Bone Marrow-Derived Mesenchymal Stem Cells Protect Islet Grafts Against Endoplasmic Reticulum Stress-Induced Apoptosis During the Early Stage After Transplantation

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Key Words. Islet • Transplantation • Apoptosis • Endoplasmic reticulum stress • Bone marrow-derived mesenchymal stem cells

ABSTRACT

Early loss of grafted islets is the main obstacle to achieve favorable outcomes of islet transplantation. Mesenchymal stem cells are known to have a protective effect; however, its mechanism remains unclear. We hypothesized that bone marrow-derived mesenchymal stem cells (BMSCs) can protect grafted islets against endoplasmic reticulum stress (ERS)-induced apoptosis. In syngeneic streptozocin-induced diabetic BALB/c mice, islet grafts decreased blood glucose levels; however, the effect was not fully functional from the immediate post-transplant phase. β-Cell apoptosis was proven on days 1 and 3 after transplantation. Ultra-structural evidence of ERS was observed along with increased expressions of marker protein BIP and apoptosis-related protein CHOP. In contrast, BMSC co-transplantation maintained glucose hemostasis, inhibited apoptosis and alleviated ERS. In ex vivo culture, BMSCs improved viability of islets and decreased apoptosis. Increased ERS were observed in cultured islets exposed to hypoxia, but not in the islets cocultured with BMSCs. Furthermore, cocultured BMSCs protected islets against ERS-induced apoptosis as well as improved their insulin secretion, and BMSCs alleviated ERS by improving Myc expression through both stromal cell-derived factor 1 signal and contact effect. In conclusion, BMSCs protected the grafted islets against ERS-induced apoptosis during the early stage after transplantation. This study opens a new arena for ERS-targeted therapy to improve outcomes of islet transplantation. Stem Cells 2018;36:1045–1061

SIGNIFICANCE STATEMENT

Early loss of grafted islets is the main obstacle to achieve favorable outcomes of islet transplantation. Mesenchymal stem cells are known to have a protective effect; however, its mechanism remains unclear. We hypothesized that bone marrow-derived mesenchymal stem cells (BMSCs) can protect grafted islets against endoplasmic reticulum stress (ERS)-induced apoptosis. In syngeneic streptozocin-induced diabetic BALB/c mice, islet grafts decreased blood glucose levels; however, the effect was not fully functional from the immediate post-transplant phase. β-Cell apoptosis was proven on days 1 and 3 after transplantation. Ultra-structural evidence of ERS was observed along with increased expressions of marker protein BIP and apoptosis-related protein CHOP. In contrast, BMSC co-transplantation maintained glucose hemostasis, inhibited apoptosis and alleviated ERS. In ex vivo culture, BMSCs improved viability of islets and decreased apoptosis. Increased ERS were observed in cultured islets exposed to hypoxia, but not in the islets cocultured with BMSCs. Furthermore, cocultured BMSCs protected islets against ERS-induced apoptosis as well as improved their insulin secretion, and BMSCs alleviated ERS by improving Myc expression through both stromal cell-derived factor 1 signal and contact effect. In conclusion, BMSCs protected the grafted islets against ERS-induced apoptosis during the early stage after transplantation. This study opens a new arena for ERS-targeted therapy to improve outcomes of islet transplantation.

INTRODUCTION

Islet transplantation is an option for the treatment of diabetes [1, 2]. However, long-term blood glucose control is still largely unachievable; only 40% to 60% of patients remain independent of insulin at 5 years post-transplantation [2–4]. Islet cell survival, insulin content, and β-cell mass was shown to decline significantly during the first 3 days after transplantation [5]. The loss of islet grafts in the immediate post-transplant phase is a key impediment to the attainment of favorable outcomes [5–8].

Evidence from in vivo and in vitro studies suggests that co-transplantation of bone marrow derived mesenchymal stem cells (BMSCs) with islets may improve survival and function of islet grafts [9–14]. However, the protective mechanisms of BMSCs against early loss of grafted islets are not known. Although differentiation of BMSCs into insulin-secreting cells can be induced [15], it requires special treatment and has low efficiency [16]. Furthermore, no β-cell proliferation and BMSCs differentiation into β cells was observed after co-transplantation of BMSCs with islets [17].
Islet β-cells are particularly sensitive to endoplasmic reticulum stress (ERS) [18]. ERS results from accumulation of unfolded proteins in the lumen of the endoplasmic reticulum. Mild ERS is amenable to restoration of ER homeostasis; however, under conditions such as surgical trauma and hypoxia, ERS may damage the cells through ERS-induced apoptosis, which involves increased expression of C/EBP homologous protein (CHOP) and subsequent activation of caspase-3 [7, 19–21]. Besides surgical trauma during the process of isolation and preparation, hypoxia during grafting (an ischemic period immediately after islet transplantation) may also induce ERS in islet cells and directly result in islet loss [22, 23]. An increase in markers of ER stress such as BIP, ATF6a and CHOP in the grafted islets was demonstrated within 3 days after transplantation in an experimental transplantation model [24] This suggests an involvement of ERS in β-cell damage. Although the role of ERS-induced β-cell apoptosis has already been investigated in the pathogenesis of β-cell dysfunction in Type 1 diabetes [25], Type 2 diabetes [26, 27], and chemical (arsenic, palmitate)-induced β-cell apoptosis [28], its role in islet transplantation remains unclear.

