Human umbilical cord matrix-derived stem cells exert trophic effects on β-cell survival in diabetic rats and isolated islets

Yunting Zhou, Qi Hu, Fuyi Chen, Juan Zhang, Jincheng Guo, Hongwu Wang, Jiang Gu, Lian Ma and Guyu Ho

ABSTRACT

Human umbilical cord matrix-derived stem cells (uMSCs), owing to their cellular and procurement advantages compared with mesenchymal stem cells derived from other tissue sources, are in clinical trials to treat type 1 (T1D) and type 2 diabetes (T2D). However, the therapeutic basis remains to be fully understood. The immunomodulatory property of uMSCs could explain the use in treating T1D; however, the mere immune modulation might not be sufficient to support the use in T2D. We thus tested whether uMSCs could exert direct trophic effects on β-cells. Infusion of uMSCs into chemically induced diabetic progression with a parallel preservation of islet size and cellularity, demonstrating the protective effect of uMSCs on β-cells. Mechanistic analyses revealed that uMSCs engrafted long-term in the injured pancreas and the engraftment markedly activated the pancreatic PI3K pathway and its downstream anti-apoptotic machinery. The pro-survival pathway activation was associated with the expression and secretion of β-cell growth factors by uMSCs, among which insulin-like growth factor 1 (IGF1) was highly abundant. To establish the causal relationship between the uMSC-secreted factors and β-cell survival, isolated rat islets were co-cultured with uMSCs in the transwell system. Co-culturing improved the islet viability and insulin secretion. Furthermore, reduction of uMSC-secreted IGF1 via siRNA knockdown diminished the protective effects on islets in the co-culture. Thus, our data support a model whereby uMSCs exert trophic effects on islets by secreting β-cell growth factors such as IGF1. The study reveals a novel therapeutic role of uMSCs and suggests that multiple mechanisms are employed by uMSCs to treat diabetes.

KEY WORDS: Stem cells, Diabetes, β-cells, Growth factors, IGF1

INTRODUCTION

Diabetes is one of the most prevailing diseases worldwide. It is characterized by hyperglycemia resulting from an absolute or relative insulin deficiency. The chronic hyperglycemia of type 1 and type 2 diabetes is associated with disabling damages of various organs such as the eye, kidneys, nerves and heart. The vast majority of cases of diabetes are type 1 diabetes (T1D) or type 2 diabetes (T2D), accounting for 5–10% and ~90–95%, respectively (American Diabetes Association, 2014). All T1D and one-third of T2D are caused by substantial deficits in insulin-producing β-cells. Insulin administration is the principal treatment for these insulin-dependent diabetic patients. However, insulin given exogenously does not exactly mimic its physiological secretion and carries the risk of the patient developing hypoglycemia or secondary diabetic complications. Replenishment of deficient β-cells through islet transplantation offers ideal therapeutic outcomes, yet the option is limited by the donor availability, graft survival and complications associated with the long-term use of immunosuppressants (Bromberg et al., 2007; Gruesnner and Gruesnner, 2013). Exploiting the regenerative capacity of stem cells has thus emerged as a novel approach to complement the current therapies.

Adult mesenchymal stem cells (MSCs), owing to their multipotent and modulatory properties, are intensely used in regenerative medicine (Volarevic et al., 2011). MSCs were first isolated from the bone marrow and have since been found in almost all postnatal tissues. Bone-marrow-derived MSCs (bMSCs) remain as the gold standard in clinical applications. However, bMSCs have specific problems that limit their use. For instance, bMSCs are present at a low frequency in the bone marrow; the cell-harvesting procedure is painful and invasive; and bMSCs exhibit a reduced ex vivo expansion capacity as the donor age increases (Batsali et al., 2013; Watson et al., 2015). On the other hand, human uMSCs can be obtained in large quantities; the cell harvesting is non-invasive; uMSCs are primitive and highly expandable (Troyer and Weiss, 2008); and they can undergo freeze-thaw for convenient off-the-shelf use. Studies have also shown that uMSCs do not spontaneously transform in culture (Tang et al., 2013) or form teratomas upon transplantation (Troyer and Weiss, 2008). These cellular features and procurement advantages make uMSCs a promising cell source in cell-based therapies.

