



Regulation of blood glucose by administration of allogenic bone marrow derived mesenchymal stem cells into diabetic rats through modulating regeneration of both α and β islet cells

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Abstract

Background: Transplanting bone marrow derived mesenchymal stem cells (MSCs) have positive effects in treating many disease conditions including diabetes. To investigate the effects of systemic administration of MSCs on blood glucose changes, we studied the changes of islet β and α cells in STZ-induced diabetic rats following systemic MSCs administration.

Methods: MSCs were isolated from rat bone marrow, characterized, expanded *in vitro* and labeled with GFP by lentivirus transfection. 2×10^6 MSCs were injected into the rats via caudal vein at 10 days following STZ administration. The fasting glucose serum levels were measured following the treatment. Animals were terminated at day 21 and 35 following STZ injection. The insulin, Pdx1, glucagon and somatostatin expressing cells in the pancreatic sections were quantified by immunofluorescence methods.

Results: We found that the blood glucose levels in the rats receiving MSCs were significantly reduced compared to the control group at day 21 following STZ treatment, and it reached to the similar high glucose level again at day 28. The injected MSCs were found in the pancreatic tissues, and the islets in the treatment group were partially restored in contrast to the control group. There were greater numbers of Pdx1+Insulin- cells in the MSCs treatment group, suggesting the ongoing regeneration of islet. The number of α cells was significantly higher in the MSCs-treated group and untreated control group than that of normal group, which may associate with higher blood glucose level in later stage of MSCs administration. In addition, MSCs were co-cultured with the isolated rat islets and islet-like cells (INS1) *in vitro* and we found that the expression of Cdk4 was up-regulated in the islets when co-cultured with MSCs.

Conclusions: We concluded that transplanting allogenic MSCs may promote the regeneration of the injured islets, but the underlying mechanisms need further investigation.

Keywords: Mesenchymal stem cells, islet regeneration, islet α and β cells, cdk4

Background

Bone marrow derived mesenchymal stem cells (MSCs) are adult stem cells, with high proliferative potential and ability to differentiate into a variety of cell types [1]. MSCs have been demonstrated to support long-term growth of HSCs hematopoietic stem cells and maintain hematopoietic and skeletal homeostasis [2]. Previous studies showed MSCs transplantation resulted in improvement of diabetes conditions, such as reducing blood glucose levels [3-5], partially because of the immunosuppressive effects of MSCs on T cells, increasing pancreatic islet β cell mass, preventing of β cell death [6] and promoting islet regeneration [7]. However, the effects of MSCs transplantation on islet cells are still not well understood. Islet is mainly consisted of α , β , δ cells, which are responsible for

secreting of insulin, glucagons and somatostatin respectively [8]. Insulin reduces the blood glucose, whereas glucagon has the opposite role of increasing blood glucose. It is known that type I diabetes is due to the reduction of absolute quantities of islets. Understanding the effects of MSCs transplantation on islet cells will help to develop more effective treatment strategies.

Islet cells were differentiated from endocrine progenitors, the Pdx1⁺ pancreatic progenitors [9,10]. Knocking out Pdx1 in mouse resulted in complete loss of whole pancreas including islets [9]. Pdx1 expression became restrictive in islet β cells during developmental process [11]. Islet β cells neogenesis from duct cells are accompanied by the re-activation of Pdx1 in the injured tissues [12]. A substantial work has been done to elucidate the mechanism of the islet proliferation and

regeneration. MSCs may exert positive effects by promoting the proliferation of islet β cells. Hess and his colleagues found that bone marrow MSCs could stimulate host pancreatic regeneration rather than direct trans-differentiation into insulin secreting cells [13].

As one of cell cycle regulators, cyclin kinase 4 (Cdk4) plays a key role in the proliferation of islet [14,15]. Expression of the p16^{INK4a}, a potent inhibitor of Cdk4 resulted in declining islet regeneration [16]. Cdk4 can be used as an indicator for islet regeneration. In the present study, we administrated GFP-labeled MSCs into the diabetic rats and found that injection of MSCs could reduce blood glucose level for a period and there were evidence of islet regeneration with increased expression of Cdk4 in pancreatic tissues. The result of our study indicated that administration of allogenic MSCs in diabetic rats could reduce blood glucose, through regulating islet α and β cells proliferation. However, the optimal dose and time of MSCs administration needs further careful investigation.