In this study, we hypothesized that BMSCs might protect islet grafts against ERS-induced apoptosis during early post-transplant stage. Co-transplantation of BMSCs and islets under renal capsule was used in a syngeneic streptozotocin-induced diabetic mouse model. The graft function, β-cell apoptosis and ERS were assessed. To further clarify the underlying mechanisms, coculture of BMSCs with islets was performed and hypoxia was used to stimulate ERS in the cultured islets. The impact of BMSCs on ERS, ERS-induced apoptosis, and islet function was examined, and the pathway through which BMSCs alleviate ERS was investigated.

**MATERIALS AND METHODS**

**Experimental Animals**

BALB/c mice aged 8–12 weeks were purchased from HFK Bioscience company animal center (Beijing, China). They were used as donors for islet cells or administered streptozotocin (Sigma-Aldrich, St Louis, MO) intraperitoneally (150 mg/kg) after fasting for 10 hours to induce diabetes before islet transplantation. Diabetes was confirmed if the blood glucose levels were >16.7 mmol/l for three consecutive days. All mice were maintained in pathogen-free conditions at the animal center and were approved by the Institutional Animal Care and Use Committee at the Tongji Medical College, Huazhong University of Science and Technology, China.

**BMSCs Isolation, Culture and Identification**

MSCs were isolated from femurs and tibias of BALB/c mice, and then were cultured to passage 4 which were used for the subsequent experiments. The surface molecular markers of MSCs were identified by staining with fluorescent isothiocyanate APC-labeled anti-CD29, PE-labeled anti-105, FITC-labeled anti-CD34 and PE-labeled anti-MHC-II (eBioscience, San Diego, CA) monoclonal antibodies using FACs Calibur instrument (BD Biosciences, Franklin Lakes, NJ). The capacity of BMSCs differentiation into adipocytes and osteoblasts was assessed by staining with oil-red O and alizarin red under appropriate culture conditions as described elsewhere [29].

**Islet Cell Isolation and Transplantation**

Isets were isolated by collagenase digestion of the pancreas and Ficoll density gradient centrifugation and then hand-picked to ensure high purity (within a size range of 150–250 μm diameter). The islets were then left freely floating for 2 hours, at 37°C with 5% CO₂, before transplantation. Islets with or without BMSCs were centrifuged at 151 g in a pipette tip attached to PE50 polyethylene tube, which delivered the grafts under the left kidney capsule.

Diabetic mice were randomly divided into four groups: (a) control group (RMP11640, n = 10); (b) BMSCs group (7 × 10⁵ BMSCs, n = 6); (c) islets-alone group (300 islet equivalents (IE), n = 6); (d) BMSCs + islets group (7 × 10⁵ BMSCs and 300 IE islets, n = 6). To evaluate the graft survival and functioning, random blood glucose levels were measured at post-operative days (POD) 0, 1, 2, 3, 5, 7, 10, 14, 17, 21, 24, and 28. At POD28, left nephrectomy was performed on the surviving animals and their blood glucose levels were recorded every day thereafter until death.

**Intraperitoneal Glucose Tolerance Test**

A separate setting of animals was divided into four groups as described above (n = 6 in each group) for intraperitoneal glucose tolerance test (IPGTT). IPGTT was performed at PODs 7, 14 and 28. Fasting blood glucose concentrations were measured before intraperitoneal injection of 2 g/kg i-g-glucose and then after 10, 20, 30, 60 and 120 minutes. Angular vein blood samples were collected at 0 and 20 minutes for measurement of insulin levels (ELISA kit, Millipore).

**Detection of Apoptosis of β Cells in Grafted Islets**

The transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (Roche Diagnostics, Indianapolis, IN) was used according to the manufacturer’s instructions. Immunofluorescence staining was performed for insulin and TUNEL to identify β-cells and apoptotic cells. At least 2,000 β cells were counted for each graft. Insulin in the cytoplasm was stained red, and TUNEL staining in nucleus appeared green, therefore, apoptotic β cells appeared as green surrounded by red fluorescence. The percentage of apoptotic β cells was defined by the following equation: [tunnel-positive cells/total cell number] × 100.

**Ultra-Structural Analysis by Transmission Electron Microscopy**

In a separate experiment, transplanted islets were harvested at PODs 1 and 3 from each group (with or without BMSCs) and prepared to semi-thin sections (0.5–1 μm thick). The sections were stained with methylene blue to select representative areas and observed under transmission electron microscope (Hitachi, Tokyo, Japan).

**Immunofluorescence for β Cells in Grafted Islets**

Specimens containing grafted islets were fixed with 4% paraformaldehyde and processed for paraffin embedding. Polyclonal mouse anti-insulin antibody (1:1,000; Abcam, Cambridge, MA), rabbit polyclonal to BIP (1:1,000; Abcam, Cambridge, MA) and rabbit polyclonal to CHOP (1:100; Abcam) were used as primary antibodies. Goat anti-mouse IgG Alexa Fluor 488 (Invitrogen, Carlsbad, CA) and goat anti-rabbit IgG Alexa Fluor 555 (Invitrogen, Carlsbad, CA) were used as secondary antibodies. Slides were examined on a Fluorescence Microscope.
Immunohistochemistry in Grafted Islets
Specimens containing grafted islets were fixed with 4% paraformaldehyde and were prepared at 4-μm thickness. The sections were then interacted with pig anti-insulin antibody (Dako, Carpinteria, CA) diluted 1:100 and counterstained with hematoxylin. Finally, the specimens were colored with a peroxidase substrate solution containing 3,3’-diaminobenzidine (Brown) (Dako Carpinteria, CA). The area of the engrafted islets was visualized using an Olympus BX-51 light microscope and measured with Image J software.