Human uMSCs are currently under clinical investigations to treat diabetes (Berezin, 2014). Although the preliminary data from both T1D and T2D studies are promising (Hu et al., 2013; Kong et al., 2014), the mode of action remains to be understood. Because there is little evidence to indicate that MSCs are capable of differentiating into insulin-producing cells (Bassini et al., 2003; Lechner et al., 2004; Lee et al., 2006; Taneera et al., 2006), the contribution of differentiation to the treatment effect is likely minimal. MSCs possess the immunomodulatory activity (Abdi et al., 2008; Nauta and Fibbe, 2007), which is shown to be associated with ameliorating hyperglycemia in autoimmune diabetic mice (Bassini et al., 2012; Ezquer et al., 2012). The immunomodulatory function of MSCs could explain the therapeutic benefits seen in T1D, which is caused by immune-mediated β-cell destruction. Yet the mere immune modulation might not be adequate to explain the efficacy seen in T2D, where the β-cell death is chiefly caused by glucotoxicity (Bensellam et al., 2012). It seems that additional mechanisms underlie the therapeutic effect of uMSCs in the treatment of diabetes.


RESEARCH ARTICLE
**TRANSLATIONAL IMPACT**

**Clinical issue**
Diabetes is one of the most prevailing diseases worldwide. All cases of type I and one-third of cases of type 2 diabetes are caused by deficits in insulin-producing β-cells. The standard of care for these patients is insulin injection. Yet the treatment has many drawbacks. For instance, patients can experience life-threatening hypoglycemia and many develop disabling diabetic complications. Stem cells, owing to their capacity to differentiate into replacement cells and repair damaged tissues, have emerged as innovative therapies to complement current treatment options. Mesenchymal stem cells from the human umbilical cord matrix (uMSCs) have already shown clinical promise for the treatment of diabetes. However, given that there is little evidence that uMSCs can differentiate into insulin-producing cells in vivo, the mechanisms that underlie this promise are not fully understood.

**Results**
Here, the authors use a chemical agent that specifically destroys β-cells to generate diabetic rats and show that infusion of uMSCs into these diabetic rats prevents hyperglycemia by improving insulin secretion and increasing islet cell numbers (85% of islet cells are β-cells). Then, using in vivo cell tracking, morphological and biochemical techniques, they show that uMSCs engraft in the chemically injured pancreas and secrete abundant β-cell growth factors, including IGF1. They subsequently show that uMSC engraftment activates the PI3K signaling pathway, which suggests that growth factors secreted by the uMSCs might be the mediators between the protective effect of uMSCs and β-cell survival. To support this assumption, they show that uMSCs promote islet cell survival and insulin secretion in an in vitro islet and uMSC co-culture model, and that reduction of IGF1 secretion from uMSCs using RNA interference diminishes the protective effects of uMSCs on islets.

**Implications and future directions**
Human uMSCs are hypoimmunogenic – they either lack, or express low levels of, cell surface molecules capable of stimulating an immune response. In addition, uMSCs suppress lymphocyte activation and induce immune tolerance. These immunosuppressive properties of uMSCs are thought to underlie the therapeutic basis of uMSCs in the treatment of diabetes. The current findings demonstrate, however, that uMSCs can also exert direct protection against β-cell death by secreting β-cell growth factors such as IGF1. Thus, this study identifies a new therapeutic role for uMSCs and suggests that uMSCs might operate through multiple mechanisms to treat diabetes.