Methods

Subjects

Adult wistar rats (male, 200–250 g) were used for MSCs isolation and diabetic model establishment. The animal experimental protocols were approved by the Animal Care and Use Committee of China Medical University, Shenyang, China and The Chinese University of Hong Kong, Hong Kong.

Study design

Rats (n=30) were fed routinely, then fasted for 12 hours, blood was taken from the caudal veins, and the blood glucose was monitored using semi-automatic biochemical analyzer (Vital Science). To establish diabetic rat model, Streptozotocin (STZ, Sigma) prepared in 0.1M citrate buffer (pH4.5) was injected intraperitoneally at the dose 50mg/kg as described previously [17]. Streptozotocin could induce diabetes within 3 days by destroying the β cells [18]. The blood glucose was then measured at 5 and 7 days after injection to confirm the onset of hyperglycemia. At day 10 following STZ injection, the GFP-MSCs (2×10^6 per rat) were injected via caudal veins. The diabetic syndromes were observed, and the fasting blood glucose level was monitored at day 21, 28, 35 following STZ injection.

Isolation and culture of bone marrow MSCs

MSCs were isolated from male adult wistar rats as previously described. In brief, the rats were terminated, and the tibias and femurs were removed aseptically. Bone marrow were collected by density gradient centrifugation (Histopaque 1077, Sigma) and cultured in LG-DMEM (Gibco) and 10% FCS (Hyclone), 5% CO₂ at 37°C. The non-adherent cells were discarded after 24 hours. Fresh media were replenished every three days. MSCs surface markers such as CD14(-), CD34(-), CD44(+), CD45(-), CD73(+), CD90(+) were confirmed by flow cytometry.

GFP labeling of MSCs

MSCs were labeled with GFP expressing gene by lentivirus transfection as previously described [19]. In brief, pGCL-GFP, pHelper 1.0 and pHelper 2.0 plasmids were cotransfected into 293T cells by Lipofectamine 2000 (Invitrogen). The supernatant containing lentivirus was harvested 48h and 72h after transfection and concentrated by centrifugal filter (Centricon Plus-20, Millipore) following manufacturer instructions. Lentivirus titer was determined by quantifying GFP-expressing cells 48h after transfection of 293T cell with serial diluted concentration. For transduction, 1×10^5 MSCs were seeded into 6-well plate and incubated with lentivirus and 8 μ g/mL polybrene in the incubator for 24 h.

