Bone marrow mesenchymal stem cells promote the repair of islets from diabetic mice through paracrine actions

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Abstract
Transplantation of bone marrow mesenchymal stem cells (MSCs) has been shown to effectively lower blood glucose levels in diabetic individuals, but the mechanism has not been adequately explained. We hypothesized that MSCs exert beneficial paracrine actions on the injured islets by releasing biologically active factors. To prove our hypothesis, we tested the cytoprotective effect of conditioned medium from cultured MSCs on isolated islets exposed to STZ in vitro and on mice islets after the experimental induction of diabetes in vivo. We assessed islet regeneration in the presence of conditioned medium and explored the possible mechanisms involved. Transplantation of MSCs can ameliorate hyperglycemia in diabetic mice by promoting the regeneration of β cells. Both β cell replication and islet progenitors differentiation contribute to β cell regeneration. MSC transplantation resulted in increases in pAkt and pErk expression by islets in vivo. Treatment with MSC-CM promoted islet cell proliferation and resulted in increases in pAkt and pErk expression by islets in vitro. The MSC-CM-mediated induction of β cell proliferation was completely blocked by the PI3K/Akt inhibitor LY294002 but not by the MEK/Erk inhibitor PD98059. Together, these data suggest that the PI3K/Akt signaling pathway plays a critical role in β cell proliferation after MSC transplantation.

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1. Introduction
Type 1 and type 2 diabetes result from a lack of functional β cells. Pancreas and islet transplantation have proven successful in restoring functional β cells. However, donor organ shortage has limited the clinical application of these transplantations and incentivized efforts to generate insulin-producing cells from other sources. Two possible solutions exist: identifying insulin-producing cells from other sources or developing exogenous sources of islets. If this method becomes possible, the limitations of in vivo regeneration method becomes possible, the limitations of in vivo regeneration could be overcome without requiring immunosuppressive therapy. Recently, different types of bone marrow-derived cells have been proposed as potential sources of cells for diabetic cell therapy (Hess et al., 2003; Banerjee et al., 2005; Hasegawa et al., 2007; Lee et al., 2006; Zhao et al., 2008; Madec et al., 2009). One strategy uses mesenchymal stem cells (MSCs). Adult bone marrow-derived MSCs are multipotent stem cells that, given their ease of isolation, low immunogenicity, and amenability to ex vivo expansion, are optimal candidates for diabetic cell therapy (Pittenger and Martin, 2004). Indeed, transplantation of bone marrow MSCs has been demonstrated to be an effective strategy for the repair of diabetic islets in experimental models (Lee et al., 2006). Seven registered
clinical trials on type 1 and/or type 2 diabetes in phase I/II can be found on the website http://www.clinicaltrial.gov (Si et al., 2011). In these clinical trials, MSCs exhibited exciting therapeutic effects in diabetic volunteers (Jiang et al., 2011). However, the mechanisms underlying these therapeutic effects have not been clearly defined. Bone marrow-derived MSCs have been shown to secrete various cytokines, including VEGF and FGF (Madec et al., 2009). Through these actions, the transplantation of MSCs has been experimentally reported to improve diabetic islets and promote the expansion of endogenous islet progenitors (Si et al., 2012; Park et al., 2010). However, the underlying mechanisms of this phenomenon are not yet fully understood. We hypothesized that MSCs exert beneficial paracrine actions on the injured islets by releasing biologically active factors. To prove our hypothesis, we tested the cytoprotective effect of conditioned medium from cultured MSCs on isolated islets exposed to STZ in vitro and on mice islets after the experimental induction of diabetes in vivo and assessed the regeneration of islets in the presence of conditioned medium.

2. Materials and methods

2.1. Animals

Six-week-old C57BL/6j mice were purchased from the Shanghai Laboratory Animal Center at the Chinese Academy of Sciences. Green fluorescent protein (GFP) transgenic mice with the C57BL/6j background were purchased from the Department of Cell Biology, Secondary Military Medical University, Shanghai. All animals were maintained in the animal facility of Zhongshan Hospital, Fudan University, Shanghai. The protocols of animal use complied with the principles of laboratory Animal Care (NIH Publication 85–23) and approved by the Ethics committee of Zhongshan Hospital, Fudan University.