MSCs secrete a broad spectrum of growth factors and extracellular matrix (ECM) molecules that are increasingly thought to be central to the tissue repair (Baraniak and McDevitt, 2010). These biomolecules can act in a paracrine fashion to promote angiogenesis and ECM remodeling (Baraniak and McDevitt, 2010). Neovascularization has been observed in the damaged pancreas after bMSC transplantation (Ito et al., 2010; Rosengren et al., 2009). Here, we tested whether uMSCs exerted direct trophic effects on β-cells by using the chemically induced diabetes model as well as the ex vivo islet and uMSC co-culture system.

**RESULTS**
Systemic administration of uMSCs prevents hyperglycemic progression and body weight loss of STZ-induced diabetic rats
The diabetic rat model was established by a single-dose injection of streptozotocin (STZ), which specifically destroys β-cells (Lenzen, 2008). The STZ administration resulted in a rise in blood glucose from euglycemic 6.06 mmol/l to 24.24 mmol/l in 7 days, representing a fourfold increase. Human uMSCs were then systemically administered (day 0). The blood glucose of untransplanted rats continued to rise, reaching 30.60 mmol/l at day 18, but stabilized thereafter (Fig. 1A). The uMSC transplantation retarded hyperglycemic progression at day 6, with the blood glucose becoming modestly lower thereafter (Fig. 1A). The cell treatment also prevented the body weight loss (Fig. 1B). The findings support our previous observation that uMSCs offer therapeutic benefits to diabetic rats (Wang et al., 2014).

The rat-specific insulin and C-peptide in the serum were measured at day 42 after transplantation. Both parameters were doubled in value after the cell treatment but neither was fully restored, with insulin at 53% and C-peptide at 79% of normal controls (Fig. 2A). Histological analysis showed that untransplanted rats had a markedly reduced islet size and cell number (Fig. 2B); uMSC transplantation partially restored the morphology, with the islet size at 63% and cell number at 42% of normal controls (Fig. 2C). Immunohistochemical analysis further supported the improved islet mass and insulin secretion in transplanted rats (Fig. 2B). Consistent with our previous report (Wang et al., 2014), expression of the human insulin gene was undetectable in the transplanted rat pancreas (data not shown). The data demonstrate that the therapeutic benefits conferred by uSMC transplantation resulted from the preservation/restoration of islet morphology and function.
Human uMSCs home to the injured pancreas and express genes and proteins of β-cell growth factors

One of the MSC characteristics is the ability to preferentially home to injured tissues upon systemic administration (Deak et al., 2010). To ascertain the pancreatic localization of uMSCs in the diabetic rats, uMSCs were labeled with the fluorescent dye CM-Dil. Studies have shown that CM-Dil does not affect cell viability, proliferation or differentiation (Weir et al., 2008). The in vitro cell labeling efficiency was >90% and labeled uMSCs were administered to the diabetic rats. The pancreas as well as the lung and liver, which are rich in blood supply, were examined at 21 and 42 days after the cell infusion. The CM-Dil fluorescence was found clustering around damaged islets at both time points (Fig. 3A). In contrast, little or no fluorescence was found in the lung or liver (Fig. 3A). Infusion of labeled uMSCs ameliorated hyperglycemia and body weight loss of diabetic rats to a similar degree as with unlabeled uMSCs (data not shown), confirming that the labeling did not affect uMSC function.

Because of the pancreatic localization of uMSCs, the pancreas of transplanted rats was screened for expression of human-specific genes of insulin-like growth factor 1 (IGF1), hepatocyte growth factor (HGF) and platelet-derived growth factor alpha (PDGFA) by qPCR. These growth factors, with the cognate receptors expressed on β-cells, have been shown to stimulate β-cell expansion and protect against STZ-induced β-cell death in mice (Chen et al., 2011; Dai et al., 2005; Garcia-Ocana et al., 2000; George et al., 2002). In addition, we examined the expression of tissue inhibitor of metalloproteinase 1 (TIMP1), which promotes β-cell survival under inflammatory conditions and is highly produced by human adipose-derived MSCs (aMSCs) (Kono et al., 2014). The mRNAs of human IGF1, HGF and PDGFA were detected at both 21 and 42 days after the cell transplantation, with the IGF1 mRNA level being the highest (Fig. 3B). Assessment of the protein secretion in the serum using the human-specific ELISA showed substantial levels at both time points, with the serum levels of human IGF1, HGF and PDGFA at 704, 142 and 55 pg/ml, respectively, at day 42 (Fig. 3C). These factors showed little change at either the mRNA or protein levels between the two time points (Fig. 3B,C).