In Vitro Coculture of Islets and BMSCs
Freshly isolated mice islets (100 IE per well) were incubated overnight in islet medium and then seeded with or without BMSCs (2 × 10^5 per well) on to six-well culture plates in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and maintained at 37°C in 5% CO2 and 95% humidity.

Evaluation of Islet Viability on Coculture with BMSC
Islets cultured with or without BMSCs were harvested at days 1 and 3. Viability of islets was assessed using Acidine Orange (AO) (Sigma-Aldrich, St. Louis, MO) and propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) staining. Dead cells were stained red and viable cells were stained green. In order to avoid the interference by cocultured BMSCs, the islets were picked out from the coculture for further experiments. Before visualization of AO/PI staining, the petri dish was shaken gently in a clockwise direction to temporarily gather the scattered islets for photography under microscopy.

In Vitro Stimulation or Inhibition of ERS
To mimic the transplant-related trauma in the in vitro islet culture, the isolated and purified islets were exposed to hypoxia (O2 < 1%) for 8 hours to induce ERS and apoptosis [30]. Subsequently, the islets were treated with BMSCs or ERS inhibitor, 4-phenylbutyric acid (4-PBA, 12 mmol/l) for 8 hours to further evaluate the protective effect of BMSCs against ERS. To directly explore the protective effect of BMSCs on ERS, the purified islets were first treated with tunicamycin (3 μg/ml) for 8 hours, followed by treatment with BMSCs or 4-PBA for 8 hours as mentioned above.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction
The islets were picked out from the coculture. RNA was isolated from islets and BMSCs + islets with tunicamycin or hypoxia to detect expression of BIP, CHOP. RNA expression was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) using iQ SYBR Green Supermix in an iCycler Real-Time PCR Detection System (Bio-Rad, Germany). Primer sets for the genes are listed below: BIP: F: 5’-GATGTTAATGTTATCTCGAGTG-3’, R: 5’-CTCAAGGTGCAGGCGGTTTG-3’; CHOP:F: 5’-GCTGAGTCCCTGCTTTCACCTT-3’, R: 5’-GATGTCGTGATGACCTCT-3’. Western Blot Analysis
The islets were picked out if cocultured with BMSCs. Expressions of CHOP and BIP proteins in the cultured islets were determined by Western blot analysis using specific antibodies to CHOP (1:1,000; Abcam, Cambridge, MA), BIP (1:1,000; Abcam, Cambridge, MA), Myc (1:10,000; Abcam, Cambridge, MA), and GAPDH (1:1,000; Abcam, Cambridge, MA). The intensity of the immunoblot bands was normalized to GAPDH (loading control).

Evaluation of Islet Cell Apoptosis on Coculture with BMSCs
The islets were picked out from the coculture, harvested by repeated aspiration, washed twice with PBS, and mildly dispersed into cells using 0.25% trypsin for 10 minutes. The cells were harvested by centrifugation. The quantitative assay of islets apoptosis was performed by flow cytometry after labeling of PI (10 μl) and Annexin V (Annexin V-FITC, 5 μl) as recommended by the manufacturer.

Islets Coculture with BMSCs Using Transwell Apparatus
Freshly isolated mice islets were cultured randomly under one of the following six settings: (1) control (300 IE islets); (2) hypoxia (O2 < 1% for 8 hours, 300 IE islets); (3) coculture (hypoxia, 7 × 10^5 BMSCs and 300 IE islets); (4) stromal cell-derived factor 1 (SDF1) blockade coculture (hypoxia, 7 × 10^5 BMSCs and 300 IE islets, given the SDF1 neutralizing antibody); (5) non-contact coculture (hypoxia, 7 × 10^5 BMSCs in a transwell apparatus and 300 IE islets); and (6) SDF1 blockade + non-contact coculture (hypoxia, 7 × 10^5 BMSCs in a transwell apparatus and 300 IE islets, given the SDF1 neutralizing antibody). The neutralizing antibody of SDF1 (ab9797, Abcam, Cambridge, MA) was controlled with a final concentration of 2–4 μg/ml in the cultures.

Glucose-Stimulated Insulin Secretion (GSIS) from Cultured Islets
Size-matched cultured islets were prepared (20 islets/well) and washed with PBS, stimulated by serum-free low glucose (L)-Dulbecco’s modified Eagle’s medium (DMEM) (5.5 mmol/l) for 1 hour. Culture media were collected. The islets were washed twice with PBS, and stimulated by high glucose (H)-DMEM (25 mmol/l) for 1 hour. The supernatant were collected and frozen until insulin content assay. Insulin content was determined by mice insulin enzyme-linked immunosorbent assay (ELISA kit, Millipore) according to the manufacturer’s instructions and was described by unit of nanogram per islet per hour (ng/islet/hour).

Statistical Analysis
All Data are presented as mean ± Standard error of the mean (SEM). For non-parametric comparisons, Mann-Whitney U test was used. Between-group differences were assessed on one-way Analysis of Variance (ANOVA) and Bonferroni post hoc correction. When applicable, the unpaired two-tailed Student t-test was performed. p < .05 were considered statistically significant.

Results
Identification of BMSCs
The cultured BMSCs at the fourth passage were flat and fibroblast-like. The cells were positive for CD29 and CD105 and negative for CD34 and MHC II. The differentiation potential was shown by adipogenic capability (Oil Red O staining) and osteogenic capability (alizarin red staining) (Supporting Information Fig. S1).
Co-Transplantation of BMSCs Improved Survival and Function of Grafted Islets from the Early Stage after Transplantation

The diabetic mice which did not receive treatment died at POD 3 (n = 1), POD 5 (n = 1) and POD 9 (n = 2). Data pertaining to the four dead mice was censored from the analysis. All animals which received treatments survived beyond the observation period of 28 days. The data shows that both BMSCs and islets enhanced the survival of diabetic mice. After undergoing left nephrectomy at POD28, all the surviving recipients died within 3 days.

