (MSC) could improve the viability of islets *in vitro* co-culture simultaneously and whether co-transplantation of MSC with islets could improve the function of islets *in vivo*.

Materials and Methods: Rat islets were cultured in either the presence or absence of rat MSC *in vitro*. Cell viability was evaluated by acridine orange/propidium iodide staining. Cell function was evaluated by glucose-stimulated insulin secretion assay and confocal fluorescence imaging. Graft function was evaluated after co-transplantation of MSC with islets in a rat islet allograft model.

Results: In the presence of MSC, 80% of the islets were still alive at day 14, while only 23% were alive when islets were cultured alone ($p < 0.01$). The insulin secretory responses to 16.7 mM glucose were significantly higher in the co-culture group when compared to islets cultured alone ($p < 0.05$). Two-photon and confocal fluorescence imaging showed that the whole-cell NAD(P)H fluorescence was >2-fold higher in the co-culture group than in the islets cultured alone group. In the rat islet allograft model, normal fasting blood glucose levels for the 1×10^6 MSCs+1000 islets group, 1×10^6 MSC+500 islets group, and the 1000 islets alone group were obtained for (21.0 ± 1.7) days, (15 ± 1.3) days, and

(10.2 ± 2.0) days; respectively.

Discussion: Our data suggest that MSC, when they were co-cultured simultaneously with islets, may promote islet survival and *in vitro* can improve the function of islets and markedly reduce the islet mass required in rat model.

INTRODUCTION

Type 1 diabetes remains a therapeutic challenge. Insulin injection therapy has been the standard treatment for type 1 diabetes since the discovery of the hormone more than 80 years ago. Although intensive diabetes treatment with insulin reduces incidence and delays progression of long-term complications, such benefits of intensive diabetes treatment come with the an increased risk of severe hypoglycemia. Islet transplantation has recently shown improved potential for treatment of type 1 diabetes¹. In 2000, using a glucocorticoid-free immunosuppressive protocol that included sirolimus, low-dose tacrolimus, and a monoclonal antibody against the interleukin-2 receptor (daclizumab), Shapiro et al² carried out islet transplantation alone for seven patients with T1DM and a history of severe hypoglycemia and metabolic instability. The expansion of clinical trials in the post-Edmonton era indicates that pancreatic islet transplantation may finally be considered an effective alternative treatment for the most severe cases of Type 1 in diabetes³⁻¹⁷.

Despite such successes, transplanted islets may undergo primary graft non-function for various reasons, such as loss of islet viability during isolation and culture, insufficient nutrient and oxygen supply due to altered islet vasculature, inflammation and immune-mediated destruction, and the toxic effects

of immunosuppressive agents^{18,19}. Therefore, great efforts have been made to develop new methods that can improve the viability of isolated islets and prolong their survival after transplantation with respect of islet shortage²⁰⁻²³.

Mesenchymal stem cells (MSC) are rare residents of the bone marrow microenvironment and have been reported to differentiate into cells of bone, adipose tissue, and cartilage²⁴. Interestingly MSC can inhibit different effector functions of immune cell populations including T cells, B cells, dendritic cells (DC), and natural killer cells *in vitro*. MSC can also down regulate immune response *in vivo* and promote the survival of allogeneic skin transplant in animals²⁵⁻²⁷. In addition, MSC have been shown to have renoprotective effects and enhance regeneration after acute kidney injury²⁸.

Inflammation and immune-mediated destruction are the prominent factors contributing to primary non-function of transplanted islets but human MSC can protect human islets from pro-inflammatory cytokine²⁹, promote graft revascularization³⁰⁻³², and modulate immune cells³³. Rackham et al³⁴ reported that pre-culturing islets using a direct contact with pre prepared MSC monolayer could improve glucose-stimulated insulin secretion *in vitro*, which correlated with superior islet graft function *in vivo*.

Subsequently, we hypothesized that MSC may promote islet survival when they are co-cultured simultaneously without a pre-prepared MSC monolayer *in vitro*. Accordingly, the current study was designed to investigate whether co-culture of MSC with islets simultaneously could enhance islet survival *in vitro* and whether co-transplantation of MSC with islet simultaneously could promote their survival *in vivo*, as indicated by islet function after transplantation.