The assay specificities were confirmed by undetectable levels of corresponding human mRNAs or proteins in the untransplanted rat (Fig. 3B,C). Neither the mRNA nor protein of human TIMP1 was detected in transplanted rats (Fig. 3B,C).

Human uMSCs activate the PI3K pathway in the injured pancreas

IGF1, HGF and PDGFA are shown to activate the PI3K and ERK1/2 pathways in isolated islets or β-cell lines (Chen et al., 2011, 2014;
Dai et al., 2005). The abundant production of the growth factors by uMSCs prompted us to assess the activation state of the two signaling pathways in the pancreas. Shown in Fig. 4A, a marked Akt, but not ERK1/2, phosphorylation was seen in the transplanted pancreas. To rule out the contribution of human pAkt owing to the uMSC engraftment, we assessed the relative mRNA levels between the rat and human GAPDHs in the pancreas. The ratio, as evaluated by qPCR, was >1000. Thus, the human pAkt, if any, was negligible in the pAkt level observed. Assessment of the downstream anti- and pro-apoptotic effectors of Bcl-2 and caspase-3 showed a 2.1-fold elevation and 52% reduction, respectively, in transplanted compared with untransplanted rats (Fig. 4A). The data demonstrate that uMSC transplantation activated the pancreatic PI3K pathway with subsequent activation of the anti-apoptotic machinery. Because of high systemic levels of the growth factors, especially uMSC-secreted IGF1, we evaluated the impact on pAkt and pERK1/2 levels in the liver. In contrast to the pancreas, the liver pAkt exhibited a modest reduction in transplanted rats as compared with the untransplanted control (Fig. 4B). The liver pERK1/2 level did not differ between the two groups (Fig. 4B).

Fig. 4. Assessment of pathway activation in the pancreas and liver of uMSC-transplanted rats. Pancreatic or liver tissues from the uMSC-transplanted and untransplanted rats (n=6/group) were collected at 42 days. (A) Levels of pAkt, pERK1/2, Bcl-2 and caspase-3 in the pancreas were analyzed by western blots and quantification was normalized against β-actin. (B) The liver pAkt and pERK1/2 were assessed as in A. *P<0.05 and ***P<0.001 were compared with the STZ group.
Human uMSCs improve the viability and function of rat islets in co-culture

To support the direct trophic effect of uMSCs on islets (containing 85% β-cells) and that the secreted factors mediated the effect, uMSCs were co-cultured with isolated rat islets in transwell plates, in which only soluble factors were freely passable between the two cell compartments. The islet culture alone exhibited a progressive cell death; the cell viability at day 6 was 37% of that of day 1 but was improved by 42% of this day-6 value in the co-culture (Fig. 5A). Similarly, the islet culture alone exhibited a progressive decline in insulin secretion; the insulin level at day 6 was 57% of that of day 1 and was enhanced by 21% in the co-culture (Fig. 5B). The data demonstrate that uMSCs exerted direct pro-survival effects on islets.

To assess the secretion of IGF1, HGF and PDGFA by uMSCs in culture, uMSCs were cultured alone or co-cultured with islets and the supernatants were taken for the measurement using the human-specific ELISA. As shown in Fig. 5C, these factors were produced with the same rank order as that observed in vivo, with IGF1 being the highest. Notably, their productions were augmented by co-culturing with islets. The IGF1 level in the co-culture at day 6 was 43 ng/ml, which was sevenfold higher than HGF and 24-fold higher than PDGFA (Fig. 5C). The assay specificity was again confirmed by undetectable levels of the human proteins in the rat islet culture alone (Fig. 5C). The TIMP1 production by uMSCs in culture was by undetectable levels of the human proteins in the rat islet culture than PDGFA (Fig. 5C). The assay specificity was again confirmed by undetectable levels of the human proteins in the rat islet culture alone (Fig. 5C). The TIMP1 production by uMSCs in culture was by undetectable levels of the human proteins in the rat islet culture.