The average level of blood glucose from POD1 to POD28 reflected the efficiency of islet function. Due to death in the control group, the survived animals were available for analysis. In the control and BMSCs groups, the blood glucose concentration remained at an extremely high level (beyond 25 mmol/l) throughout the observation period; no significant difference was observed between the two groups in this respect. In the islets-alone group, blood glucose concentrations were markedly decreased but were still higher than the normal level. Blood glucose concentrations in the BMSCs + islets group were significantly lower than that in the islet-alone group and consistently maintained within the normal range, which showed the resolution of diabetes (Fig. 1A). However, after the left nephrectomies at POD28, the blood glucose of all the surviving recipients in both the BMSCs + islets and islet-alone groups exceeded 25 mmol/l in the next day, and all these animals died within 3 days. These findings suggest that the grafts modulated blood glucose but were partly impaired and not fully functional which is indicative of islet loss in the immediate post-transplant phase; further, co-transplantation of BMSCs protected the islets and improved the outcomes.

IPGTT was performed at PODs 7, 14 and 28. Two animals in the control group died on POD4 and POD 6, respectively; data pertaining to these mice were censored from the analysis. The rest of animals failed to tolerate glucose and died during IPGTT on POD28, therefore, no data pertaining to IPGTT was available for the control group. Glucose levels (both blood concentration and IPGTT area under the curve (AUC)) in the BMSCs + islets and islets-alone group were significantly lower than those in the control and BMSCs group at the time-points between 10 to 120 minutes at POD7, 14 and 28. Furthermore, the glucose level in BMSCs + islets group was significantly lower than that in the islets-alone group at POD14 and 28 (Fig. 1B, 1C). Serum insulin concentration in the BMSCs + islets group was significantly higher than those in the control, BMSCs and islets-alone groups (Fig. 1D), which suggests superior islet secretion function. Therefore, under an additional glucose metabolic stress, BMSCs stably improved glycemic control, and insulin-secretion by the islets grafts.

Apoptosis and Endoplasmic Reticulum Deformation Rose in the Early Stage after Transplantation and Was Reduced by Co-Transplanted BMSCs

To investigate the reasons for islet graft hypo-function (islet loss) from the early stage after transplantation, we evaluated apoptosis in the islet grafts. In a separate experiment, the grafted islets in the BMSCs + islets (n = 6) and islets-alone (n = 6) groups were collected on POD1 and POD3 to detect β-cell apoptosis by double staining with insulin and TUNEL (Fig. 2A, 2B). The apoptotic β-cells exhibited green surrounded by red fluorescence. The numbers of β-cells and the apoptotic β-cells were counted to calculate the percentage of apoptotic β-cells in the islets-alone group and BMSCs + islets group. The percentage was 19.34% ± 1.46% and 15.35% ± 0.78%, respectively (p < .05), at POD 1, and 22.1% ± 1.5% and 16.34% ± 0.88%, respectively (p < .05), at POD3 (Fig. 2C). The data show that β-cell apoptosis enhanced during the early stages after transplantation and co-transplantation of BMSCs had a protective effect against apoptosis.

β-Cell apoptosis results in islet loss and decreases the success of engraftment of islets, an outcome which can be identified by the area change in the engrafted islets at POD28. At POD 1, the area of the engrafted islets was (6.75 ± 0.59) × 10⁶ μm² and (7.28 ± 0.65) × 10⁶ μm² (p < 0.05), respectively, in the islets-alone (n = 6) and BMSCs + islets (n = 6) groups. However, at POD28, the area was (4.67 ± 0.42) × 10⁶ μm² and (6.23 ± 0.53) × 10⁶ μm² (p < .05), respectively, as shown in Figure 2D and 2E. The data show that BMSCs protected the survival of islet grafts against β-cell apoptosis.

To further explain the occurrence of β-cell apoptosis, we examined the ultra-structure of the grafted β-cells by TEM at the early stage after transplantation (Fig. 2F). In the islets-alone group, at POD1, cisternae of the Golgi complex and rough endoplasmic reticulum showed obvious swelling, increased degranulation of the rough endoplasmic reticulum was observed, and only slight swelling of the mitochondrial matrix was observed. At POD3, the abovementioned degenerative characteristics were more obvious. In the BMSCs + islets group, TEM showed similar results at POD1 and POD3. Rough endoplasmic reticulum showed normal morphology, with no obvious swelling and degranulation. Most of the mitochondrial cristae were normal. These findings show that ERS occurred immediately after transplantation in the islets, and it was alleviated by co-transplanted BMSCs.

Co-Transplanted BMSCs Alleviated ERS Protein Expression of Islet Grafts

BIP is a typical ERS protein and represents the severity of ERS. We examined BIP expression in grafted β-cells at PODs 1 and 3 by immunofluorescence staining. The fluorescence intensity increased sharply at POD1 and POD3 in the islets-alone while BMSCs remarkably decreased the intensity in the BMSCs + islets groups, (Fig. 3A–3C). The intensity in islets-alone group and BMSCs + islets group was 0.115 ± 0.010 and 0.064 ± 0.007, respectively (p < .05) at POD1, and 0.172 ± 0.014 and 0.087 ± 0.017, respectively, (p < .05) at POD3. These data suggest that ERS occurred immediately after transplantation in the islets, and it was alleviated by co-transplanted BMSCs.