Immunocytochemistry and islet area measurement

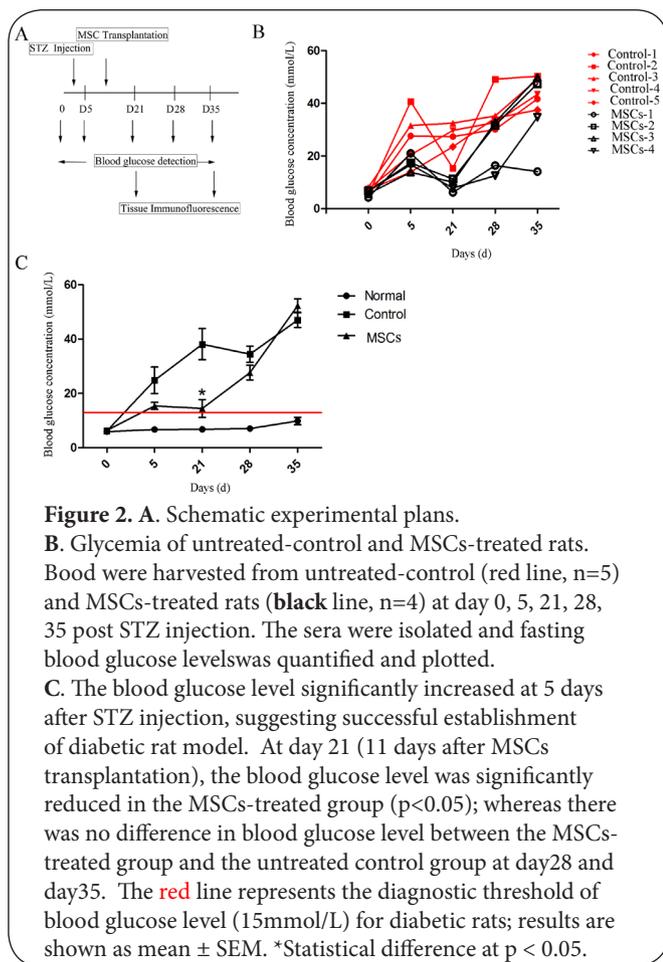
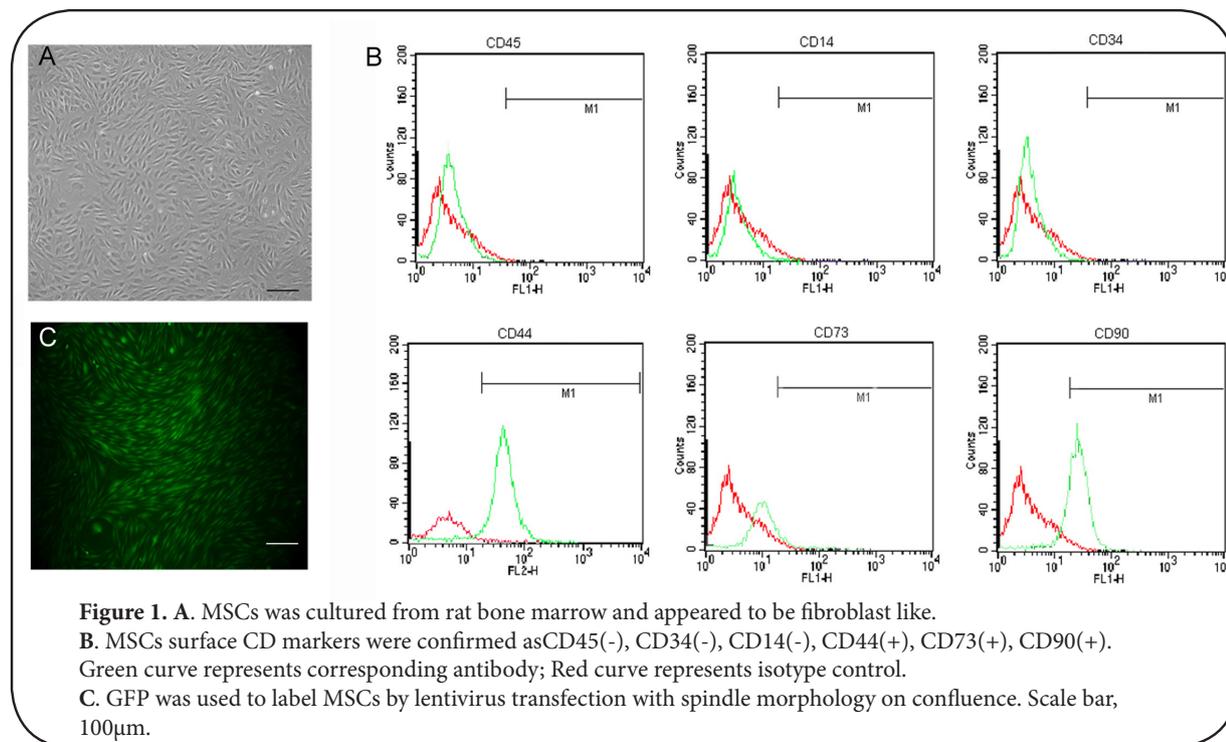
Rats were sacrificed at day 21 and day 35 following STZ injection. Pancreas were dissected and fixed with 4% paraformaldehyde. Frozen sections (8 μ m) of pancreas were blocked with 5% BSA for 1 hour, and incubated with the primary antibodies overnight at 4°C. After washing, the sections were then incubated with secondary antibodies at room temperature for 1 hour. Counterstained with DAPI, sections were observed under the fluorescence microscope (Olympus BX51). Primary antibodies were mouse anti-insulin (dilution 1:200, Santa Cruz, USA), rabbit anti-insulin (dilution 1:200, Santa Cruz), mouse anti-somatostatin (1:200 dilution, Santa Cruz), rabbit anti-Glucagon (1:200 dilution, Santa Cruz) and rabbit anti-Pdx1 (1:1000 dilution, Abcam). The insulin positive cells areas from the sections were randomly selected and measured using the software Image Pro-plus 6.0. The statistical analysis for significance was done with Students t-test; p < 0.05 was considered statistically significance.

Isolation and culture of rat islets

The rat islet isolation procedure was followed as previously reported [20]. Briefly, the common bile duct was isolated and injected with collagenase P (Roche). The pancreas were isolated and incubated at 37°C for 15 min. The digested tissues were suspended in histopaque 1077 (Sigma) and overlaid using Hanks solution and centrifuged at 1750g 20min. The islets at the interface were collected after centrifugation and cultured in 10% FBS 1640 medium.

