Characterization of insulin-producing cells derived from PDX-1-transfected neural stem cells

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Abstract. Islet cell transplantation is a promising treatment strategy for type-1 diabetes. However, functional islet cells are hard to obtain for transplantation and are in short supply. Directing the differentiation of stem cells into insulin-producing cells, which serve as islet cells, would overcome this shortage. Bone marrow contains hematopoietic stem cells and mesenchymal stem cells. The present study used bone marrow cells isolated from rats and neural stem cells (NSCs) that were derived from bone marrow cells in culture. Strong nestin staining was detected in NSCs, but not in bone marrow stromal cells (BMSCs). *In vitro* transfection of the pancreatic duodenal homeobox-1 (PDX-1) gene into NSCs generated insulin-producing cells. Reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) analysis confirmed that PDX-1-transfected NSCs expressed insulin mRNA and released insulin protein. However, insulin release from PDX-1-transfected NSCs did not respond to the challenge of glucose and glucagon-like peptide-1. These results support the use of bone marrow-derived NSCs as a renewable source of insulin-producing cells for autologous transplantation to treat type-1 diabetes.

Introduction

According to the website of the World Health Organization (http://www.who.int/diabetes/en/), 346 million individuals worldwide have diabetes. The chronic cardiovascular and metabolic complications of diabetes carry significant risks of morbidity and mortality, requiring that the disease is carefully managed. Type-1 diabetes, an autoimmune disorder, is characterized by the loss of insulin-producing β cells. Pancreatic islet transplantation is one of the most successful therapeutic strategies for diabetes (1-4). However, there are several obstacles for islet transplantation that must be overcome, including the adverse effects resulting from treatment with immunosuppressive drugs, preservation of islet function and the shortage of islet donors (5). Therefore, generating functional β cells would be a promising strategy in providing adequate numbers of β cells for transplantation.

Stem cells, including embryonic and adult stem cells, possess the potential for self-renewal, proliferation and differentiation into various types of cells. Stem cells may be isolated from embryo, pancreas, liver and bone marrow tissue and used as β cells for transplantation (6,7). Hepatic stem cells have the highest potential for differentiation into insulin-producing cells, as the pancreas and liver have common precursor cells during embryogenesis. A study by Yang *et al* demonstrated the production of insulin-secreting β cells from hepatic oval stem cells, this may be a therapeutic strategy for autologous stem cell transplantation (8). However, this application is limited since stem cells are difficult to obtain.

Bone marrow stromal cells (BMSCs), which have high plasticity and are therefore easily manipulated *in vitro*, are abundant in bone marrow and are readily obtained (9,10). BMSCs may be isolated from other cells and cultured *in vitro*. Previous studies have shown that BMSCs differentiate into chrondrocytes, osteocytes, adipocytes and fibroblasts (11,12). Tang *et al* demonstrated that mouse BMSCs differentiate into insulin-producing cells (13). Moreover, genetically manipulated human BMSCs show stable transgene expression from over 17 passages *in vitro* and over 3 months *in vivo* (14). Therefore, BMSCs are a promising source of insulin-producing cells for autologous transplantation.

Nestin, a type VI intermediate filament protein, promotes the phosphorylation-dependent disassembly of vimentin intermediate filaments during mitosis (15) and is also used as a marker of neural precursor cells (16) and pancreatic stem cells (17). Studies suggest that cerebral microglia expressing nestin and NG2, a chondroitin sulfate proteoglycan, have high plasticity similar to neonatal brain neurons (18,19). Moreover, Milanesi *et al* demonstrated that nestin-positive cells differentiate into pancreatic endocrine cells *in vitro* (20). Taguchi and Otsuki reported that PDX-1 staining was detected in small evaginations of the main pancreatic duct and in the nuclei of islet cells; nestin-positive staining was also detected in small

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evaginations of the main duct, islets and spindle-shaped cells in the connective tissue around the main duct (17). These authors proposed that the nestin and PDX-1 double-positive cells were pancreatic stem cells. PDX-1 plays a significant role during the formation of the pancreas by regulating insulin secretion. PDX-1 has been reported to be capable of reprogramming extrapancreatic tissue towards a β-cell phenotype (21). When implanted under the renal capsule of NOD-SCID mice, PDX-1-transfected liver cells transdifferentiated into insulin-producing cells, which reduced hyperglycemia (22). Therefore, the PDX-1 gene is significant in regulating the production of insulin.