CHOP, as one ERS protein, contributes to ERS-induced apoptosis. At POD1, the fluorescence intensity of CHOP in the islets-alone and BMSCs + islets groups was 0.104 ± 0.020 and 0.058 ± 0.012, respectively (p < .05). At POD3, the intensity was 0.140 ± 0.010 and 0.093 ± 0.011, respectively (p < .05); (Fig. 3D–3F). The findings indicate that transplant trauma-related ERS-induced apoptosis was initiated at day 1 and increased at day 3, while BMSCs played protective effect against ERS-induced apoptosis.

Coculture with BMSCs Improved Islets Viability and Protected Islets against Apoptosis In Vitro

To directly investigate the impact of BMSCs on islets, in vitro coculture of BMSCs and islets was performed. After isolation,
Figure 1. Co-transplantation of bone marrow-derived mesenchymal stem cells (BMSCs) improved survival and function of grafted islets. (A): Random glucose levels at 0, 1, 3, 5, 7, 10, 14, 17, 21, 24, and 28 days after transplantation in control, BMSCs, islets-alone, and BMSCs + islets groups. BMSCs + islets group showed significantly lower glucose level as compared to those in the other groups ($p < .01$: vs. islets-alone; $p < .01$: vs. BMSCs; $p < .01$: vs. control; $p = .476$: BMSCs vs. control). (B): Intraperitoneal glucose tolerance test (IPGTT) was performed at 7, 14, and 28 days after transplantation. Fasting blood glucose concentrations were measured before intraperitoneal injection of 2 g/kg D-glucose and at 10, 20, 30, 60, and 120 minutes post-injection. Glucose levels in BMSCs + islets and islets-alone group were significantly lower than those in control and BMSCs groups at the time-points from 10 to 120 minutes at postoperative days 7, 14, and 28. (C): Glucose AUC at 7, 14, and 28 days after transplantation. (D): Serum insulin levels after IPGTT at 7, 14, and 28 days after transplantation. Angular vein blood samples were collected at 0 and 20 minutes for insulin measurement. $n = 6$ per group; *, $p < .05$; **, $p < .01$. Abbreviations: BMSC, bone marrow-derived mesenchymal stem cell; POD, postoperative days; IPGTT, intraperitoneal glucose tolerance test; AUC, area under the curve.
Figure 2. Apoptosis and endoplasmic reticulum deformation rose in the early stage after transplantation and was reduced by co-transplanted bone marrow-derived mesenchymal stem cells (BMSCs). (A): Double immunofluorescence staining for transferase-mediated dUTP nick-end labeling (TUNEL) (green) and insulin (red) at postoperative day (POD) 1 in islets-alone group and BMSCs + islets group. Scale bar = 50 μm, magnification: ×200. (B): Double immunofluorescence staining for TUNEL (green) and insulin (red) at POD3 in the islets-alone and islets + BMSCs groups. Scale bar = 50 μm, magnification: ×200. (C): The rate of apoptotic β cells at POD1 and 3. Ten fields at a 200-fold magnification were randomly selected from each group, the numbers of β-cells and the apoptotic β cells were counted to calculate the percentage of apoptotic β cells in BMSCs + islets and islets-alone groups. (D): Immunohistochemistry staining for insulin (the brown area circled by the orange lines) at POD1 and POD28 in the islets-alone and BMSCs + islets groups. Scale bar = 50 μm, magnification: ×200. (E): Quantitative analysis of the area of the engrafted islets at POD1 and POD28. (F): Endoplasmic reticulum stress emerged in the early stage after transplantation and was alleviated by co-transplanted BMSCs. In islets-alone group, at POD1, cisternae of the Golgi complex and rough endoplasmic reticulum showed obvious swelling and increased degranulation; the mitochondria showed mild swelling; at POD3, the abovementioned degenerative characteristics were more obvious. In BMSCs + islets group, at POD1 and POD3, rough endoplasmic reticulum showed normal morphology with no obvious swelling and degranulation, and only slight swelling of the mitochondrial matrix was observed. Scale bar = 0.5 μm. n = 6 per group, *, p < .05. Abbreviations: —: rough surfaced endoplasmic reticulum; BMSC, bone marrow-derived mesenchymal stem cell; M: mitochondria; POD, postoperative days.
islets were cultured with or without BMSCs for 3 days. At days 1 and 3, the cell viability of the cultured islets was determined by AO/PI staining. The dead cells in the cultured islets increased from days 1 to 3 in both BMSCs-islets coculture and islets culture. However, the dead cells in BMSCs-islets coculture were significantly less than that in the islets culture at each time-point (Fig. 4A). BMSCs coculture improved the viability of the cultured islets.
Figure 3. Co-transplanted bone marrow-derived mesenchymal stem cells (BMSCs) alleviated endoplasmic reticulum stress protein expression of islet grafts. (A): Double immunofluorescence staining for BIP (green) and insulin (red) at postoperative day (POD) 1 in the islets-alone and BMSCs + islets groups. Scale bar = 50 µm, magnification: ×200. (B): Double immunofluorescence staining for BIP (green) and insulin (red) at POD 3 in the islets-alone and BMSCs + islets groups. Scale bar = 50 µm, magnification: ×200. (C): Quantitative analysis of fluorescence intensity of BIP using ImageJ. Significance between group difference was observed at PODs 1 and 3. (D): Double immunofluorescence staining for CHOP (green) and insulin (red) at POD1 in the islets-alone and BMSCs + islets groups. Scale bar = 50 µm, magnification: ×200. (E): Double immunofluorescence staining for CHOP (green) and insulin (red) at POD3 in the islets-alone and BMSCs + islets groups. Scale bar = 50 µm, magnification: ×200. (F): Quantitative analysis of fluorescence intensity of CHOP using ImageJ. Significance between group difference was observed at PODs 1 and 3. n = 6 per group, *, p < .05. Abbreviations: BMSC, bone marrow-derived mesenchymal stem cell; CHOP, C/EBP homologous protein; POD, postoperative days.
We then quantitated the rate of apoptosis in the cell cultures. The islets were picked out from the coculture and then separated into single cell suspension and examined for apoptosis on flow cytometry. At day 1, the percentages of early and late apoptotic cells in the islet culture were 4.31% ± 0.72% and 4.24% ± 0.69% as against 3.20% ± 0.14% and 2.86% ± 0.33% in the BMSCs + islets coculture. At day 3, the percentages of early and late apoptotic cells in the islet culture
were 6.65% ± 0.99% and 7.89% ± 0.69% respectively, as against 4.46% ± 0.80% and 5.64% ± 0.75% in the BMSCs + islets coculture (Fig. 4B). The total rate of dead cell in the islet culture was 9.33% ± 1.00% as against 6.67% ± 0.80% in the BMSCs + islets coculture at day 1; the total rate of dead cell in the islet culture is 15.26% ± 1.31% as against 11.11% ± 1.25% in the BMSCs + islets coculture at day 3 (Fig. 4C). The dead cells include early apoptotic, late apoptotic, and necrotic cells. Therefore, BMSCs coculture protected islets against apoptosis.