Co-culture of islets and MSCs

The fourth passage MSCs were plated at the density of 4×10^4 per well in six-well plastic culture plates, supplemented with 3ml 10% FBS in 1640 medium. The cells became adherent after 6 hours and expanded to monolayer. The medium was changed to the mixture of LG-DMEM and 1640 equally containing 10% FBS after 3 days. Simultaneously, rat islets were inoculated in the insert (BD) and subsequently placed in the well with MSCs at the bottom.



Quantitative PCR of Cdk4

Islets co-cultured with MSCs for 5 days were harvested. Total RNA of islet and INS1 rat insulinoma cell line (CCTCC, China Center for Type Culture Collection) was then extracted using the Trizol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized by reverse transcription by Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega). Quantitative PCR was performed to using *SYBRPremix Ex Taq* (Takara) to examine the expression of Cdk4 (5'-ACAGCTACCAGATGGCCCTCAC-3') according to the manufacturer's instructions. The relative expression of Cdk4 was analyzed by ddCt method by normalizing to endogenous GAPDH.

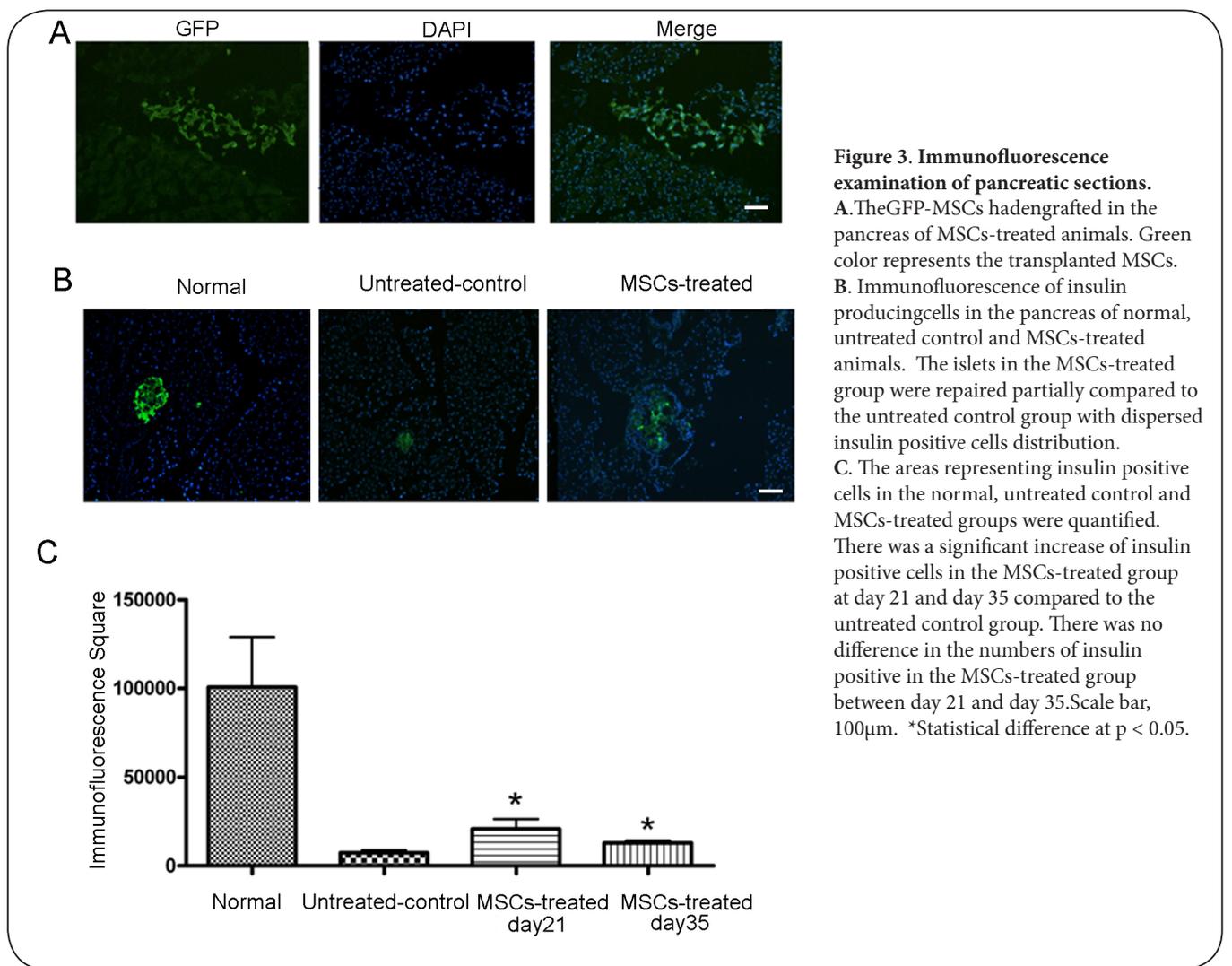
Results

Isolation, identification and transfection of MSCs

MSCs isolated from rat bone marrow presented fibroblast like morphology and proliferated fast (Figure 1A). Passage 3 or 4 MSCs were used for GFP labeling by lentivirus transfection and MSCs were high efficiently labeled with GFP (Figure 