The objective of the current study was to examine bone marrow-derived neural stem cells (NSCs) as a renewable source of insulin-producing cells for autologous transplantation.

Materials and methods

Isolation and culture of BMSCs and neuron-like cells. Sprague-Dawley rats 3-4 weeks old (60-70 g) were obtained from the Laboratory Animal Center of South Medical University (Guangzhou, China). The rats were sacrificed by cervical dislocation and placed in 75% alcohol for 1 min. The two femurs were removed and bone marrow cells were flushed from the marrow with a 1-ml syringe filled with L-DMEM containing 100 U/ml heparin. The cell suspension was centrifuged at 400 rpm for 8 min. The supernatant was discarded and the cells were resuspended with L-DMEM; cells were then layered over lymphocyte isolation reagent (Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences) and centrifuged at 2,500 rpm for 20 min at 25˚C. Mononuclear cells were removed from the gradient interface and washed twice with PBS. For BMSCs, the pellet was resuspended with L-DMEM (containing 10% FBS) and plated in tissue-culture flasks at a density of $1x10^6$ cells/ml. For neuronal stem cells, the pellet was resuspended with neuronal stem cell culture medium (patent no. 02134314.4, Neurosurgery Lab, South Medical University) and plated in tissue-culture flasks at a density of $1x10^7$ cells/ml. Half of the culture medium was removed following the first 48 h. The cells were then passaged until cell growth reached 70-80% confluence. The study was approved by the ethics committee of Shenzhen Longgang Central Hospital, Shenzhen, China.

Immunocytochemical staining. The fourth passage of MSCs and first passage of NSCs were fixed with 4% paraformaldehyde for 15 min, washed 3 times with distilled water and incubated with 0.03% Triton X-100 for 10 min. Cells were washed 3 times with PBS and subsequently incubated with 30% H_2O_2 in methanol (1:50) for 30 min. Cells were then washed with distilled water 3 times and non-specific binding was blocked with 5% BSA for 20 min at room temperature. Cells were then incubated with primary antibodies against nestin (Beijing Biosysnthesis Biotechnoloy Co., Ltd., Beijing, China) with 1:100 dilutions for 1 h at room temperature, washed 3 times with PBS and incubated with SABC reagent (Beijing Biosysnthesis Biotechnology) for 20 min at room temperature. The cells were counter-stained with propidium iodide (PI) to detect nuclei, observed using an Olympus phase contrast microscope (CK-2; Olympus Optical Co. Ltd., Tokyo, Japan) and images were captured with an Olympus PM-CBAD exposure control unit (Olympus, Centre Valley, PA, USA). Fluorescence of cells was observed with an inverted fluorescence microscope (Leica, Mannheim, German).

Reverse-transcription polymerase chain reaction (RT-PCR). Total RNA of BMSCs and NSCs was extracted with TRIzol (Gibco, Carlsbad, CA, USA) and reverse transcribed to cDNA with an RT-kit (Tiangen Biotech, Beijing, China). RT-PCR was performed as follows: the amplification reaction was carried out with preliminary denaturing at 95˚C for 3 min followed by 35 cycles of the following conditions: 94˚C for 55 sec, 55˚C for 45 sec, 72˚C for 1 min and 72˚C for 8 min. The following primers for nestin were used: forward, 5'-GCGGGGCGG TGCTGATAC-3'; reverse, 5'-AGGCAAGGGGGAAGA GAAGGATGT-3' and the expected size of the PCR product was 326 bp. PDX-1 primers were forward, 5'-CGGCCACAC AGCTCTACAAGG-3'; reverse, 5'-GAGGTTACGGCA CAATCCTGA-3' and the expected size of the PCR product was 667 bp. The insulin primers were forward, 5'-CGGGAG GATGGGCTTTTCTG-3'; reverse, 5'-AGCTGCTTTTGG TTGAGCACAG-3' and the expected size was 191 bp.

Transfection. The fourth passage of MSCs and 7-day cultures of nestin-positive cells were dissociated from culture dishes with 0.25% trypsin-EDTA. Cells were washed and resuspended at a density of 3 to $4x10^6$ cells/ml in Nucleofector® solution. Cell suspension (100 µl) and 5 µg pBluescript-EGFP-C2-PDX-1 plasmid were mixed and transferred into electroporation cuvettes. The A33 Nucleofector® Program was selected to transfect cells. Following transfection, 4 ml L-DMEM was added for culture and after 24 h, cellular morphology was observed by fluorescence microscopy.