ERS Stimulated by Hypoxia in Cultured Islets Was Alleviated by Cocultured BMSCs

During transplantation, the islets were exposed to ischemic or hypoxic conditions. To mimic the transplant-related trauma, in vitro islet culture was exposed to hypoxic conditions. Islets were cultured in an environment of O₂ < 1%, and the mRNA and protein expressions of BIP and CHOP were quantitated on real-time RT-PCR and Western blot analysis (Fig. 5A, 5B). Both mRNA and protein expressions of BIP and CHOP were found to have increased. To further verify that the cultured islet were under ERS, we added 4-PBA, a potent ERS inhibitor, to the culture. The increase in mRNA and protein expressions was accordingly reversed. These findings confirmed hypoxia-induced ERS. Instead of 4-PBA, we cocultured islets with BMSCs under hypoxic conditions, and an effect similar to that of ERS inhibition was observed. Therefore, ERS was induced by hypoxia and cocultured BMSCs appeared to alleviate the ERS.

Besides induction of ERS, hypoxia could have other effects on islets. To focus on the ERS-based mechanism of BMSCs, we replaced hypoxia by tunicamycin, a potent stimulator of ERS, in the islet culture system. As shown in Figure 5C and 5D, tunicamycin-stimulated ERS in the cultured islets was alleviated by cocultured BMSCs. The results are highly similar to those observed under hypoxic condition. Collectively, as a transplant-related trauma, hypoxia potently induced ERS in cultured islets, and cocultured BMSCs alleviated its effect.

BMSCs Coculture Protected Islets Against ERS-Induced Apoptosis and Improved Their Function

To prove the effect of BMSCs on ERS-induced apoptosis, after 8 hours of hypoxia exposure, islets were cultured for 1 day and examined for signs of early and late apoptosis. During the culture, the cells were treated with either BMSCs or 4-PBA. The mean percentages ± SD of cells with early apoptosis under no treatment, hypoxia, hypoxia + BMSCs, and hypoxia + 4-PBA were found to be 5.9% ± 1.3%, 19.4% ± 1.2%, 11.7% ± 1.5%, and 12.5% ± 1.7%, respectively. The total rate of apoptotic cells under no treatment, hypoxia, hypoxia + BMSCs, and hypoxia + 4-PBA were found to be 5.9% ± 1.3%, 19.4% ± 1.2%, 11.7% ± 1.5%, and 12.5% ± 1.7%, respectively. The total rate of apoptotic cells under no treatment, hypoxia, hypoxia + BMSCs, and hypoxia + 4-PBA was found to be 10.2% ± 1.5%, 40.5% ± 1.0%, 24.6% ± 7.2%, and 28.1% ± 3.3%, respectively. No difference was observed between BMSCs and 4-PBA (p > .05) (Fig. 6A). These results indicated that BMSCs
significantly protected islets against both early and late apoptosis induced by hypoxia. Instead of hypoxia, tunicamycin was used to stimulate ERS in islet culture. Early apoptosis under conditions of no treatment, treatment with tunicamycin, tunicamycin + BMSCs, and tunicamycin + 4-PBA was found to be 5.9% ± 1.3%, 20.5% ± 1.2%, 12.7% ± 3.0%, and 10.7% ± 3.0%, respectively. The total rate of apoptotic cells under no treatment, tunicamycin, tunicamycin + BMSCs, and tunicamycin + 4-PBA was found to be 10.2% ± 1.5%, 42.5% ± 1.7%, 25.6% ± 2.0%, and 24.3% ± 3.5%, respectively. No difference was observed between BMSCs and 4-PBA (p > .05) (Fig. 6B). These results indicate that BMSCs significantly protected islets against both early and late apoptosis induced by tunicamycin. In conclusion, BMSCs coculture was verified to protect the cultured islets against ERS-induced apoptosis.

