Original Article
Partially repair damaged Islets of diabetic rat model via insulin-producing cells differentiated from human umbilical cord mesenchymal stem cells infusion

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Abstract: Pancreatic islet transplantation is the most effective treatment for type 1 diabetes, but the shortage of donor islets and risk of immunological rejection prevent it from being used widely. Mesenchymal stem cells in the Wharton's jelly of human umbilical cords are accepted as a promising alternative transplant source. The current method of inducing differentiation is time consuming. The aim of this study was to develop a novel method combining genetic and chemical approaches to tackle this problem and minimize the potential risk of rejection. Freshly isolated and expanded human umbilical cord mesenchymal stem cells (HUMSCs) were induced to differentiate into insulin-producing cells by the novel two-step method. In this method, HUMSCs were first transfected with neuronal differentiation 1 (NEUROD1) for 2 days and afterwards treated with chemical agents for 8 days to induce differentiation. The differentiated cells expressed the pancreatic cell markers, which were pancreatic and duodenal homeobox 1 (PDX1), glucagon, insulin, and NK2 homeobox 2 (NKX2-2), and released insulin both spontaneously and after high-glucose stimulation. Then, differentiated cells were transplanted via tail vein into a diabetic rat model induced by streptozotocin. Hyperglycemia, body weight and survival rate of the rats was improved, and serum insulin levels increased after transplantation. More importantly, it was histologically confirmed that transplanted cells homing to the pancreatic gland of diabetic rats and improved the morphology of pancreatic islets of the recipient rats. Overall, the novel two-step method successfully induced HUMSCs to differentiate into insulin-producing cells and effectively treat the diabetic rat via vascular delivery.

Keywords: Diabetes, human umbilical cord, insulin-producing cells, mesenchymal stem cells

Introduction

Type 1 diabetes is an insulin-dependent auto-immune disorder characterized by the destruction of insulin-producing β-cells in the pancreas [1]. Islet transplantation is the most effective therapy for type 1 diabetes [2, 3], while its application is virtually impossible due to high risk of immune rejection and the shortage of donor islets [4].

Stem cells, including human embryonic stem cells (hESC) [5], induced pluripotent stem cells (IPS) [6] and mesenchymal stem cells (MSCs) [7], have the ability to differentiate into functional insulin-producing cells, which could be developed as promising sources of islet cells for transplantation. Among the various types of adult and embryonic stem cells, human umbilical cord mesenchymal stem cells (HUMSCs) are readily obtained, isolated, and expanded [8]. When expanded in vitro, even after more than 10 passages, HUMSCs maintain their multiple differentiation abilities [9]. HUMSCs are less immunogenic because they express to stem cell surface markers such as cluster of differentiation CD29, CD44, CD59, CD90, and CD105, with little or no expression of the important graft-versus-host disease markers CD80, CD86, and CD40 [9]. In vitro, HUMSCs have been induced to differentiate into neuron-like [10], chondrogenic [11], osteogenic [12], adipo-

genic [13], myogenic [14], or insulin-producing cells [15-17].

Current methods used to induce various types of stem cells to differentiate into insulin-producing cells in vitro are called one-step methods. In these methods, either chemicals, genetic reprogramming, or co-culturing with rat pancreatic cells is used. These methods are relatively simple and easy to be applied for laboratories. Chemical induction has been used to differentiate HUMSCs into islet-like clusters that expresses pancreatic β cell-related genes and secrete insulin [15, 16]. However, the whole procedure take about one month, and such long-term culture increase the risk of malignant transformation [18].

Genetic reprogramming has been used to induce rat bone marrow-derived mesenchymal stem cells (MSCs) to differentiate into insulin-producing cells [7]. In this method, the rat pancreatic and duodenal homeobox factor 1 (PDX1) gene was introduced by infecting cells with Pdx1-bearing adenovirus. Seven days after viral infection, the mRNA levels of insulin and pancreatic glucagon genes were detected.

Co-culturing with pancreatic islets has also been used to induce MSCs derived from rat bone marrow to differentiate into insulin-producing cells [19]. However, it is difficult to separate the insulin-producing cells from the culture for transplantation. Therefore, more efficient methods are needed to induce stem cells to differentiate into insulin-producing cells.

Neuronal differentiation 1 (NEUROD1) is a transcription factor that regulates the expression of the insulin gene (INS) in pancreatic β cells [20]. Over-expression of NEUROD1 in adult human pancreatic duct cells induces them to express insulin and growth hormone although no pancreatic β cells are found [21].

To develop an effective method of inducing HUMSCs to differentiate into insulin-producing cells, we evaluated a two-step method in which NEUROD1 was overexpressed in HUMSCs, and then the cells were chemically treated to promote differentiation. The mRNA level of pancreatic cell-specific genes and insulin production were measured in vitro, and the cells' therapeutic effect was determined in vivo in STZ-induced diabetic rats after transplanted via the tail vein.