1B). Transfection did not alter cell morphology of MSCs. The cell surface markers were examined by flow cytometry and confirmed that the expression of CD45, CD34, CD14 was negative, whereas the expression of CD44, CD73 and CD90 was positive (Figure 1C).

Changes of blood glucose level following MSCs administration

The detailed experimental procedures are highlighted in Figure 2A. For STZ-induced diabetic rat model, when the



blood glucose level was greater than that of normal rat (e.g. $>15\text{mmol/L}$) for two times, the rats were regarded as diabetes [17]. All STZ given rats were considered diabetic at day 10, and the GFP-MSCs were then injected at day 10. Blood glucose level was monitored weekly thereafter till termination at day 21 and 35 following STZ injection. The fasting blood glucose level of each rat from untreated-control and MSCs-treated group were plotted (Figure 2B). We found that the blood glucose level in the MSCs treatment group was significantly lower than that of the untreated control group at 11 days following MSCs injection (day 21 after STZ injection), although it was still higher than that of the normal level. However, the blood glucose level increased again at day 28 following STZ injection, with no significant difference between the MSCs treated and non-treated control group (Figure 2C).

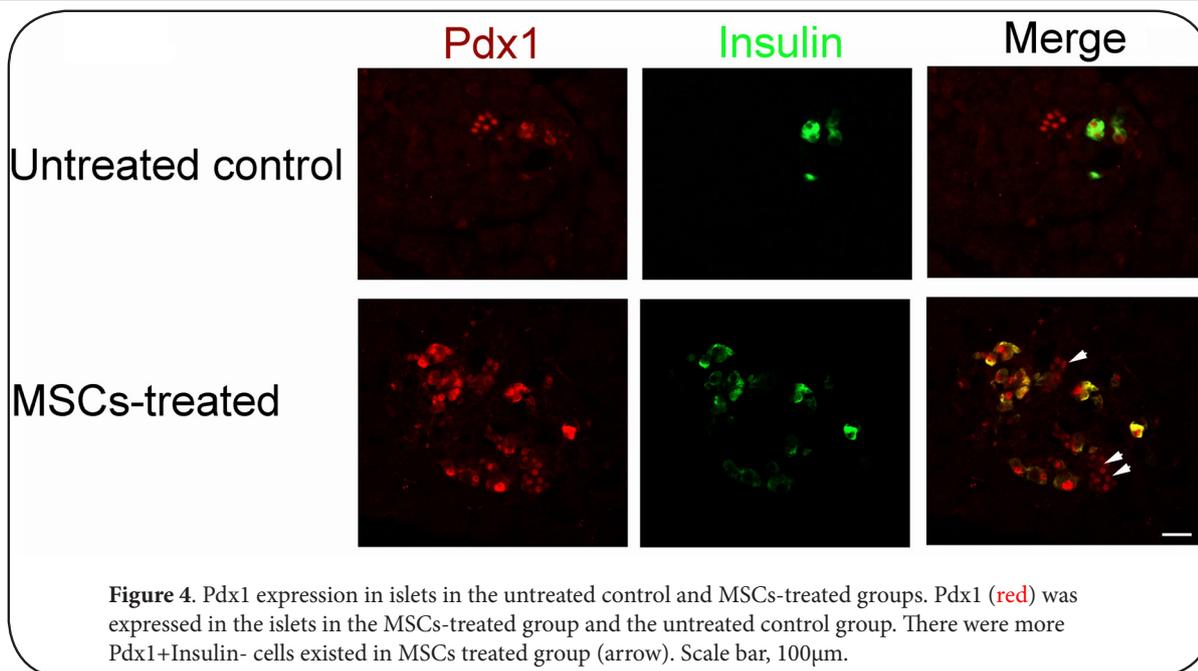
Injected MSCs engrafted the pancreas and promoted the regeneration of islets