MATERIALS AND METHODS

ANIMALS

Male Sprague Dawley (SD) rats and Lewis rats (8-10 weeks old; body weight, 250-350 g) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. All rats were bred and maintained under specific pathogen-free conditions. The animal handling and experimental procedures were approved by the Animal Care and Use Committee of our hospital.

ISOLATION AND PURIFICATION OF MSC

Bone marrow (BM) was obtained from SD rats. Briefly, rats were scarified, and femurs and tibiae

were aseptically removed. Bone marrow was flushed from the shaft of the bone with DMEM medium containing 0.1% fetal bovine serum (FBS, Hyclone) plus penicillin/streptomycin and then filtered through a 200- μ m sterile filter to produce a single-cell suspension. Mononuclear cells were isolated by Ficoll-Paque (Sigma-Aldrich, USA) density-gradient centrifugation from the filtered BM cells. Cells were then plated in DMEM plus 10% FBS and penicillin (100 U/ml)-streptomycin (0.1 mg/ml) and allowed to adhere for 48 h. Non-adherent cells were then removed. Medium was changed regularly every 3 d. At 80% confluence, cells were harvested with 0.25% trypsin and 0.02% EDTA and re-plated in the culture. Cells of passages 4-6 were used for both *in vitro* and *in vivo* experiments. Cells were phenotypically characterized by flow cytometry and their differentiation potential was evaluated by alizarin red and oil red O stained.

ISLET PREPARATION AND CULTURE WITH OR WITHOUT MSC

Male Lewis rats were used as islet donors. Islets were isolated as previously described by Ricordi and Rastellini with some modifications³⁵. Briefly, rats were scarified with a cut in the heart under pentobarbital anesthesia (50 mg/kg, i.p.). A midline abdominal incision was made to expose the pancreas. The pancreatic duct was injected with 8-10 ml of 1 mg/ml collagenase P (Roche Molecular Biochemicals, Indianapolis, IN) in Hank's buffered saline solution (HBSS) (Farco Chemical Supplies, Beijing, China). The pancreas was surgically removed and incubated at 37 °C for 10 min, and then passed through a 500- μ m mesh. The digested pancreas was rinsed with HBSS, and islets were separated by density gradient in Ficoll400 (Pharmacia). Islets were extensively washed in sterile HBSS and cultured in RPMI 1640 medium (GIBCO) supplemented with 0.2% NaHCO₃, 1% (v/v) penicillin/-streptomycin, 5.6 mM D-glucose, and 2% fetal bovine serum (pH 7.4). Islets of approximately equal size (200-250 μ m in diameter) were handpicked and placed in the 6-well plate. To standardize the islet mass for transplantation, one islet equivalent (IEQ) was defined as one 125- μ m-diameter islet. Approximately 30 islets/well were maintained at 37 °C in a humidified atmosphere with 5% CO₂ for 20 days in either the presence or absence of MSC. The medium was changed every 24 h. In the islet alone group, 30 islets per well were cultured without MSC. In the co-culture group, 5 \times 10⁴ MSCs

were plated together with 30 islets per well.

ISLET VIABILITY ANALYSIS

Islets viability was determined with acridine orange (AO)/propidium iodide (PI) staining. The numbers of AO and PI positive islets were counted under a fluorescence microscope. The percentage of live islets (survival rate) was determined by the number of AO-positive islets divided by the sum of PI and AO-positive islets.

GLUCOSE-STIMULATED INSULIN RELEASE ASSAYS

At days 1, 3 and 7 of the culture, the islets were evaluated for their glucose-stimulated insulin secretion. The assays were performed in triplicates as described previously³⁶. For both islets alone and islet + MSC group, survival islets, which were intact under microscope, were handpicked to eppendorf tubes. Each tube contained 30 islets and 400 μ l serum- and glucose-free RPMI 1640 medium. The tubes were kept at 37 °C for 30 min, and then the medium was completely removed by centrifugation (1,200 rpm, 2 min) and replaced with serum-free RPMI 1640 medium supplemented with 2.8 mM glucose. Following a 60-min incubation, the medium was completely removed and kept at -20 °C for assay. These eppendorf tubes with islets were then incubated with serum-free RPMI 1640 containing 16.7 mM glucose for an additional 60-min. After a 60-min incubation, the medium was removed and kept at -20 °C for assay. Insulin concentrations in the media samples were measured by immunoradiometric assay. The amount of insulin secreted at high glucose (16.7 mM) was divided by the amount at low glucose (2.8 mM), and the mean insulin stimulation index was calculated.