Islets (20 islets per well) were exposed to hypoxia or tunicamycin, and the insulin secretion under low and high glucose stimulation was examined. In the setting of hypoxia-induced ERS experiment, glucose-stimulated insulin secretion in vitro was 2.62 ± 0.33, 1.01 ± 0.11, 1.43 ± 0.23, and 1.49 ± 0.30 ng/islet/hour in the no treatment, hypoxia, hypoxia + BMSCs, and hypoxia + 4-PBA groups, respectively (Fig. 6C). In the setting of tunicamycin-induced ERS experiment, the insulin concentration was 2.64 ± 0.55, 0.78 ± 0.26, 1.51 ± 0.56, and
Figure 5. Endoplasmic reticulum stress stimulated by hypoxia in cultured islets was alleviated by cocultured bone marrow-derived mesenchymal stem cells (BMSCs). (A, B): mRNA and protein expressions of CHOP and BIP under hypoxia, hypoxia + BMSCs, and hypoxia + treatment with 4-phenylbutyric acid (4-PBA). (C, D): mRNA and protein expressions of CHOP and BIP after treatment with tunicamycin, tunicamycin + BMSCs, and tunicamycin + 4-PBA. n = 3 per group, * p < .05 ** p < .01. Abbreviations: BMSC, bone marrow-derived mesenchymal stem cell; CHOP, C/EBP homologous protein; 4-PBA, 4-phenylbutyric acid.
BMSCs Alleviated ERS by Improving Myc Expression Through Both SDF1 Signal and Contact Effect

It is known that the protective effect of MSCs on target damaged cells depends on both secreting soluble factor (non-contact) and contact effect. Myc was recently proven to serve as a master regulator for ERS alleviation. Therefore, we used antibody blockade and transwell apparatus to further investigate the mechanism, as shown in Figure 7. First, after hypoxia was induced, the Myc expression of the cultured islets was downregulated, while ERS-induced apoptosis (CHOP, BIP) was elevated. However, the expression of Myc, CHOP, and BIP recovered remarkably when the islets were cocultured with BMSCs, verifying the protective effect of BMSCs against ERS-induced apoptosis through Myc pathway. Interestingly, SDF1 blockade lessened the protective effect with results of Myc expression.

Figure 6. Bone marrow-derived mesenchymal stem cells (BMSCs) coculture protected the cultured islets against endoplasmic reticulum stress-induced apoptosis and improved their function. (A): Cell early apoptosis of the cultured islets under conditions of hypoxia, hypoxia + BMSCs, and hypoxia + treatment with 4-phenylbutyric acid (4-PBA). (B): Cell early apoptosis of the cultured islets after treatment with tunicamycin, tunicamycin + BMSCs, and tunicamycin + 4-PBA. (C): Insulin secretion by the cultured islets under low or high glucose stimulation in hypoxic setting with BMSCs or 4-PBA. (D): Insulin secretion by the cultured islets under low or high glucose stimulation in the setting of tunicamycin with BMSCs or 4-PBA. n = 3 per group, *, p < .05 **, p < .01. Abbreviations: BMSC, bone marrow-derived mesenchymal stem cell; 4-PBA, 4-phenylbutyric acid.

1.90 ± 0.29 ng/islet/hour in the no treatment, tunicamycin, tunicamycin + BMSCs, and tunicamycin + 4-PBA, respectively (Fig. 6D). These results show that BMSCs improved insulin secretion by the islets suffering ERS.
downregulation and CHOP and BIP upregulation compared with BMSCs coculture, suggesting that the SDF1 secreted by BMSCs played critical roles for islet protection. Meanwhile, using transwell apparatus to avoid BMSCs contacting with islets also lessened the protective effect. Therefore, BMSCs alleviated ERS by Myc through both SDF1 signal and contact effect. We then simultaneously gave SDF1 blockade and transwell apparatus into the coculture, and found the protective effect was further lessened but still existed, indicating more mechanisms need investigation.

**DISCUSSION**

A massive β cell loss immediately after transplantation has remained one of the major obstacles to the widespread application of islet transplantation. More than half of transplanted β cells undergo apoptotic cell-death triggered by hypoxia and nonspecific inflammatory reactions, especially during the first 3 days, which results in poor outcomes of islet transplantation [5, 31, 32]. Protection of islet grafts against apoptosis in the early post-transplantation phase is essential to improve outcomes of islet transplantation. Therefore, unraveling the underlying mechanisms of apoptotic cell-death in the early post-transplant phase is a key research imperative.

In this study, the STZ-induced diabetic BALB/c mice suffered from high glucose level and failed to survive beyond 1 month. Although islet grafts improved glucose control and insulin secretion, the relatively high glucose level (>10 mmol/l) suggests that the function of grafted islets were impaired from the very beginning, which indicates islet losses at the early stage post transplantation. To explain the islet losses, dynamic assessment of β-cell apoptosis of grafted islets was performed, which revealed a high apoptosis ratio at POD1 and 3. This result is consistent with that reported elsewhere [33, 34]. To find the underlying mechanism for the apoptosis, we further used ultra-structure examination and observed severe ERS in the transplanted β-cells at POD1 and 3. Of note, only mild swelling of mitochondria was observed, which indicated that mitochondria-mediated apoptosis pathway contributes little to β-cell apoptosis induced by transplant-related trauma. Subsequently, we identified significant upregulation of BIP (an index for ERS) and CHOP (a key ERS protein that induces β-cell apoptosis). It is worthwhile pointing out that the ERS is initiated mainly by transplant-related trauma but not by high blood glucose level since hyperglycemia was remarkably ameliorated even at POD1 while ERS remained processing afterward. These findings suggest that islet grafts develop ERS and ERS-induced apoptosis at the very beginning post-transplant, which results in β-cell loss and poor outcome.