The uMSC-secreted IGF1 promotes the islet cell survival and function

Because of the abundant production of human IGF1 both in vivo and in vitro, we investigated the contribution of uMSC-secreted IGF1 to islet viability and function by using siRNA knockdown. Our preliminary studies showed that transfection of the IGF1-specific siRNA to uMSCs gave the best protein knockdown at 48 h (data not shown). Thus, uMSCs transfected for 48 h with either the specific or scrambled siRNA were utilized in the co-culture studies. As shown in Fig. 6A, the IGF1 production was persistently reduced by the IGF1 siRNA in the 6-day co-culture. The area under the curve of the IGF1 production in the 6-day specific knockdown co-culture was reduced by 27% as compared with the scrambled control (Fig. 6B). In parallel assays, the islet viability was diminished in both 3-day and 6-day co-cultures, with a 17% reduction in the 6-day co-culture (Fig. 6C); the insulin secretion was reduced by 14% in the 6-day co-culture (Fig. 6D). Thus, the data establish the crucial contribution of uMSC-secreted IGF1 to islet survival and function.

DISCUSSION

In this study, the diabetic model was generated by a single large-dose injection of STZ. Under such conditions, STZ destroys β-cells via DNA alkylation (Lenzen, 2008). The model differs from the autoimmune diabetic model of non-obese diabetes (NOD) or multiple low-dose STZ-induced diabetes, where islet inflammation is the salient pathological feature (Bassi et al., 2012; Ezquer et al., 2012; George et al., 2002; Leiter, 1982; Paik et al., 1980). The non-inflammatory diabetic model used here, which was confirmed by the lack of inflammatory cell infiltrates in the islet of untransplanted but STZ-treated rats (Fig. 2B), allows us to better examine the direct trophic effect of uMSCs on β-cell survival with fewer complications from their immunomodulatory activities. In this study, uMSC transplantation retarded the hyperglycemic progression 6 days after the treatment, yet the treatment only modestly reversed hyperglycemia despite the extended 42-day treatment. Multiple factors might influence the efficacies. Because the transplanted cells were apparently alive and functional for the entire 42 days (discussed below), we suspect that the delayed treatment intervention (i.e. the glucose level was already fourfold higher than normal) limited the efficacy. An earlier intervention with more residual islets left for regeneration might produce better therapeutic outcomes.

Human uMSCs were found localized to the injured pancreas but not the lung or liver. The finding concurs with the general homing characteristics of MSCs to injured tissues (Deak et al., 2010) and the homing pattern of human bMSCs in diabetic mice (Lee et al., 2006). Notably, the xenogeneic graft survived long-term in the absence of immunosuppressants. The successful engraftment was further supported by the stable expression of human genes in the rat pancreas. Because of the lack of inflammatory infiltrates in and around the islet of transplanted rats (Fig. 2), uMSCs did not seem to have provoked the host immune response. Our data support the long-held notion that uMSCs are hypoimmunogenic (Weiss et al., 2008, 2006), which, coupled with the immunosuppressive property...
IGF1 siRNA

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in the whole-tissue extracts used in the analysis. 

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has also been observed (Kono et al., 2014). Although the molecular 

islet cells. An augmentation of aMSC-secreted factors by islet cells 

remains to be elucidated, the induced production by islets, in 

in conjunction with the long-term survival of uMSCs in the injured 

pancreas, could argue in favor of cell-based over factor-based 

strategies in diabetes treatment (Baranaki and McDevitt, 2010). 