TWO-PHOTON AND CONFOCAL FLUORESCENCE IMAGING

Islets were handpicked and placed on 35-mm glass dishes (MatTek Corp, USA). In the islet + MSC group, 5×10^4 MSCs were plated together with 30 islets per dish. In the islet alone group, islets at 30 islets per dish were plated without MSC. They were cultured in RPMI 1640 media supplemented with 11 mM glucose, 200 mM Hepes and 10% FBS (Hyclone) at 37°C in a humidified atmosphere containing 5% CO₂.

At day 7 the islets were placed on two-photon excitation microscopy. Two-photon imaging of the intensity NAD(P)H autofluorescence was performed on a Zeiss LSM 510 META laser scanning microscopy with 150-fsec pulses of 710-nm light from a Coherent Mira laser focused through a Plan-Apochromat 63 \times

(N.A.=1.4) oil immersion objective (Zeiss).

ISLET TRANSPLANTATION

Diabetes was induced in SD rats by the intravenous injection of streptozocin (60 mg/kg body weight). Blood glucose levels were monitored daily from day 3 in the rats by using the Accu-Chek Advantage glucometer (Roche). Rats with fasting glucose level greater than 16.7 mmol/L for 5 continuous days were used. Islets of approximately equal size (200-250 μ m in diameter) were handpicked for transplantation. The diabetic rats were randomly allocated to 4 groups. Rats in groups 1, 2, and 3 received 1000 islets + 1×10^6 MSC (n=8), 500 islets + 1×10^6 MSC (n=8), and 1000 islets (n=8), respectively through portal vein to the liver. Rats in group 4 received saline injection and served as a control (n=5). After transplantation, samples for fasting blood glucose and insulin levels were measured at days 5, 10, 20, and 30, respectively.

GRAFT FUNCTIONAL OUTCOME ANALYSIS

The primary end point of the experiment was the number of days normoglycemia was maintained. The islet lost function was defined as fasting blood glucose levels >16.0 mM.

STATISTICAL ANALYSIS.

Statistical analyses were performed using SPSS 13.0 for Windows (Chicago, IL, USA). Kaplan-Meier analysis was performed in each group. Differences between groups were tested using the log-rank statistic. Data are presented as the means \pm SD in each group. Data were analyzed using univariate analysis. If the F ratios were significant, post hoc tests were applied to assess significance. Results were considered significantly different if $p < 0.05$.

RESULTS

MSC AND ISLET PREPARATION

MSC were prepared as previously described. Characterization of MSC in culture was analyzed by flow cytometry and differentiation tests. Results showed that the isolated MSC were negative for CD34, CD14 and CD45, while positive for CD105, CD73 and CD29 (Figure 1 A). They could be readily induced to differentiate into osteoblast and adipose cells (Figure 1 B, C). The islets of rats were round, and blood-red with staining of DTZ (Figure 1 D). Diameters were 50-200 μ m. The range of the number of islets was 330-550 islets/rat. Islet purity was over 95%.

MSC ENHANCE ISLET SURVIVAL IN VITRO

Approximately, 30 islets/well were cultured in ei-

ther the presence or absence of MSCs. When islets were cultured alone, most of them died at day 14 as assayed by AO-PI staining (Figure 2, A). In the presence of MSC, 80% of the islets were still alive at day 14, while only 23% were still alive in the islet alone group ($p < 0.05$). The islet viability rates were significantly higher in the islet + MSC group than those without MSC at each time points ($p < 0.05$) (Figure 2, B).