Fluorescent-activated cell sorting (FACS). Cells were detached from the dish with 0.25% trypsin-EDTA 24 h following transfection. Cells were then washed and resuspended in 500 ml PBS buffer. Transfection efficiency was determined by the percentage of green fluorescent-positive cells using a FACS system (Beckman Coulter, Miami, FL, USA).

Western blotting. Cells were collected and washed with PBS 3 times. Cell extraction solution (100 µl) was then added and the mixture was sonicated at 240 W for 6 sec and the cells were centrifuged at 13,000 x g for 10 min. Cell pellets were placed in 4X denatured buffer and boiled for 3-5 min. Protein sample (15 µl) was then added into each well of a 12% polyacryamide gel for electrophoresis and the blot was transferred onto a PVDF membrane at 90 V for 90 min. Non-specific binding was blocked with 5% non-fat milk/PBS for 30 sec and subsequently 4 µl of the primary antibody in PBS was added and incubated for 90 min at RT. The mixture was washed with PBS 3 times and then incubated with 4 µl secondary antibody in PBS for 90 min at room temperature. This mixture was then washed 3 times with PBS and the signal was developed with 50 µg/ml BCIP + 100 µg/ml NBT developing buffer and incubated for 5-10 min.

Enzyme-linked immunosorbent assay (ELISA). Insulin and C-peptide were determined by ELISA kit (Diagnostic Systems Laboratories, Webster, TX, USA). Standards and equal culture

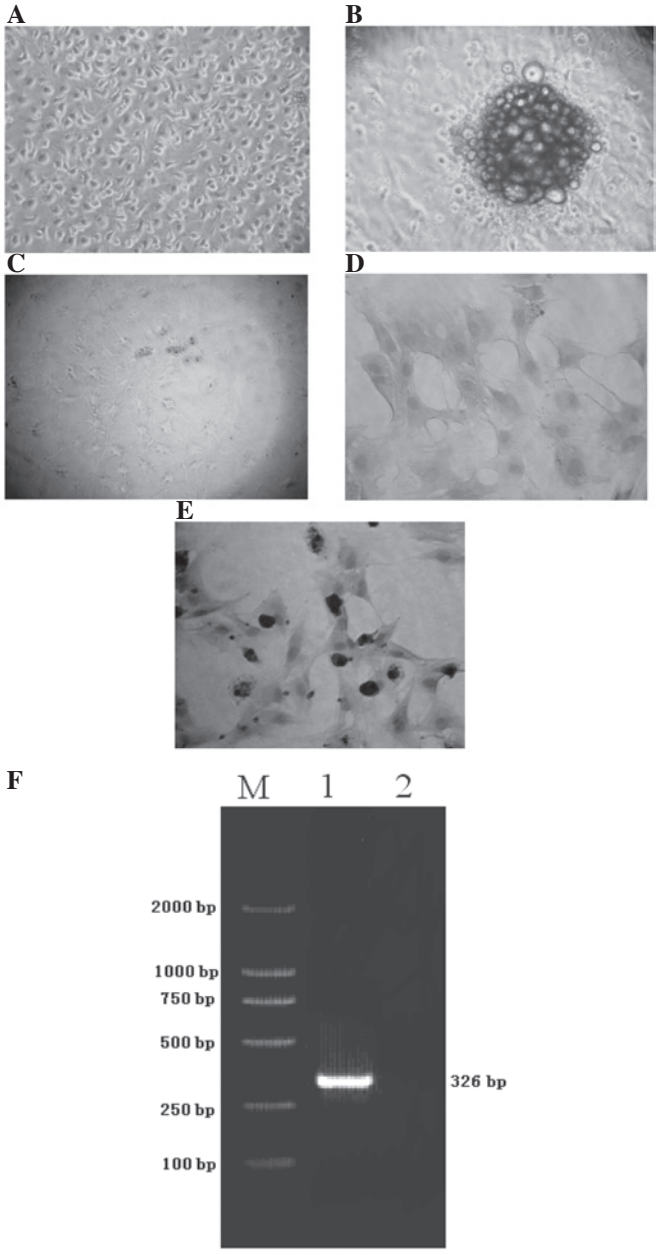

Figure 1. Primary culture of BMSCs and NSCs. Bone marrow cells were isolated from rat femurs and cultured *in vitro*. (A) Cell morphology of BMSCs after 6 days. (B) Neurosphere of NSCs after 3 days. (C) Extended dendrites were observed in the NSCs after 8-10 days. (D) BMSCs and (E) NSCs were analyzed by immunocytochemical staining using nestin antibody. (F) Nestin mRNA expression in BMSCs and NSCs was determined by RT-PCR analysis. M, DNA ladder; lane 1, NSCs; lane 2, BMSCs. BMSCs, bone marrow stromal cells; NSCs, neural stem cells.