To investigate the fate of injected MSCs, pancreas sections

were examined. The GFP labeled MSCs can be found engrafted into the pancreatic tissues (Figure 3A). The islets in the MSCs group were significantly restored compared to the control group, as shown by the pancreatic tissue immunofluorescence (Figure 3B). Under $\times 400$ magnifications, 5 immunofluorescence areas were selected randomly, measured and analyzed using the Imaging Pro-6 software. The immunofluorescence areas in the MSCs group at day 21 (following STZ injection) were significantly larger than that of the control group, while the areas remained unchanged at day 35 following STZ injection (Figure 3C).

MSCs administration enlarged the Pdx1+ cell population within the islets

Pdx1 is an important transcription factor for the development of islet and islet function. It is only expressed in the pancreatic progenitor cells and become demolished in the mature islets. Pdx1 was expressed in the insulin expressing cells both in the control and MSCs injection group (Figure 4). There were



a proportion of Pdx1⁺ cells in the insulin⁻ cells within the islet. The numbers of Pdx1⁺insulin⁻ cells were substantially greater in the MSCs treatment group than that of the control group (Figure 4, arrows and arrow heads), suggesting that Pdx1 was re-activated in the insulin⁻ cells that may contribute to islets restoration.

The imbalance between α and β cells contributes the re-elevated blood glucose level

Blood glucose is mainly regulated by insulin and glucagons. After a short-term reduction of blood glucose following MSCs transplantation, glucose level re-elevated again. We hypothesized that the glucagons secreting α cells may also increase following the MSCs transplantation. To examine this, we compared cell numbers of glucagon secreting α cells and glucose irrelevant somatostatin secreting δ -cells. We found that the numbers of glucagon-expressing cells (α cells) was greatly increased in the MSCs-treated group compared to the normal animals (Figure 5A), while the number of somatostatin expressing cells within the islets remained the same between the control and MSCs-treated group (Figure 5D). To further confirm this, we semi-quantified α cells and β cells area by software. Data showed α cells area in the MSCs-treated group was significantly higher than that of untreated control and normal group. This indicated that MSCs administration greatly promote proliferation of α cells as well as β cells. We also found that α cells area of control group was significantly higher in the diabetic rat groups than that of the normal group (Figure 5B). These data suggest that islet injury or β cells destruction may lead to improper growth of α cells, while MSCs administration accelerate this process. We next quantified the ratio of α cells area to β cells, which were dramatically higher in the MSCs

treated and untreated group than that of normal group. In morphology, the α cells were mainly seen at the borders of islets in the normal animals, whereas the α cells were mixed with the β cells in the untreated control and MSCs-treated group; and the α cells became predominant cell type within the islets in the MSCs-treated group (Figure 5A).