INSULIN SECRETORY ACTIVITY

To evaluate the insulin secretory activity of islets, the glucose stimulated insulin secretion (GSIS) in a two-hour static incubation assay at 2.8 mM and 16.7 mM was used. We compared the insulin secretion stimulating index (insulin secretion at 16.7 mM divided by insulin secretion at 2.8 mM) between the islet + MSC and the islet alone groups. The insulin secretion response to high glucose gradually decreased from day 1 to day 7 in both groups. Stimulating index (SI) was 3.1 ± 0.52 and 2.0 ± 0.44 at days 1 and 3, respectively in the islet alone group, and it was 3.3 ± 0.62 and

2.5 ± 0.51 at days 1 and 3, respectively in the islet + MSC group ($n=3$, $p > 0.05$; Table 1). However, SI of 1.6 ± 0.36 in the islet + MSC group was significantly higher than SI of 1.0 ± 0.08 in the islet alone group at day 7 ($n=3$, $p < 0.05$; Table 1).

NAD(P)H RESPONSE IN PANCREATIC β CELLS

Glucose-induced insulin secretion is coupled to the metabolic state of the β -cells. After glucose is transported into the cell, glucose is phosphorylated and shunted into glycolysis, which increases metabolic flux. This altered metabolic state can be detected by NAD(P)H's autofluorescences which increases and reflects the functional activity of islets^{37,38}. Two-photon and confocal fluorescence imaging showed that the whole-cell NAD(P)H fluorescence was >2-fold higher in the islet + MSC group than in the islet alone group by quantitative image analysis (data was not showed), indicating a better function of

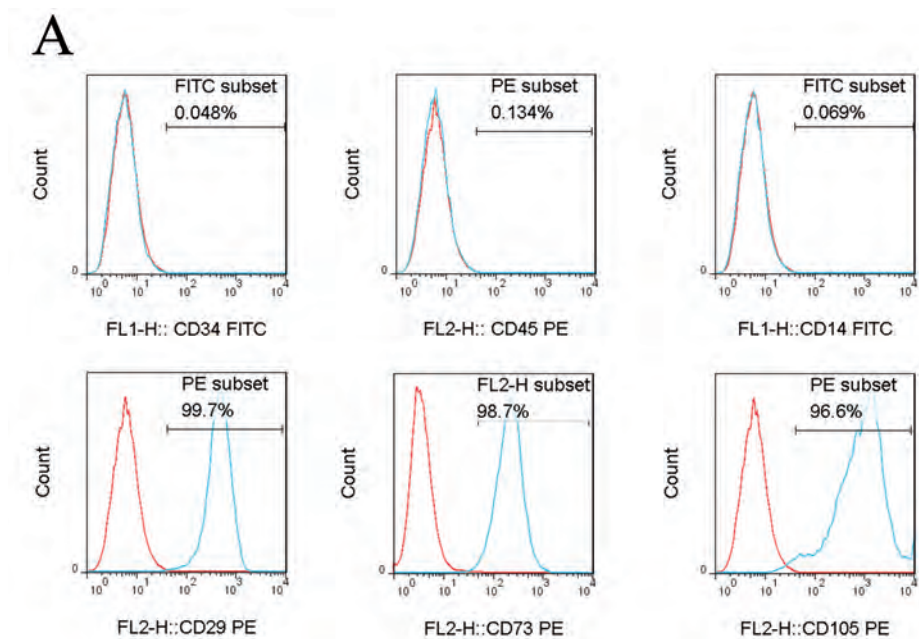
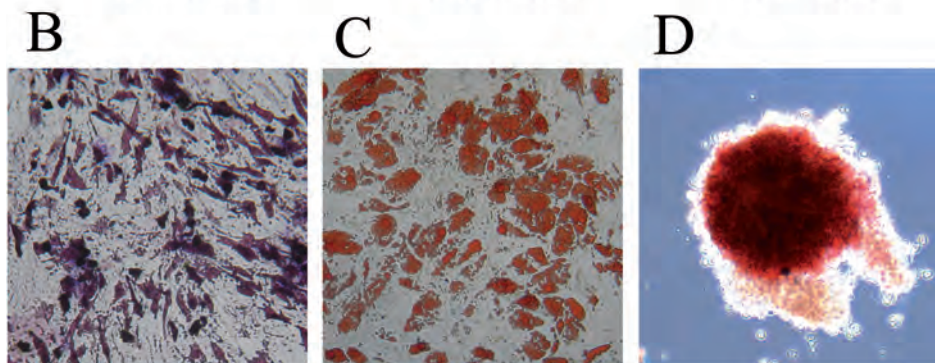