2.2. Antibodies

Guinea pig (GP) anti-mouse insulin (Invitrogen, Carlsbad, CA, USA), rabbit anti-GFP (Millipore, Billerica, MA, USA), goat anti-mouse insulin, rabbit anti-mouse glucagon, rabbit anti-mouse insulin, goat anti-mouse nestin, goat anti-mouse PDX-1, goat anti-Pax4, goat anti-Pax6, goat anti-Nkx6.1, goat anti-Nkx2.2, goat anti-Ngn3, and goat anti-NeuroD antibodies were all purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Mouse monoclonal anti-BrdU antibody was purchased from Sigma–Aldrich, St. Louis, MO, USA. FITC conjugated chicken anti-mouse secondary antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Rabbit anti-mouse CD29, FITC anti-mouse CD44, PE anti-mouse SCA-1, PE anti-mouse CD117, PE Cy2-conjugated donkey anti-rabbit and Cy3-conjugated donkey antibody was purchased from Santa Cruz. Rhodamine-conjugated donkey anti-APC secondary antibody was purchased from Millipore. Cy2-conjugated donkey anti-rabbit and Cy3-conjugated donkey anti-goat secondary antibodies were purchased from Jackson ImmunoResearch, West Grove, PA, USA. PE anti-mouse CD117, PE anti-mouse CD29, FITC anti-mouse CD44, PE anti-mouse SCA-1, and PE anti-mouse CD34 antibodies were purchased from Biolegend, San Diego, CA, USA. 4-6-diamidino-2-phenylindole (DAPI) was purchased from Sigma–Aldrich. Rabbit anti-phosphorylated Akt (pAkt, Ser473), rabbit anti-Akt, rabbit anti-phosphorylated Akt (pAkt, Ser473), rabbit anti-Akt, rabbit anti-phosphorylated Akt (pAkt, Ser473), and rabbit anti-Erk1/2 (pErk, thr202.tyr204) and rabbit anti-Erk antibodies were purchased from Cell Signaling, Beverly, MA.

2.3. Induction of diabetes

Mice were injected intraperitoneally (i.p.) with 50 mg/kg of streptozotocin (STZ; Sigma–Aldrich) daily for 7 consecutive days. STZ was solubilized in sodium citrate buffer, pH 4.5, and injected within 15 min of preparation. Non-fasting blood glucose was measured using a One Touch Sure Step meter (Johnson & Johnson, Shanghai, China) between 9:00 and 11:00 a.m. every 3 days after STZ injection and then weekly after bone marrow MSC transplantation.

2.4. Mesenchymal stem cell purification and transplantation

Bone marrow was collected from the femurs of mice or GFP-transgenic mice. The mononuclear fraction of bone marrow was isolated from a Ficoll density gradient. The nucleated cells were plated in plastic culture dishes (Corning, NY, USA) and incubated in Dulbecco’s modified Eagle’s medium (DMEM; Sigma–Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C in a 5% humidified CO2 atmosphere. MSCs were selected based on their adherent property of preferentially attaching to culture dishes. The cells were continuously cultured as MSCs until passage 3. The cells were then incubated with PE anti-mouse CD117, PE anti-mouse CD29, FITC anti-mouse CD44, PE-Cy anti-mouse SCA-1, or PE anti-mouse CD34 antibodies (Biolegend, San Diego, CA, USA). Isotype-identical antibodies served as controls. Passage 3 MSCs were incubated to differentiate into adipocytes, osteoblasts in corresponding induction medium for 3 weeks. MSCs adipogenic differentiation medium kit (Cyagen, Santa Clara, CA, USA) includes medium A and B. Medium A includes basal medium A 175 ml, FBS 20 ml, Glutamine 2 ml, Insulin 400ul, Indomethacin 200ul, Dexamethasone 200ul. Medium B contains Basal medium 175 ml, FBS 20 ml, Glutamine 2 ml and Insulin 400ul. For adipogenesis, we followed the instruction of adipogenesis protocol. MSC osteogenic differentiation kit (Cyagen, Santa Clara, CA, USA) contains basal medium 175 ml, FBS 20 ml, Glutamine 2 ml, Ascorbate 400ul, β-Glycerophosphate 2 ml and Dexamethasone 20ul. For osteogenic differentiation, we followed the instruction of osteogenesis protocol. Eight hours before transplantation, recipient mice were irradiated (500 cGy), and MSCs (6.5 x 106) were transplanted into the diabetic mice through their tail veins.