Co-transplantation of BMSCs has been proved to improve transplanted islet survival and islet function in diabetic animals by us and others [9–14]. In this study, although treatment with only BMSCs improved the survival, it failed to regulate glucose metabolism. Therefore, the effect of BMSCs + islets co-transplantation is not due to BMSCs themselves alone but rather to their impact on islet grafts. We verified that BMSCs + islets co-transplantation achieved glucose homeostasis and maintained favorable islet function even under high glucose metabolic stress (IPGTT) throughout the study. BMSCs significantly decreased β-cell apoptosis of the grafted islets on POD1 and 3, and the protection ensures superior outcome of transplanted islets at POD28 with a higher area of engraftment. Moreover, BMSCs stabilized the ultra-structure of ER, decreased...
Figure 7. Bone marrow-derived mesenchymal stem cells (BMSCs) alleviated endoplasmic reticulum stress by improving Myc expression through both stromal cell-derived factor 1 (SDF1) signal and contact effect. (A): Freshly isolated mice islets were cultured randomly under one of the following six settings: control (normal islets); hypoxia; coculture with BMSCs (suffering hypoxia); SDF1 blockade coculture (suffering hypoxia); non-contact coculture (suffering hypoxia); SDF1 blockade + non-contact coculture (suffering hypoxia). The expression of Myc, CHOP, BIP, and GAPDH was analyzed by Western blot. (B-D): The intensity of the immunoblot bands (Myc, CHOP, BIP) was normalized to GAPDH. n = 6 per group, *, p < .05. Abbreviations: CHOP, C/EBP homologous protein; SDF1, stromal cell-derived factor 1.
the expression of markers of ERS (BIP) and downregulated apoptosis-inducing ERS protein (CHOP). All these findings show that BMSCs protect the function of islet grafts, alleviate ERS, and decrease ERS-induced apoptosis during the early stage after transplantation.

Using ex vivo culture system, ERS can be modulated directly to avoid uncontrollable factors during the process of transplantation. Therefore, islets culture was performed to focus on the ERS-based protective mechanism of BMSCs. In the first step, we showed that BMSCs improved the viability and decreased apoptosis of cultured islets. Then, we introduced hypoxia into the culture. Because the process of islet preparation and the transplant microenvironment has a mean pO2 of only 5–10 mmHg (<1% O2) as compared to 40 mmHg in healthy pancreas [35], islet grafts undergo critical trauma by hypoxia. To mimic the transplant-related conditions, we exposed the islet culture to hypoxic culture. BIP, CHOP, and islet cell apoptosis were all found increased. After addition of specific ERS-inhibitor 4-PBA, the protein expression and apoptosis were found decreased in the culture accordingly. Use of ERS-stimulator tunicamycin, instead of hypoxia, yielded consistent results. These data show that hypoxic insult stimulates ERS and ERS-induced apoptosis. The effect of BMSCs coculture on ERS and ERS-induced apoptosis (especially early apoptosis which could be reversed by treatment) was similar to that of 4-PBA, which proves that the direct protective effect of BMSCs on islet is based on ERS modulation. As a function test, GSIS showed that BMSCs improved insulin secretion by cultured islets suffering from ERS. These findings confirm that cocultured BMSCs modulate ERS and ERS-induced apoptosis and had a protective effect on the islets.

Recently, SDF1 has been proven to play critical roles in endometrial regenerative cell-based therapy [36]. It is already known that SDF1 receptor (chemokine(C-X-C motif) receptor 4) is expressed on β cells [37] and SDF1 signal can modulate Myc expression [38]. Myc modulates cell cycle progression and apoptosis [39, 40], and interacts with SIRT7 to target the promoters of ribosomal proteins and thus alleviate ERS [41]. Therefore, we supposed that the underlying mechanism in islet protection against ERS is related with SDF1 signal. In this work, we found that SDF1 blockade downregulated Myc expression, upregulated CHOP and BIP expression and finally lessened the protective effect of the cocultured BMSCs. While BMSCs contacting with islets also contributed to the protective effect, simultaneously giving SDF1 blockade and transwell apparatus (non-contact) into the coculture failed to fully delete the protective effect, indicating that more mechanisms beyond SDFD signal exist. As an attempt, we neutralized other kind of secretion factors such as VEGF, yielding negative findings for islet protection (data not shown). In conclusion, BMSCs alleviated ERS by improving Myc expression through both SDF1 signal and contact effect, and more mechanisms still keep further elucidation.

**Conclusion**

Taken together, for the first time, we have shown that (a) ERS can be stimulated by transplant-related trauma and thereafter induces β-cell apoptosis during early stage after transplantation; (b) BMSCs alleviate trauma-induced ERS through both SDF1 signal and contact effect, resulting in decrease in ERS-induced apoptosis and improvement of islet function. In conclusion, BMSCs protected the grafted islets against ERS-induced apoptosis during early stages after transplantation. Although the effect of BMSCs on ERS requires more in-depth research, our findings open a new arena for development of ERS-targeted therapy to improve outcomes of islet transplantation.

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**Author Contributions**

Y.H. and Y.Z.: performed almost all molecular biological, biochemical experiments and all animal surgeries; D.Z.: performed almost all molecular biological, biochemical experiments and all animal surgeries, provided continuous support and conceptual advice for this study, wrote the paper; J.M., J.W., and H.G.: analyzed the data and discussed the results; J.Z. and M.W.: preceded the preliminary experiments; W.Z.: designed the research; N.G.: designed the research, provided continuous support and conceptual advice for this study, wrote the paper, is the guarantor of this work and, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Disclosure of Potential Conflicts of Interest**

The authors indicated no potential conflicts of interest.

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