Figure 1. MSCs and islets preparation and identification. *A*, Flow cytometry identification of rat bone marrow MSCs, positive for CD29, CD73, and CD105, negative for CD34, CD45, and CD14. *B*, MSCs can be induced to differentiate into osteoblast (alizarin red stained) and (*C*) adipose cells (oil red O stained). *D*, The islet of rat with staining of DTZ.



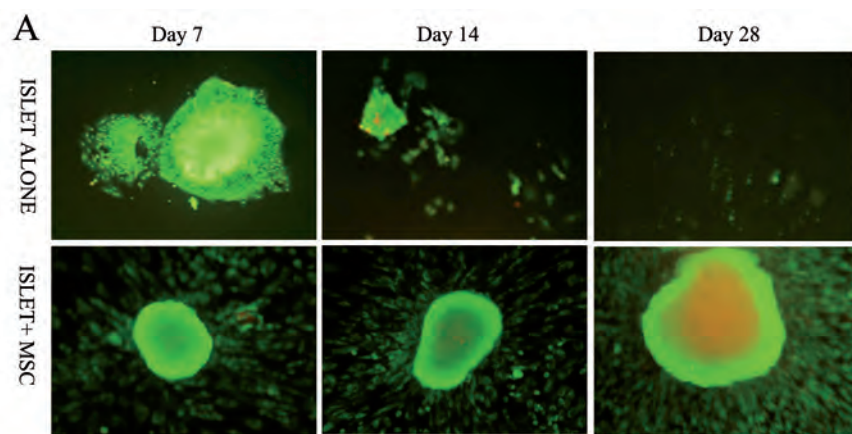


Figure 2. MSCs enhances survival of isolated rat islets. Islets of approximately equal size (200-250 μm in diameter) were handpicked and placed in the 6-well plate. The medium was renewed every 48 h. In co-culture group, MSCs at 5×10^4 cells were plated together with islets at 30 islets per well. *A*, Representative examples of AO (green)/PI (red) staining in both groups. Magnification, $\times 100$. *B*, Cell survival rate (percentage of AO-positive cells) decreased with time in both groups, but it was higher in the co-culture group for each time point ($p < 0.01$).

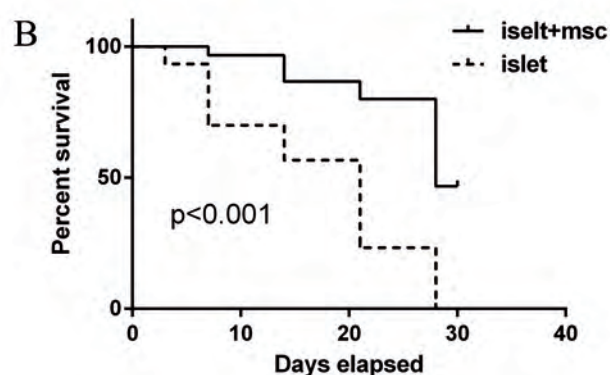


TABLE 1 Insulin secretory responses of islets to glucose challenge (expressed as mg/mL)

Duration of culture (day)	Islet alone (n=3)			Islet and MSC (day)			P
	Basal glucose	Elevated glucose	SI	Basal glucose	Elevated glucose	SI	
1	11.9 \pm 1.6	36.3 \pm 3.0	3.1 \pm 0.52	11.2 \pm 1.8	36.7 \pm 3.1	3.3 \pm 0.62	0.595
3	14.2 \pm 1.7	28.6 \pm 2.7	2.0 \pm 0.44	13.5 \pm 2.2	33.2 \pm 3.4	2.5 \pm 0.51	0.156
7	16.9 \pm 2.0	16.7 \pm 1.3	1.0 \pm 0.08	15.5 \pm 2.1	24.6 \pm 2.9	1.6 \pm 0.36	0.03