2.5. Conditioned medium

Conditioned medium was generated as follows: 80% confluent, passage-3 MSCs in 10-cm tissue culture dishes (Corning) were fed with 5 mL of serum-free DMEM per dish for 12 h in a chamber. The conditioned medium was further concentrated (50 times) by ultra-filtration using 5-kDa cut-off centrifugal filter units (Millipore) following the manufacturer’s instructions.

2.6. Isolation of islets

After the mice were killed, their pancreata were inflated via the pancreatic duct with type V collagenase (0.7 mg/ml in Hank’s balanced salt solution, Sigma), excised, and digested at 37 °C for 7–10 min. The resulting digest was washed twice with cold Hank’s balanced salt solution containing 5% bovine serum albumin, and the islets were separated using a Histopaque density gradient. The interface-containing islets were removed and washed with Hank’s balanced salt solution containing bovine serum albumin, and the islets were resuspended in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 5% bovine serum albumin, 100 U/mL penicillin, and 100 μg/mL streptomycin. Following 2 h of incubation at 37 °C, the islets were handpicked into fresh media and washed twice in PBS. The islets were fed with RPMI 1640 medium containing 10% fetal bovine serum albumin on plastic dishes at 37 °C in a 5% humidified CO2 atmosphere. Each dish (30 mm) contained approximately 40 islets. For islet injury, we prepared the solution of STZ (10−5 mmol/L). And each dishes added about 100 μl solution of STZ. After an hour later, we changed the medium and stop the injury of STZ. For MSC-CM treatment, we changed the
medium every 3 days and added about 100 μl concentrated MSC-CM each time.

2.7. BrdU incorporation

The thymidine analog 5-bromo-2'-deoxyuridine (BrdU; Sigma–Aldrich) was injected intraperitoneally at 0.05 g per gram of body weight 24 h before harvesting the pancreata. The pancreata were then isolated and processed for fluorescent immunohistochemical studies. In vitro, BrdU (40 μmol/L) was added to the culture 24 h before staining. To calculate the number of BrdU-positive cells among the islets, we microscopically examined the entire surface of all dishes.

2.8. Histological and morphological analyses

Mice pancreata were excised and fixed overnight in 4% paraformaldehyde at 4 °C. Fixed tissues were processed for paraffin embedding, and serial 5-μm-thick sections were prepared and stained with hematoxylin/eosin (HE; Beyotime Institute of Biology, Suzhou, China) to assess pancreatic islet histology and morphology in the experimental animals. Every tenth, twentieth, and thirtieth section of the islets were observed. The histology and morphology of the islets were scored using a Carl-Zeiss Axiosvert 200 microscope (Carl-Zeiss, Jena, Germany) and a computer-assisted image analysis program (AxioVision Ver. 4.0; Carl-Zeiss).

2.9. Fluorescent immunohistochemistry of the pancreas

The slides were deparaffinized and rehydrated, and then antigen retrieval was performed using antigen unmasking buffer; the slides were then blocked for 40 min at room temperature in 5% bovine serum albumin (BSA) and phosphate-buffered saline (PBS). To stain for insulin, the sections of paraffin-embedded pancreata were incubated overnight with GP anti-mouse insulin antibody (1:400) at 4 °C, following which the sections were incubated in secondary antibody with rhodamine (1:500) at 37 °C for 30 min. Twenty fields were examined for each section, and the number of islets and insulin-positive cells in each field was determined. For double staining, the sections were incubated overnight at 4 °C with the appropriate antibody. Labeled cells were visualized with the appropriate biotin-conjugated secondary antibody with rhodamine and Cy2. To stain for BrdU, the sections were pretreated with 2 N HCl for 30 min at 37 °C and then incubated overnight with mouse monoclonal anti-BrdU (1:50). Labeled cells were visualized with the appropriate secondary antibody with FITC (1:200). Isotype-matched antibodies and PBS were used as controls for the stained sections. Nuclear regions were stained by DAPI counterstaining.