The β-cell growth factors secreted by uMSCs are pleiotrophic 

(Clemmons and Maile, 2003; Nakamura et al., 2011; Ronnstrand, 

2004). Whether their systemic exposure leads to undesired effects 

should be of clinical concern. Transplantation of uMSCs reduced 

the pAkt level and had no effect on pERK1/2 in the liver of 

transplanted rats, which might suggest a low potential in the 

proliferative growth of normal tissues. IGF1 is noted to act mainly 

on injured tissues (Agudo et al., 2008). Nevertheless, the liability 

remains to be elucidated, the induced production by islets, in 

consistent with the stable mRNA expression in the pancreas, 

human IGF1, HGF and PDGFA proteins were stably detected in the 

serum of transplanted rats, among which IGF1 was most abundant. 

The local concentrations in the pancreas, where the proteins were 

produced, could be even higher and might thus act effectively as the 

β-cell trophic factors. In support, a marked activation of the 

pancreatic PI3K pathway and downstream anti-apoptotic machinery 

was evident. We also analyzed the pro-growth potential of the PI3K 

activation by assessing the downstream cell-cycle genes such as 

cyclin D1, cyclin D2, p21 and p27. Expression of these genes is 

involved in 

β-cell proliferation (Heit et al., 2006). We saw ~twofold upregulation of the cyclin D2 mRNA level but expression of the 

other genes was unchanged in the pancreas of transplanted rats (data 

not shown). The limited expression changes of cell-cycle genes 

imply that the β-cell expansion was restrained, which agrees with the 

modest reversal of hyperglycemia observed. These biochemical 

data suggest that the anti-apoptotic activity of uMSCs is the main 

contributor to the improved islet size found in the histological study. 

Despite the marked activation of the pancreatic PI3K pathway, we 

did not see pancreatic ERK1/2 activation. We suspect that, in 

contrast to the fundamental role of PI3K in β-cell survival (Bernal- 

Mizrahi et al., 2001; Tuttle et al., 2001), the role of ERK might be 

auxiliary and that a modest activation in β-cells could be masked by the 

whole-tissue extracts used in the analysis. 

The siRNA knockdown experiment showed that a 27% reduction 
in uMSC-secreted IGF1 led to a 17% reduction of β-cell survival. 

Although the study illustrates the major role of uMSC-secreted 

IGF1, the disproportionate reduction implies that other β-cell 

trophic factors, although playing minor roles, are required to fully 
support the β-cell survival. The uMSC-secreted HGF and PDGFA, 

both produced in the nM range in co-cultures and detectable in 

transplanted rats, could be the other possible mediators. It is worthy 
of noting that secretion of these factors by uMSCs was induced by 
islet cells. An augmentation of aMSC-secreted factors by islet cells 

has also been observed (Kono et al., 2014). Although the molecular 

mechanism underlying the cross-talk between uMSCs and islets 

remains to be elucidated, the induced production by islets, in 

Table 1. The human primer sequences used in qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5′-3′)</th>
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<tbody>
<tr>
<td>IGF1</td>
<td>Sense TGGATGCTTCTTACGTTGCTG</td>
</tr>
<tr>
<td></td>
<td>Antisense TGTTAGAGGGGCTGCTAGAC</td>
</tr>
<tr>
<td>HGF</td>
<td>Sense GAAAACTACGCTCTGTTTCC</td>
</tr>
<tr>
<td></td>
<td>Antisense TGTGTTGCTGATGAATGGA</td>
</tr>
<tr>
<td>PDGFA</td>
<td>Sense CCCCCGGCATTCGGGGAAAGGAAGG</td>
</tr>
<tr>
<td></td>
<td>Antisense TCCTGTACCTTGGATGCAATT</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Sense CAGACCACCTTATACACGCGT</td>
</tr>
<tr>
<td></td>
<td>Antisense GATAAACAGGGGAAACACTGTCG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense CACCATCTTCCAGAGGCGGA</td>
</tr>
<tr>
<td></td>
<td>Antisense TCAGCAGAGGGGGCCAGGA</td>
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shown to stimulate regulatory T (Treg) cells and suppress autoimmune diabetes in mice (Anguela et al., 2013; Bilbao et al., 2014). In the study, the recombinant human IGFI1 delivered by minipumps to maintain a serum level of ∼350 pg/ml is efficacious in the treatment of diabetes (Bilbao et al., 2014). Using this dose as a gauge, IGFI1 stably produced by uMSCs with the serum level of 704 pg/ml and possibly higher levels in the pancreas might exert dual trophic and immunomodulatory functions to preserve/ regenerate islets. Notably, IGFI1 might also contribute to the efficacy by enhancing insulin sensitivity in diabetic individuals (Clemmons, 2012).