P values were compared with islet alone SI. SI=stimulation index

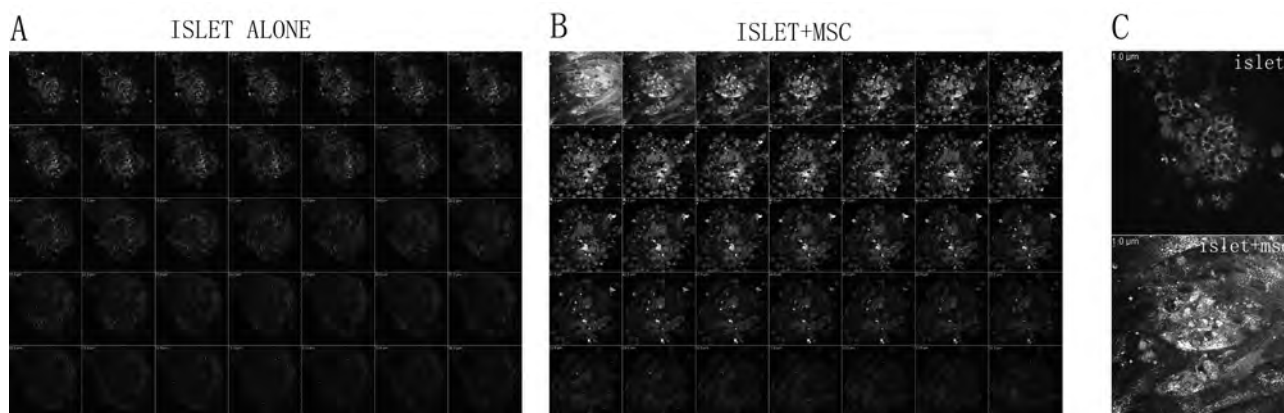


Figure 3. Imaging of NAD(P)H auto fluorescence of islets with or without MSC. Islets were imaged with a 710-nm (two-photon) excitation microscope in the absence (*A*) or in the presence of MSC (*B*). The same section was magnified (*C*).

islets cultured in the presence of MSC (Figure 3).
CO-TRANSPLANTATION OF MSC AND ISLETS

Having established that MSC can enhance islet function and survival *in vitro*, we tested next whether co-transplantation of MSC and islets could promote islet function in an allogeneic islet transplant model. Glucose monitoring was begun the day after transplantation. As shown in Figure 4 normal fasting blood glucose levels were obtained for (21.0±1.7) days, (15±1.3) days, and (10.2±2.0) days in the 1×10⁶ MSCs + 1000 islets group, 1×10⁶ MSCs + 500 islets group, and 1000 islets alone group, respectively.

As an additional indicator for functional islets at post-transplant, fasting serum insulin levels were measured at 0, 5, 10, 20 and 30 days of post-transplantation. Data in Figure 5 illustrates that animals of islet co-transplanted with MSC had higher fasting serum insulin levels (μU/ml) of (13.54±0.36), (13.81±0.29), (6.03±0.97), and (3.62±0.43) in the MSCs + 1000 islets (group 1) and of (9.52±0.32), (10.18±0.37), (3.89±0.30), and (2.04±0.06) in the MSC + 500 islets (group 2) at post-transplant days 5, 10, 20 and 30, respectively, while compared to the fasting serum insulin levels of (12.43±0.24), (5.30±0.54), (2.94±0.54), and (2.04±0.12) in the 1000 islets alone (group 3) and of (2.10±0.08), (2.11±0.07), (2.09±0.07), and (1.86±0.14) in the control (group 4) at post-transplant days 5, 10, 20 and 30, respectively. Fasting serum insulin levels in the MSC + 1000 islets (group 1) was significantly higher than in the 1000 islets alone (group 3) ($p < 0.001$) and in the control (group 4) ($p < 0.001$) at each time point.