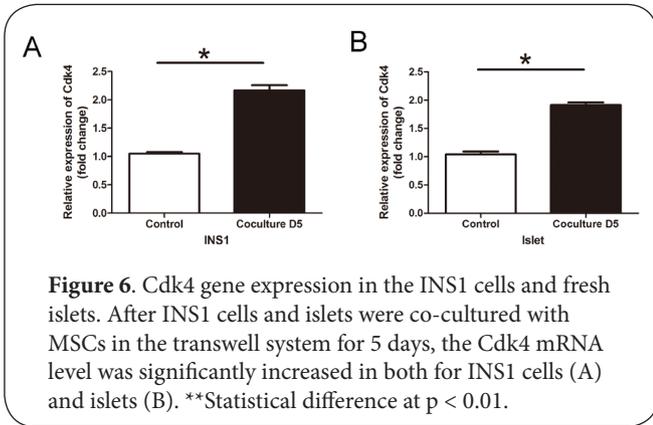
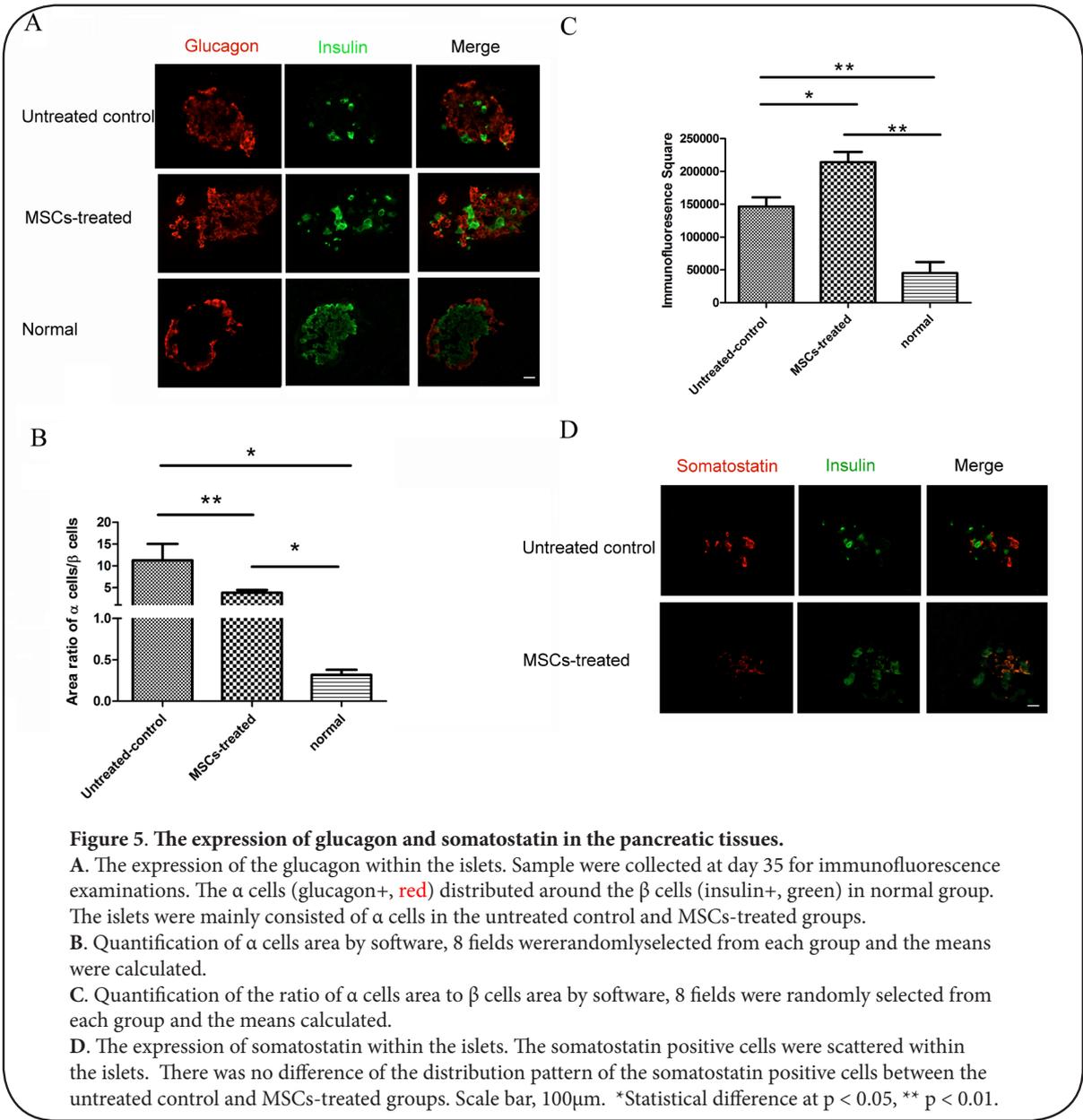
The expression of Cdk4 was upregulated in islets and INS1 cells when co-cultured with MSCs

The coculture system was used to classify the underlining mechanisms of the regeneration of β cells. We cocultured MSCs with freshly isolated rat islets and a rat β cells cell line INS1 cells in transwell system. After coculturing with MSCs for 5 days, the expression of Cdk4 was significantly upregulated by quantitative PCR examination (Figure 6).

Discussion

The present study indicates that administration of the bone marrow derived MSCs in diabetic rats can alleviate the high blood glucose syndromes for a short period, with signs of islets regeneration. The transplantation of MSC triggered expression of Pdx1 within the islets, and co-culture of the isolated islets with MSCs also induced expression of Cdk4, suggesting that MSCs may assist islets regeneration. However, the glucagon secreting α cells increased significantly in the MSCs-treated group, leading to increased blood glucose level again. These results suggest that administration of undifferentiated MSCs for the treatment of diabetes needs careful consideration.