In conclusion, we have two key novel findings in this study: (1) uMSCs exert trophic effects on β-cell survival by activating the pancreatic PI3K pathway; and (2) the trophic effect is mediated by uMSC-secreted factors, among which IGFI1 might play a major role.

MATERIALS AND METHODS

Animals

Sprague Dawley (SD) rats were purchased from the Animal Center of Shantou University Medical College. Animals were housed under standard conditions with food and water given ad libitum. All experimental procedures were conducted in accordance with the guidelines published in the Ministry of Science and Technology of China for the Care and Use of Laboratory Animals and approved by the Animal Care and Welfare Committee of Shantou University Medical College.

Isolation and expansion of uMSCs

Protocols for obtaining human umbilical cords were reviewed and approved by the Medical Ethics Committee of Shantou University Medical College (SUMC2013XM-0036). The umbilical cord donors gave informed consent. Isolation of uMSCs was carried out as described (Tang et al., 2013). Isolated uMSCs were cultured at 37°C with 5% CO2 in growth medium containing GlutaMAX™-1, 10% FBS and 1% penicillin-streptomycin (all from Gibco, USA). The cells were expanded for 2–4 passages before use.

Induction of diabetes and cell transplantation

Eight-week-old SD rats were given the intraperitoneal injection of STZ at 65 mg/kg body weight (Sigma, USA) dissolved in 0.01 M citrate buffer at pH 4.2 or the buffer alone. Blood glucose was measured daily with the Glucotrend glucose meter (Roche Diagnostics, Switzerland) for the first 7 days to confirm the establishment of diabetic conditions. At day 7 after the STZ treatment, rats were administrated, via the tail vein, either 3×10^6 uMSCs in 200 μL PBS or the vehicle. Blood glucose and body weight were measured every 3 days thereafter for 42 days.

Tissue collection

Rats were euthanized using 10% chloral hydrate at 0.4 ml/100 g body weight (Sigma, USA) and decapitated. The pancreas and liver from each rat were quickly removed. A main portion of the pancreas was fixed with 4% paraformaldehyde and embedded in paraffin for histological examination. Paraffin sections (4-µm thickness) were stained with hematoxylin and eosin (HE) for histological examination. For the immunohistochemical detection of insulin, paraffin sections were incubated with the rabbit anti-rat insulin antibody (cat#4590, Cell Signaling Technology, USA) at 1:100 dilution, followed by incubation with the HRP-conjugated anti-rabbit secondary antibody (cat#PV-9000, ZSGB-BIO, China) and development with the HRP-AEC kit (GENMED Sciences, USA). These sections were counterstained with hematoxylin and examined under the light microscope (Leica DM2000, Germany). For measurement of the islet area, islets in HE sections were identified and the islet area was measured using Image-Pro software (Media Cybernetics, USA). The average islet area from each rat was determined from 4–5 sections spaced >200 μm apart. The cell number per islet area in the same section was counted using Image-Pro (Media Cybernetics, USA) and the average cell number per islet from each rat was determined in the same manner as that of the islet area.