2.10. Fluorescent immunohistochemistry of the islets

Islets cultured in dishes were fixed with 2% paraformaldehyde on ice for approximately 20 min; the dishes were then washed twice, and the islet cell pretreated with 2 N HCl for 30 min at 37 °C. The dishes were again washed twice and then exposed to 1% BSA. Anti-insulin antibody (1:1000) and anti-BrdU (1:100) antibody were added, and the islets were incubated overnight at 4 °C. The dishes were then incubated with secondary fluorescent antibodies against insulin and BrdU. The dishes were evaluated and photographed using a confocal fluorescent microscope (Leica TCS SP2, Heidelberg, Germany).

2.11. Western blotting

The isolated islets were analyzed using western blotting. Samples were lysed in detergent lysis buffer (20 mmol/L Tris–HCl, PH 7.5; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L EGTA; 1 mmol/1 Na3VO4; and 1 μg/ml leupeptin), and the lysates were centrifuged at 14,000 rpm for 20 min at 4 °C. Protein concentrations were measured using a bicinchoninic acid assay (Sigma–Aldrich, St. Louis, MO, USA). Ten micrograms of protein were resolved on a 7.5–10% SDS–PAGE gel and transferred to nitrocellulose membranes (Millipore, Billerica, MA). The membranes were then blocked with 0.1% Tween-20 Tris-buffered saline containing 5% nonfat dry milk. The membranes were then incubated with antibodies against either pAkt, Akt, pErk, Erk, or GAPDH (1:1000 dilution) overnight at 4 °C. The membranes were then incubated with a secondary peroxidase-conjugated antibody (1:5000 dilution) after washing. The immunoreactions were visualized using ECL Plus or ECL Western Blotting Detection. The resulting images were scanned, and the densities were measured using Image J software. Protein expression levels were corrected based on GAPDH density.

2.12. Statistical analysis

Data are expressed as the means ± SE. Differences between experimental groups were evaluated using the unpaired Student’s t test for several independent observations. A value of p < 0.05 was considered to be statistically significant. The P values indicated in the graphs were obtained using Student’s t test.

3. Results

3.1. Characterization of bone marrow mesenchymal stem cells

Most cultured adherent cells exhibited a fibroblastic morphology that is characteristic of MSCs. Fluorescence-activated cell sorting (FACS) analysis of our bone marrow MSCs showed that they were negative for CD117 and CD34 and that they strongly expressed typical surface antigens, such as CD44, CD29, and Sca-1 (Fig. 1A). When cultured in adipogenic or osteogenic medium, the cells differentiated into adipocytes (Fig. 1B) or osteoblasts (Fig. 1C), respectively.

3.2. Bone marrow MSCs repair the pancreatic islets of diabetic mice

Six-week-old C57BL mice were administered STZ, which induced pancreatic injury and hyperglycemia. To assess the effect of bone marrow MSC-transplantation on diabetic islets, we randomly divided the diabetic mice into two groups at day 10. One group (n = 24) received bone marrow MSCs from GFP mice, and the other group (n = 24) received PBS. During the study period, the untreated mice exhibited persistent hyperglycemia. However, the blood glucose level of the MSC-transplanted diabetic mice was significantly lower on days 7, 14, 21, 28, 35, and 42 (Fig. 2A). These results demonstrate that MSC transplantation can ameliorate hyperglycemia in diabetic mice. We analyzed the untreated islets and β cells of mice in the two groups on days 7 (n = 8) and 42 (n = 16). STZ induced an inflammatory reaction and decreased the mass of the pancreatic islets. Microscopic examination of the hematoxylin/eosin-stained pancreatic sections on day 7 revealed that islet size and number were markedly decreased in the STZ-treated mice. After MSC transplantation, both the size and number of the islets were partially restored, and the mass of the β cells was partly recovered on day 7. In contrast, the numbers of islets and β cells remained very low in the untreated mice on day 7 (Fig. 2B–D). On day 42, blood glucose levels continued to decline in the MSC-transplanted mice. Furthermore, compared with the untreated mice, the size and number of islets, and the mass of β cells were increased significantly on day 42 after MSC transplantation (Fig. 2E–G).
3.3. Transdifferentiation of bone marrow MSCs

To investigate whether bone marrow MSCs transdifferentiated into insulin-positive cells in our model, pancreata from MSC-transplanted mice were immunostained with anti-insulin antibody; then, an intensive search for both insulin- and GFP-positive cells was conducted. GFP-positive cells were located around or in the islets; however, no double-positive cells were detected. To further confirm this result, we double-stained the sections for GFP and PDX-1, Ngn3, Nestin, Nkx6.1, Pax4, NeuroD, and Pax6, all of which are expressed in different stages of islet \( \beta \) cell development (Fig. 3). Again, no double-stained cells were evident. Thus, we suggest that the observed repair of the diabetic islets in our mouse model was not due to differentiation of the transplanted MSCs.