DISCUSSION

The primary findings of the present study are MSC can

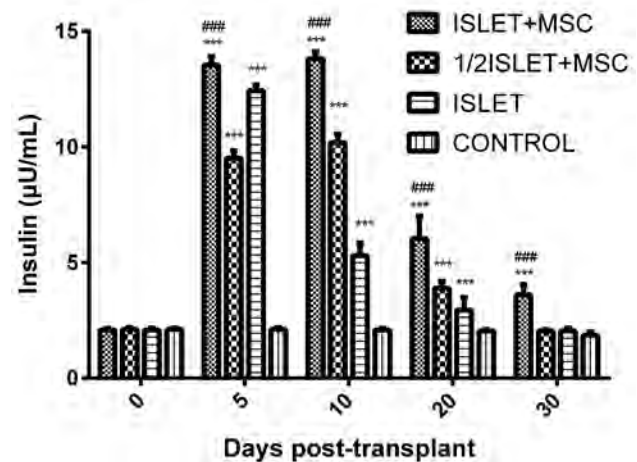


Figure 5. MSC improves graft function. Fasting serum insulin levels were measured as a marker of islet graft function. Rats in Group 1 (received 1000 islets with 1×10⁶ MSCs) had significantly higher insulin levels compared with rats in Group 3 (received 1000 islets alone) (### $p < 0.001$) and controls group (received saline) (***) ($p < 0.001$) at each time points.

promote survival of islets *in vitro* in a co-culture simultaneously system without the need of pre-prepared MSC monolayers, which is different from the previous report³⁴, and that most importantly, co-transplantation of MSC and islets simultaneously resulted in a significant improvement of islet allograft engraftment.

MSC are pluripotent cells capable of differentiation into many cell types. Furthermore, β cell-specific differentiation has been reported^{39,40}. MSC are particularly appealing to us in islet transplantation because these cells have immunomodulatory properties and the ability to protect cells from apoptosis. Moreover, they can be easily obtained from

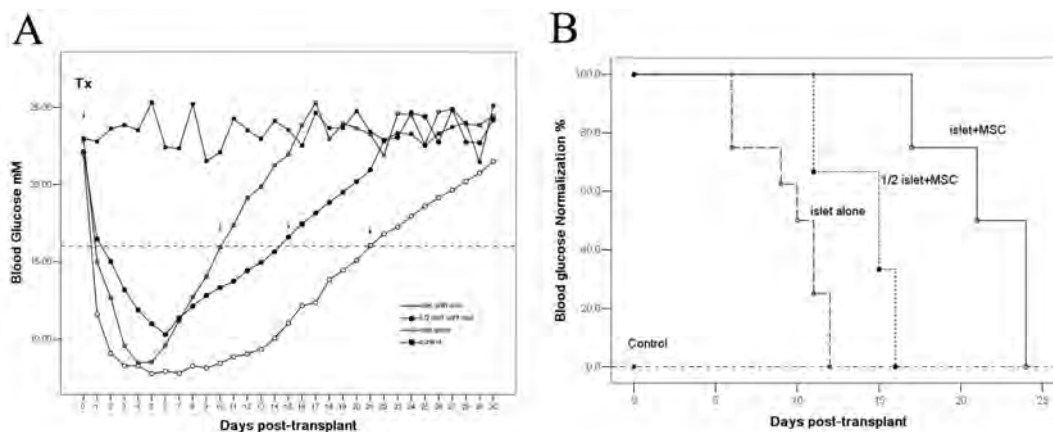


Figure 4. Co-transplantation of MSC improves islet function in STZ-treated rats. STZ-treated diabetic rat received transplant of islets on day 0. **A**, Fasting blood glucose levels in transplant recipients ($n = 8$ for each group). **B**, Blood glucose normalization curves for transplant recipients, expressed as percentage of animals normalized. The day of islet lost function was considered where blood glucose was >16 mM.

bone marrow and expanded in culture.

Islets transplanted often fail to function (primary non-function) because of various reasons, including immunologic rejection, hypoxia, pro-inflammatory cytokines, and free radicals released from macrophages in the microenvironment of the transplanted islet, that will finally result in islet β -cell apoptosis⁴¹⁻⁴⁴. Therefore, most of the recipients have to be transplanted with islets from more than one donor². Different methods have been tried to promote islet survival in order to meet this clinical needs. For example, *ex vivo* gene transfer of *bcl-2*, an antiapoptotic gene, was cytoprotective for transplanted islets⁴⁵. Gunther showed that carbon monoxide, a product of heme oxygenase activity, protects β -cells from apoptosis through guanylate cyclase activation and when mouse islets were exposed to CO for 2 h they functioned significantly better after transplantation than islets not exposed to CO⁴⁶.