Quantitative PCR

Total RNAs of the pancreases were extracted using TRIzol according to the manufacturer’s instructions (Life Technologies, USA). Total RNAs (1 μg) were reverse-transcribed using the Transcriptor First-strand cDNA Synthesis kit (Takara, Japan) and qPCR was performed using the SYBR® Premix Ex Taq™ II kit (Takara, Japan). Human-specific primers for the indicated genes were designed using the GenBank database and are listed in Table 1. Reactions were run in the 7500 Fast Real-Time PCR System (Applied Biosystems, USA) under the following conditions: 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 30 s. Quantification of relative mRNA levels was performed using the ΔΔct method (Livak and Schmittgen, 2001) with the 7500 Software v2.0.6 (Applied Biosystems, USA).

Western blot analysis

Proteins were extracted from tissues using the RIPA buffer (Cell Signaling Technology, USA) containing 1 mM NaF and 1 mM phenylmethylsulfonyl fluoride (PMFS; Solarbio, China). Protein concentrations were determined using the BCA assay (Thermo Scientific, USA). Equivalent amounts of protein samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The primary antibodies used were rabbit anti- phospho-Akt (cat#4060s), rabbit anti-phospho-ERK1/2 (cat#4370s), rabbit anti-caspase-3 (cat#9664) and rabbit anti-Bcl-2 (cat#2870) (all from Cell Signaling Technology, USA; used at 1:1000 dilution) or the mouse anti-actin (TA09, ZS-Bio, China; used at 1:2000). The secondary antibodies used were goat anti-rabbit IgG (cat#926-08511) or rabbit anti-mouse (cat#926-32211) antibodies (LI-COR, USA) containing 1 mM NaF and 1 mM phenylmethylsulfonyl fluoride (PMFS; Solarbio, China). A total of 70-100 rat islets were used per well. For co-culture experiments, 1×10^5 uMSCs were seeded into the lower chamber of the Transwell plate and grown to 70-80% confluence. The human-specific siRNA knockdown

Human uMSCs were labeled with CM-Dil according to the manufacturer’s instructions (Invitrogen, USA). The labeling efficiency was examined under fluorescent microscopy and the viability of labeled cells was confirmed by their ability to grow in vitro. After labeling, cells were washed with PBS and 3×10^6 cells were injected into the STZ-treated rats as above. Rats were euthanized at the indicated times and paraffin sections (4-µm thickness) of selected tissues were stained with DAPI (Vectorshield, USA) for 5 min at room temperature. The CM-Dil and DAPI staining was visualized and acquired by the fluorescent microscopy (Olympus f1000, Japan).
Cell viability and ELISA

Cell viability was measured by the colorimetric MTS assay (absorbance at 490 nm) according to the manufacturer’s instructions (Promega, USA). Rat insulin and C-peptide in sera and culture supernatants were measured using the ultrasensitive rat-specific ELISA kit as specified (MeliBio, China). The 24-h basal insulin secretion in rat islet cultures was assessed as follows: the medium was changed on the day specified and the rat insulin level in the supernatant was measured 24 h later. The levels of human IGF1, HGF, PDGFA in the serum and supernatants were quantified using the human-specific ELISA kits according to the manufacturer’s instructions (Abcam, USA).

Statistical analysis

The Student’s t-test and one-way ANOVA were used for group comparisons (GraphPad Software Inc., USA). Data were expressed as the mean±standard error of the mean (s.e.m.). P<0.05 was considered statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.Z. and O.H. designed and performed the experiments, analyzed data, and prepared the manuscript. F.C. and J.Z. contributed to cell culture, tissue collection and data analysis. J.Guo contributed to siRNA knockdown. L.M., J.Gu and H.W. contributed to the siRNA knockdown. L.M., J.Gu and H.W. contributed to the data analysis. G.H. conceived, designed and directed the study, and wrote the manuscript. F.C. and J.Z. contributed to cell culture, tissue collection and data analysis. Y.Z. and Q.H. designed and performed the experiments, analyzed data, and wrote the manuscript.

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