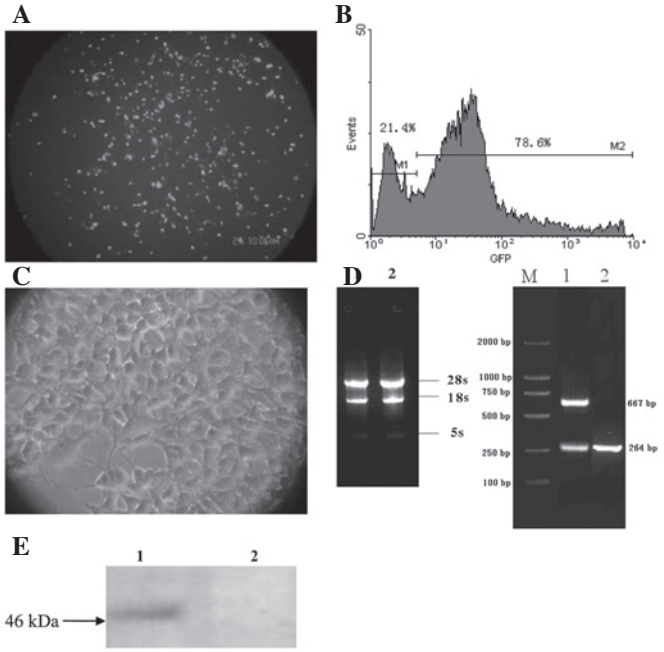

Figure 2. PDX-1 expression in NSCs. NSCs were transiently transfected with pEGFP-C2-PDX-1 plasmids (30 μg) for 24 h. (A) Fluorescent images of transfected cells were captured with a fluorescence microscope. (B) The transfection efficiency was quantified by flow cytometry. The M2 region indicates the percentage of GFP expression in total cells. (C) The cell morphology of pEGFP-C2-PDX-1-transfected NSCs was observed on the third day. (D, left) Total RNA was extracted 2 days following transfection and underwent electrophoresis. (D, right) The expression of PDX-1 mRNA on pEGFP-C2 and pEGFP-C2-PDX-1-transfected NSCs was analyzed by RT-PCR. Lane M, DNA ladder; lane 1, pEGFP-C2-PDX-1-transfected cells; lane 2, pEGFP-C2-transfected cells. (E) Protein expression of PDX-1 was analyzed by western blotting. Lane 1, pEGFP-C2-PDX-1-transfected cells; lane 2, pEGFP-C2-transfected cells. PDX-1, pancreatic duodenal homeobox-1; NSC, neural stem cell; GFP, green fluorescent protein.

supernatant were added to the 96-well plates. The absorbance was then measured with GENios spectrometer (Tecan Group Ltd., Männedorf, Switzerland) at 450 nm. A relative optical density value was plotted using these standards and the concentration of insulin and C-peptide in the culture supernatant derived.

Results

Nestin mRNA is expressed in NSCs. Bone marrow cells from the femurs of SD rats were harvested and separated by Ficoll. Cells were cultured in L-DMEM with 10% FBS for the generation of BMSCs and cultured in neurostem cell medium for the generation of NSCs. BMSCs adhered to the culture dishes and grew rapidly following 6 days of culture (Fig. 1A). However, the NSCs formed neurospheres after 3 days (Fig. 1B) and dendrites of the NSCs appeared after 8-10 days (Fig. 1C). To characterize BMSCs and NSCs, immunocytochemical staining and RT-PCR of nestin were performed. Strong staining of nestin was observed in NSCs but not BMSCs (Fig. 1D and E). The RT-PCR analysis also confirmed that nestin mRNA was expressed in NSCs, but not in BMSCs (Fig. 1F).