3.4. The islet regeneration pathway

In theory, \( \beta \) cell regeneration occurs by the replication of precursor cells and by neogenesis from precursor cells within the pancreas. To test whether the observed regeneration of \( \beta \) cell was derived from the replication of \( \beta \) cell, we double-stained for insulin and BrdU. We observed insulin-positive cells expressing BrdU in the MSC-treated mice, but were unable to find these cells in the untreated mice (Fig. 4A). Some studies have indicated that PDX-1 plays crucial roles in the differentiation of endocrine lineages, and we noticed that some insulin-BrdU\(^+\) cells stained positive for PDX-1 (Fig. 4B). We also performed double staining using antibodies against insulin and glucagon. Cells co-expressing insulin and glucagon were found in the MSC-treated mice, but not in the untreated mice (Fig. 4C).

The bHLH transcription factor Ngn3 is first observed in the embryonic pancreatic epithelium at E9.5, then reaches maximum expression by E15.5, and declines after birth; Ngn3 is not expressed in adult mice (Watada, 2004). Ngn3 is considered a marker of endocrine progenitor cells (Gu et al., 2002). To assay for putative pancreatic progenitor cells during pancreatic \( \beta \) cell regeneration, we double stained the samples for insulin and Ngn3. No Ngn3\(^+\) cells were found in the untreated mice, but Ngn3\(^+\) cells were found in the islets of the MSC-treated mice (Fig. 4D).

3.5. Bone marrow MSC-conditioned medium improves function of diabetic islets in vivo and in vitro

Immunohistochemistry indicated the presence of GFP-positive cells in and around diabetic islets, but these cells were unable to differentiate into insulin-expressing cells. Furthermore, some studies have shown that bone marrow MSCs can produce large amounts of growth factors (Kinnaird et al., 2004; Gnegchi et al., 2005, 2008; Poll et al., 2008; Liu et al., 2006). This led us to suspect that a paracrine mechanism underlies the supportive effect of MSCs on islet repair.

To test our hypothesis, we first studied the effects of conditioned medium obtained from cultured MSCs (MSC-CM) in vivo. Concentrated conditioned medium was injected into the tail veins of diabetic mice (MSC-CM). We found that the blood glucose levels of the MSC-CM mice were significantly lower than those of the MSC-treated mice on days 17, 24, 31, 38, 45, and 52 (Fig. 5A), and the numbers of islets and \( \beta \) cells were partly restored in the MSC-CM mice (Fig. 5C and D). We also found cells that co-expressed insulin and BrdU (Fig. 5E). To strengthen these morpholog-
ical observations and further investigate the protective effect of the conditioned medium, we also studied the effects of the conditioned medium in vitro. Isolated islets from normal mice were cultured in 30-mm plastic dishes for 2 weeks, and then STZ (5 × 10⁻⁴ mmol/L) was added to the islets. One hour later, the medium was changed, and the islets were divided into 2 groups, one with normal medium (STZ, \( n = 6 \)) and the other with concentrated conditioned medium and normal medium (MSC-CM, \( n = 6 \)). Ten days later, we found more BrdU positive cells in the MSC-CM islets. To further confirm these results, we double-stained for insulin and BrdU antibodies (Fig. 5F). The results showed that there were more insulin-positive cells and doubly stained (insulin and BrdU) cells in the MSC-CM islets (Fig. 5G).

3.6. MSCs activate Akt signaling in vivo and in vitro

Recent contradictory results suggest that the signaling pathway of islet regeneration remains unclear (Wang et al., 2004; Schrader et al., 2007; Chen et al., 2013; Hayes et al., 2013). Some studies indicate that the Akt and Erk signals may play a part in this pathway. To clarify which signal was activated after MSC transplantation, we tested the expression of pAkt in diabetic islets. Protein was extracted from islets collected at 6 weeks after MSC transplantation; we found that the expression levels of pAkt and pErk were significantly increased in the islets of MSC-transplanted mice (Fig. 6A–D).