In this study we tested the effects of MSC on the survival of islets based on the following facts: (i) MSC have been shown to secrete a lot of growth factors that may favor the survival of cells²⁹. (ii) MSC are capable of exerting a strong immune suppression^{33,47}. (iii) MSC have been shown to prevent cell apoptosis³⁰⁻³².

In a severe hyperglycemia model produced in NOD/scid mice with daily low doses of streptozotocin, Lee et al. showed that intracardiac infusion of MSC lowered blood glucose levels in the diabetic mice⁴⁸. In the MSC-treated diabetic mice, there were increases in pancreatic islets and β cells producing mouse insulin. Very recently Ding et al. reported that administration of MSC significantly reduced delayed-type hypersensitivity responses to allogeneic antigen and profoundly prolonged the survival of fully allogeneic islet grafts in transplant recipients⁴⁹. They found that MSC can secrete matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9 that reduce surface expression of CD25 on responding T-cells and suppresses effects of MSC on T-cell responses to alloantigen. Interestingly, we also found lower blood glucose levels in rats receiving only MSC transplantation when compared to the control group, indicating that MSC may promote islet regeneration after streptozotocin treatment (data not shown). Our results are consistent with findings in previous reports including a cynomolgus monkey model of islet/bone marrow transplantation, where Berman et al⁵⁰ demonstrated that MSC co-transplantation significantly enhanced islet engraftment and function at 1 month post-transplant compared with animals that received islets without MSCs. Addi-

tional infusions of donor or third-party MSCs might also decrease the risk of rejection. The authors also found that stable islet allograft function was associated with increased numbers of regulatory T-cells in peripheral blood, although it was not clear whether this was a direct effect of the MSC infusion. Similar results were obtained when syngeneic MSC were co-transplanted with islets into an omental pouch in a streptozotocin-induced diabetic rat model⁵¹.

More recently, several innovations have been developed to further facilitate islet transplantation. Kerby et al⁵² co-encapsulated mouse islets and kidney MSC in alginate capsules and transplanted the capsules intraperitoneally into diabetic mice. They showed that MSCs could improve graft outcome in this microencapsulated/isolated-graft model of islet transplantation. Luo et al⁵³ co-cultured human islets and bone marrow (BM) to support human islet survival and function after transplantation into the left subrenal capsule of diabetic severe combined immunodeficient mice. The co-cultured human islets with MSC before transplantation demonstrated improved viability, increased size, and migration capacity *in vitro*. After 4 months, animals transplanted with precultured BM/islets exhibited euglycemia and detectable human insulin levels (157 μ U/mL), whereas no human insulin was detected in the islet-only transplanted group. Because BM is readily available from the patients, it may represent an alternative cell source for MSC to enhance the survival and function of transplanted islets.

The limitations of this study include not addressing the paracrine actions contributed to the protective effects of MSC in β cells due to difficulties in collecting sufficient transplanted islets for analysis. Other investigators⁵⁴ have found that MSC improve cardiac function after myocardial infarction or kidney function after acute renal injuries through secreting angiogenic and antiapoptotic factors. Secondly, we did not evaluate whether the infused MSC contributed, via differentiation and engraftment, to the cells of islets. Nevertheless, as autologous MSC have been shown to be safe when they are systemically infused and MSC can be produced in large quantity with an aspiration from bone marrow, MSC might be a useful therapeutic tool for improving islet transplantation in humans⁵⁵.

In conclusion, we have shown that exposure of rat islets to MSC enhance their survival *in vitro*, and most importantly, co-transplantation of MSC and islets resulted in significantly improved functional performance after transplantation to a diabetic allogeneic recipient. Treatment with adult MSC offers a new approach to promote β -cell survival in addition to pharmacological

interventions. Further study is needed to fully characterize the integration of MSC with transplanted islets, optimal cell number, and proper delivery technique.

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