Cells transfected with pBluescript-EGFP-C2-PDX-1 express mRNA (667 bp) and protein (46 kDa). To induce cell differentiation, NSCs were transfected with pBluescript-EGFP-C2-PDX-1. Following 7 days of transfection, the green fluorescence of pBluescript-EGFP-C2-PDX-1-transfected cells was observed by fluorescence microscope (Fig. 2A) and the transfection efficiency was 78.6% by FACS analysis (Fig. 2B). The cells became round with larger nuclei and, 3 days after PDX-1 transfection, the nuclei-to-plasma ratio increased (Fig. 2C). The RNA of vector-only (pBluescript-EGFP-C2) or pBluescript-EGFP-C2-PDX-1-transfected cells was extracted and the expression of PDX-1 mRNA was examined by RT-PCR and PDX-1 protein was determined by

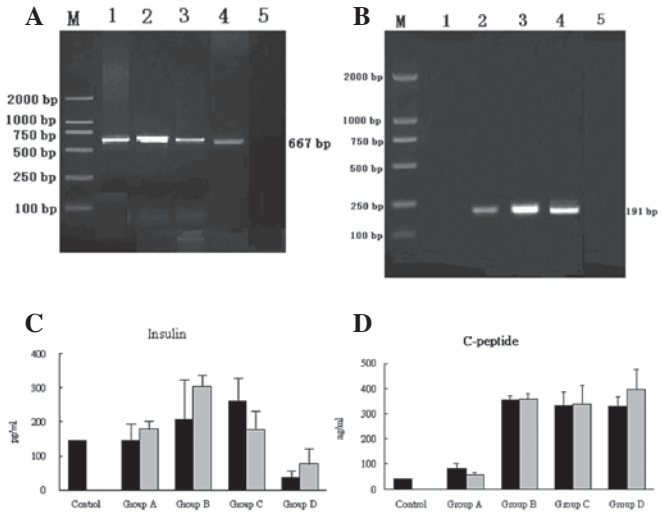

Figure 3. Effects of glucose and GLP-1 on PDX-1-transfected NSCs. (A) The PDX-1-transfected nestin-positive NSCs were treated with glucose and/or GLP-1. After 2 h, the cells were harvested and the mRNA expression levels of (A) PDX-1 and (B) insulin in NSCs after various treatments were determined by RT-PCR. Lane M, DNA ladder; lanes 1-4, EGFP-C2-PDX-1-transfected NSCs with treatment with medium (lane 1), glucose (lane 2), GLP-1 (lane 3), GLP-1 and glucose (lane 4); lane 5, EGFP-C2-transfected NSCs + GLP-1 and glucose. Before (black bar) and after (grey bar) treatment of glucose, GLP-1, or glucose + GLP-1, (C) production of insulin and (D) C-peptide was determined by ELISA. Control and groups A-C were EGFP-C2-PDX-1-transfected NSCs with treatment of medium (control), glucose (group A), GLP-1 (group B) and GLP-1 + glucose (group C). Group D, EGFP-C2-transfected NSCs treated with GLP-1 and glucose. GLP-1, glucagon-like peptide-1; PDX-1, pancreatic duodenal homeobox-1; NSC, neural stem cell.

western blot analysis (Fig. 2D and E). Cells transfected with pBluescript-EGFP-C2-PDX-1 expressed mRNA (667 bp) and protein (46 kDa). Neither PDX-1 mRNA nor protein expression was observed in vector-transfected cells.

Insulin mRNA was induced in the presence of glucose, GLP1 and glucose + GLP1. To determine whether the PDX-1-transfected NSCs were responsive to glucose or glucagon-like peptide 1 (GLP1) challenges, insulin mRNA and insulin protein were measured using RT-PCR and ELISA. Following 7 days of culture, NSCs were transfected with vector or pBluescript-EGFP-C2-PDX-1. Cells were then cultured in 20% FBS/L-DMEM only (control group) or with glucose (group A), GLP1 (group B) or glucose + GLP1 (group C). Vector (pBluescript-EGFP-C2)-transfected cells were cultured with glucose + GLP1 (group D) as a control. The PDX-1 mRNA was detected by RT-PCR in all EGFP-C2-PDX-1-transfected cells (Fig. 3A). Subsequently, whether insulin was induced by glucose or GLP1 was examined. As shown in Fig. 3B, insulin mRNA was induced in the presence of glucose (lane 2), GLP1 (lane 3) and glucose + GLP1 (lane 4). Neither pEGFP-C2-PDX-1-transfected cells cultured with medium (lane 1) nor vector-transfected cultured with glucose + GLP1 (lane 5) produced insulin. To determine whether the PDX-1-transfected NSCs synthesized insulin protein, the supernatants were collected and determined by ELISA. Fig. 3C shows that insulin was detected in the control group (145 pg/ml), group A (144±57.28 pg/ml), group B (208±117 pg/ml), group C (261.25±69.88 pg/ml) and group D (271.25±89.68 pg/ml) prior to glucose/GLP-1 stimulation. However, the insulin was not significantly released following a glucose/GLP-1 challenge. Similar results were observed in the production of C-peptide (Fig. 3D).