To verify the importance of Akt signaling in vitro, we isolated islets, injured them by exposing them to STZ, and then divided them into 2 groups as previously described (termed the untreated and MSC-CM groups). We tested the expression of pAkt and pErk at different time points after MSC-CM was added to the islets and found that the expression levels of pAkt and pErk were significantly increased in the MSC-CM islets (Fig. 6E–G).

To determine the role of the Akt and Erk signaling pathways in pancreatic islet cell proliferation, the effects of LY294002 and PD98059 on BrdU incorporation in pancreatic islet cells were examined. As shown in the figures, the MSC-CM islets exhibited significantly increased BrdU incorporation compared to the untreated islets; pretreatment with LY294002 (50 nmol/L) completely inhibited the MSC-CM-mediated BrdU incorporation compared to the untreated islets (Fig. 7A and B). Conversely, PD98059 (50 nmol/L), a Mek/Erk inhibitor, did not attenuate the MSC-CM-mediated BrdU pathway during beta-cell proliferation. We double-stained for insulin and BrdU in islet cells pretreated with LY294002 and/or PD98059. A significant increase in cells that exhibited insulin and BrdU double-staining was noted in the MSC-CM-treated islets. Pretreatment with LY294002 also...
completely inhibited the BrdU incorporation, whereas pretreatment with PD98059 did not inhibit this incorporation (Fig. 7C and D). To confirm the specific inhibitory effects of LY294002, we analyzed the MSC-CM-mediated phosphorylation of Akt in the islets. Pretreatment with LY294002 completely blocked the MSC-CM-mediated phosphorylation of Akt (Fig. 7E).

4. Discussion

Currently, a number of studies and clinical trials have revealed that the transplantation of MSCs can successfully treat hyperglycemia in animals or subjects with type 1 or type 2 diabetes (Lee et al., 2006; Zhao et al., 2008; Madec et al., 2009; Jiang et al., 2011; Si et al., 2012). Heretofore, it was believed that the mechanisms underlying the therapeutic effects of MSCs on hyperglycemia involved islet regeneration, immunoregulation, and an improvement in insulin sensitivity. Different mechanisms have been indicated by different diabetic animal models, but both type 1 and type 2 diabetes are characterized by β cell loss and dysfunction. Therefore, a major goal of diabetes research is to discover methods of promoting the regeneration of β cells. In this study, we found that MSCs could mobilize into diabetic pancreata and repair diabetic islets by promoting β cell regeneration in vivo. We excluded the possibility that MSC-derived functional β cells were generated in vivo because we were unable to detect cells co-expressing GFP and...
specific beta cell makers in the pancreas. However, the question of whether beta cell regeneration occurs mainly via the replication of preexisting beta cells or via neogenesis from stem cells in the islets remains a major focus of interest. In recent years, doubts about the existence and importance of beta cell neogenesis, especially by stem cells, have been raised. Lineage-tracing studies have only found evidence for beta cell replication (Dor et al., 2004; Nir et al., 2007), and strong evidence to support the presence of a population of stem cells that gives rise to pancreatic endocrine cell types in the adult pancreas remains lacking. Our studies found that the blood glucose reduction was accompanied by an increase of insulin+ BrdU+ cells; this finding indicates that beta cell proliferation occurred during the process of islet regeneration. Most importantly, several lines of evidence in our study indicate that islet progenitor's differentiation contributed to the regeneration of beta cells. First, BrdU+insulin+ PDX-1+ cells were found in the islets of the MSC-treated mice. Previous studies have demonstrated that BrdU+ Insulin+ PDX-1+ cells can differentiate into beta cells in vivo (Duvillié et al., 2003). Second, Ngn3 was activated during the course of beta cell regeneration. Ngn3 is a key transcription factor for the differentiation of the endocrine pancreas (Watada, 2004). Previous studies have shown that Ngn3+ cells are endocrine progenitor cells and can differentiate into beta cells in adults mice (Xu et al., 2008). Third, insulin and glucagon double-stained cells were also found during beta cell regeneration.
cell regeneration, and, in vitro, this staining pattern is only found in embryonic stem cells (D’Amour et al., 2006) and pancreatic ductal cells (Bonner-Weir et al., 2000) that have differentiated into β cells. A major debate exists concerning the origin of stem cells that can produce β cells via neogenesis. Most reports have suggested that such cells originate in or adjacent to the pancreatic duct epithelium (Gnecchi et al., 2008; Inada et al., 2008), but a recent study did not find any sign of β cell through lineage tracing after partial duct ligation (Solar et al., 2009). In this study, we noticed that these endocrine progenitor cells were located in the islets and did not find any cells expressing stem cell markers in the pancreatic duct. Based on the results of our study, we propose that progenitor cells in the islets were the main source of beta cell neogenesis in our diabetic mouse model.