Bone marrow MSCs transplantation has been used for diabetes therapy [13,21]. Hess reported that bone marrow derived stem cells (C-kit⁺) can initiate the regeneration of the endogenous islets, the donor derived endothelial cells



(PECAM⁺) participating the regeneration. Lee *et al.*, [3] reported that the human MSCs could engraft into the mouse pancreas and kidney, the islet became larger after MSCs transplantation and appeared to bud off pancreatic ducts. The alleviation of the high glucose syndromes after bone marrow transplantation may be a necessary step associated with islets regeneration, which has been reported previously that administration of MSCs could reverse diabetic mice hyperglycemia for at least 2 months, accompanying with reduced albuminuria and renal histopathologic alterations [4]. In our study, the GFP-MSCs were found in the pancreas, proving that MSCs engrafted in the pancreas. We found that the injured islets were partially restored in the MSCs-treated rats, although the size was smaller

than the normal islets. The blood glucose level reduced for a short period (11 days) following MSCs administration, then elevated again. This finding is consistent with recent studies reported by Si *et al.*, [6]. The regenerated islets may contribute to the reduction of blood glucose level, as a substantial number of Pdx1⁺Insulin⁻ cells were found within the islets in both the untreated control and MSCs-treated groups. Pdx1 is a key regulator for islet formation [10] and it has been reported that the injury in the pancreas can lead to islet regeneration accompanied by transient expression of Pdx1 [22]. The Pdx1⁺Insulin⁻ cells may be precursor β cells suggested by the previous reports [23]. MSCs transplantation obviously accelerated the islets regeneration process. The role and fate of these Pdx1⁺Insulin⁻ cells still need further investigation.

The therapeutic effect of MSCs administration for the treatment of type 1 diabetes (T1D) and type 2 diabetes (T2D) was intensively studied. Substantial immunosuppressive factors like TGF- β , IL-10, HGF and chemoattractants and trophic factors like CXCL-1, HGF, VEGF may be produced by MSCs *in vivo*, which were thought to modulate the immune attack by T cell in T1D and support the survival of local existing islet β cells [7,24]. In our study, we found that MSCs administration promoted regeneration of islet insulin-secreting cells. However, another concern is that the uncontrolled proliferation may cause dysfunction of β cells and lack of coordination between β , α cells or other cell types. We found that α cell numbers increased significantly in the untreated control and MSCs-treated rats than that of normal rats and the increased α cells may contribute to elevated high blood glucose levels following a short period of decline, as the production of α cells might have outnumbered the β cells in the MSCs-treated group. Moreover, the ratio of α cells to β cells was dramatically higher in the diabetic rats than those of the normal ones, indicating that the increased number of glucagon-secreting α cells may be responsible for increasing the blood glucose level in the present study. Since the MSCs implanted in the present study were undifferentiated and the local environment in the injured pancreas may not be right for insulin-producing β cells differentiation and proliferation, the exact conditions for promoting MSCs β cells differentiation *in vivo* are still unknown. Pre-programmed MSCs *in vitro* to induce their differentiation towards β cells lineage may be desirable for regenerating β cells *in vivo*, this will be the future research direction.

Cdk4 gene plays a key role in regulating islets regeneration. Lee *et al.*, reported recently that Cdk4 stimulates the quiescent β cells and the β cells progenitors to proliferate [14]. Experimental evidence showed that postnatal islets grow mainly through replication of β cells rather than differentiation from pancreatic progenitors both in human and mouse [25,26]. It is reasonable that Cdk4 is indispensable for islet regeneration, as loss of Cdk4 expression resulted in insulin-deficient diabetes [27]. Furthermore, Cdk4 together with cyclinD1 enhance β cells proliferation by improving

retinoblastoma protein phosphorylation (pRb) [15]. In this study, we found that islets co-cultured with MSCs can up-regulate expression of Cdk4 in islet. This indicates Cdk4 may contribute to MSCs inducing islet regeneration. Nevertheless, how MSCs influenced Cdk4 expression in islet and INS1 still need further investigation.

Conclusion

The current study found that transplantation of allogenic MSCs in diabetic rats can reduce the blood glucose level in a short period, and apparently promoted the regeneration of the injured islets. However, the transplantation strategies need to be optimized to improve the survival of the transplanted MSCs *in vivo* and suppress a cell abnormal proliferation.

Competing interests

The authors declare that they have no competing interests.

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