Discussion

Previous studies have shown that insulin-producing cells are generated from progenitor cells from various sources, including liver (8), pancreas (23,24), intestinal epithelium (25) and embryonic stem cells (26). However, overcoming the rejection of transplanted β cells remains a challenge. Moreover, it is difficult to obtain sufficient stem cells from these organs. Bone marrow is an abundant source of adult stem cells. This study examined the possibility of using bone marrow cells that differentiate into NSCs by transfecting the PDX-1 gene into BMSCs to generate insulin-producing cells. The production of insulin from PDX-1-transfected NSCs was confirmed by RT-PCR and ELISA. The current study suggests that bone marrow contains pluripotent cells that are capable of being reprogrammed to differentiate into insulin-producing cells.

In pancreatic β cells, GLP-1 regulates numerous genes that control insulin, glucokinase, glucose transporter-1 (GLUT-1), GLUT2 and hexokinase I (27-29). GLP-1 also enhances insulin secretion in a glucose-dependent manner. However, significantly increased insulin release was not observed in the presence or absence of glucose, GLP-1, or with glucose + GLP-1 in the PDX-1-transfected NSCs of the current study. The PDX-1-transfected NSCs spontaneously released insulin without glucose/GLP-1 challenge. These data suggest that PDX-1-transfected NSCs are β-like cells and further induction, including with nicotinamide and exendin 4, is required to reach a higher degree of differentiation, as observed in native pancreatic β cells (13,30).

Campbell and Macfarlane reported that glucose, GLP-1 and insulin positively regulate the PDX-1 gene promotor in pancreatic β cells (31). In the current study, glucose or GLP-1 induced the expression of mRNA and insulin in PDX-1-transfected nestin-positive cells, but no synergistic effect was identified. There are three possible reasons for this. Firstly, the expression level of plasmid DNA may have changed as the transfection system is different. In the current study, the plasmid was transfected by Nucleofector into the nucleus directly, not into the cytoplasm. Secondly, the concentration of glucose or GLP-1 was too high to induce a synergistic effect and thirdly, PDX-1 may be regulated through a pathway independent of glucose and GLP-1.

Multiple growth factors, including insulin, were added to the cell culture prior to transfection. However, neither PDX-1-transfected nestin-positive cells nor nestin-positive cells in the presence of glucose and GLP-1 produced insulin, suggesting that insulin production was induced by glucose and GLP-1 stimulation in PDX-1-transfected nestin-positive cells. Conversely, insulin production was not induced in nestin-positive cells in the presence of GLP-1 and glucose. That PDX-1 is necessary for glucose and GLP-1 to induce insulin expression was consistent with the results of previous studies (32,33).

The length of time required for BM-derived nestin-positive cells to differentiate into insulin-producing cells was shorter in the present study compared with that in others (13,34). There

are two reasons for this difference. Firstly, the plasmid DNA was transfected into the nucleus directly and the expression reached a peak after 24 h. Additionally, the amount of time for PDX-1 expression was shorter in the current study compared with that of others. Secondly, in the study by Gao *et al*, high concentrations of serum inhibited the differentiation of β cells (35). However, in the current study, high concentrations of serum enhanced cell proliferation and cell-cell contact, resulting in a more rapid differentiation.

This study provides evidence that PDX-1-transfected BM-derived nestin-positive cells differentiated into insulin-producing cells following stimulation with glucose and GLP-1. However, more research is required to clarify the mechanisms of a single or synergistic effect of growth factors. Based on these findings, the source of insulin-producing cells appears to be unlimited for autologous transplantation for type-1 diabetes.

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