In conclusion, we suggest that both the replication of β cell and the differentiation of islet progenitors contribute to β cell regeneration after bone marrow MSC transplantation. Replication and differentiation are not mutually exclusive. Both processes often occur simultaneously, and eventually, one or both of these pathways may be manipulated for the therapeutic treatment of diabetes.

The mechanisms involved in the process of β cell regeneration after MSC transplantation remain unknown. Many studies have confirmed that MSCs can secrete a variety of cytokines, such as VEGF, EGF, and HGF (Gnecchi et al., 2008). Some of these cytokines, such as EGF and HGF, are known to be crucial for the multiplication and apoptosis of β cells. The transplantation of MSCs has been shown to improve the repair of infarcted hearts (Gnecchi et al., 2005) and skin wounds (Liu et al., 2006) through the secretion of paracrine factors. However, whether MSCs promote the repair of islet function by paracrine stimulation is unclear. Support for our paracrine hypothesis was provided by the results obtained from our MSC-conditioned medium experiments. We first injected concentrated medium from MSCs into diabetic mice and obtained remarkable islet protection in vivo, similar to that observed with MSC transplantation. Furthermore, the number of islets and β cells in the MSC-CM-treated mice were the same as those in the MSC-treated mice. We also observed proliferation of β cells in the MSC-CM-treated mice. We then showed that conditioned medium from MSCs exerts a striking protective effect on isolated islets exposed to STZ in vitro. Also, conditioned medium from MSCs promoted β cell proliferation. These new data strongly support the concept that the effects of MSC-transplantation in diabetic mice are, to a great extent, attributable to paracrine protection and the functional recovery of pancreatic islets.

Akt and Erk are potent regulators of beta cell proliferation. Some studies have reported that the overexpression of active Akt1 in mouse β cells substantially affects compartment size and function (Tuttle et al., 2001). Another study demonstrated that the anti-proliferative effect of pro-inflammatory cytokines in cultured β cells is associated with an extracellular signal that is regulated by kinase 1/2 pathway inhibition (Blandino-Rosano et al., 2008). Thus, which signal is involved in beta cell proliferation remains unknown. This study provides pioneering evidence linking stem cell paracrine action to the activation of Akt signaling in the host. After MSC transplantation into diabetic mice, the expression of pAkt and pErk was significantly increased in mouse pancreatic islets; pAkt, in particular, increased by approximately 1.8 times. We also confirmed that MSC-CM could increase the expression of pAkt and pErk in vitro. Meanwhile, the proliferation of β cells was strengthened accordingly. To further confirm whether the Akt and Erk pathways were involved in beta cell regeneration after BMSC transplantation, we observed the proliferation of islets after blocking the signal pathway. We found that the islets stopped proliferating after the Akt pathway was blocked with LY294002 but not with PD985009. Therefore, activation of the Akt pathway appears to be critical for stimulating the proliferation of islets, and pancreatic β cell in particular.
In conclusion, our study suggests that MSCs exert direct salutary effects on diabetic islets via paracrine mediators. The therapeutic benefits of MSCs appear to be attributable primarily to diffusible factors released by cells that promote β cell regeneration. The PI3K/Akt pathway plays an essential role in β cell proliferation after MSCs transplantation. And MSCs or MSCs derived molecules may promise novel therapeutic approaches in the treatment of diabetes. The future identification of the exact nature and mechanism of action of the secreted factors may have important implications for the development of islet injury and regeneration. And these new findings would bring new ideas for the treatment of diabetes.

**Author contributions**

X.G. and L.S.: conception and design, data analysis, manuscript writing; K.S. and H.W.: collecting of data and data analysis; M.Q.: collecting of data; W.N. and X.Q.: conception and design, financial support, manuscript writing and editing.

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