Materials and methods

Cell culture and characterization

The institutional review board of Shantou university medical college gave ethical approval for this study. HUMSCs were obtained and expanded as described previously [10]. Briefly, human umbilical cords from consenting patients were collected into sterilized 50 ml tubes immediately after delivery of full-term infants by caesarean section. The specimens were washed with phosphate buffered saline (PBS), and after dissecting out arteries, veins, and remaining tissue, Wharton's jelly was diced into small fragments and transferred to a 75 cm^2 flask containing Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, Australia), 100 µg/mL penicillin/streptomycin (Shanghai Bioscience, China), 1 g/mL amphotericin B (Gilead Sciences, Inc. San Dimas, CA, USA), 5 ng/mL epidermal growth factor (EGF; Invitrogen Life Technologies, Carlsbad, CA, USA), and 5 ng/ml basic fibroblast growth factor (bFGF, Sigma-Aldrich, St. Louis, MO, USA). The culture was incubated at 37°C with 5% CO$_2$ to allow HUMSCs to migrate from the explants. Typically, HUMSCs migrated out from fragments of Wharton's jelly after 5-7 days in culture and the primary culture were established within 10-14 days. HUMSCs were maintained in the growth medium by splitting every 3-5 day at a ratio of 1:3. To confirm their stem cell properties, proliferating HUMSCs at passage 3 was evaluated for expressions of cluster of differentiation CD29, CD59, CD80, CD86, CD40, and CD40 (BD, USA) by flow cytometry as described previously [10, 22].

HUMSCs induced to differentiate into insulin-producing cells in vitro

HUMSCs (5 × 10^6 per well) were plated on 6-well tissue dishes. After twenty-four hours, 10 µg/µl plasmid DNA (NEUROD1, under the control of a cytomegalovirus promoter) mixed with 20 µl Lipofectamine (Invitrogen Life Technologies, Carlsbad, CA, USA) in 500 µl DMEM was added to each well. Cells were incubated at 37°C for 2 days for transfection. Transfected cells were cultured in high-glucose DMEM (DMEM-HG, 25 mM glucose) supplemented

with 10% FBS and 10-6 mM retinoic acid for 24 hours, then in DMEM-HG with 10% FBS for 2 days, and finally in low DMEM (DMEM-LG) supplemented with 10% FBS and 10 mM nicotinamide for 6 days. For the undifferentiated control, HUMSCs were cultured in DMEM-LG containing 10% FBS only [17].

Reverse transcriptase PCR and real-time PCR

Total RNAs from HUMSCs or differentiated cells were extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was reverse-transcribed into cDNA using a Quantscript RT kit (Tiangen, Bejing, China). Complementary DNA samples were amplified with EX Taq polymerase (TaKaRa, Japan) using a PCR protocol in accordance with the manufacturer's instructions and then analyzed on agarose gel. To quantitatively determine the gene expression in cells, cDNA samples underwent real time PCR with SYBR premix (TakaRa, Japan) in an ABI 7300 system. The primer pairs used for reverse transcriptase PCR and real time PCR were: human β-actin, forward 5'-tggcaccacacc-ttctacaatgagc-3' and reverse 5'-gcacagcttctcct-taatgtcacgc-3'; human pancreatic and duodenal homeobox 1 (PDX1), forward 5'-tcccatggat-gaagtctacc-3' and reverse 5'-gccgtgagatgtactt-gttg-3'; human insulin, forward 5'-agcctttgtga-accaacacc-3' and reverse 5'-gctggtagagggag-cagat-3'; glucagon, forward 5'-cagagcttaggaca-cagagcacatc-3' and reverse 5'-acgttgccagctgcc-ttgta-3'; and NK2 homeobox 2 (NKX2-2), forward 5'-acaaaccgtcccagcgtta-3' and reverse 5'-ggctgacaatatcgctactcacaca-3; and NEURO-D1, forward 5'-aggtggtgccttgctattc-3' and reverse 5'-ttctcaaactcggcggac-3'.

Glucose challenge test

Differentiated cells were washed twice with PBS and incubated for 1 h in DMEM-LG (4.5 mM glucose). The medium was collected and stored at -20°C. These cells were then washed twice with PBS, and incubated for 1 h in DMEM-HG (17 mM glucose; Gibco, USA). The medium was collected and stored at -20°C. Insulin concentrations of these collected samples were measured by radioimmunoassay.

Diabetic rats model

To induce diabetes, male Sprague Dawley rats at age of 8-10 weeks received intraperitoneal injections of 70 mg/kg streptozotocin (STZ; Sigma, USA). Blood glucose levels of treated rats were monitored every 3 days. Rats with blood glucose levels higher than 16.7 mMs for 3 consecutive measurements were used for transplantation study.

Cell transplantation and physiological monitoring

STZ-induced diabetic rats were divided into three groups of 6-8 rats in each group. Each rat was transplanted with five million insulin-producing cells differentiated from HUMSCs using the two-step method (described above) in 200 μl PBS via tail vein injection. Before transplantation, harvested cells were dispersed in single cells suspension by gently pipetting. The body weight, survival rate, and the levels of blood glucose and concentration of serum insulin of transplanted rats in random condition were measured and recorded before and after cell transplantation. Blood samples were collected from the tail vein. The glucose levels in blood samples were measured with a blood glucose meter (Bayer, Germany). Serum insulin levels were measured by radioimmunoassay in the Department of Radioimmunoassay at Second Affiliated Hospital of Shantou University Medical College.

Histopathological and immunohistochemical analyses

Pancreatic tissues were fixed in 10% neutral formalin, dehydrated in ethanol, and embedded in paraffin. For histopathological analysis, paraffin sections (4 μm) were stained with hematoxylin and eosin (H&E). For immunohistochemical analysis, paraffin sections were treated with a blocking solution for 30 mins and then incubated with mouse anti-human insulin monoclonal antibodies (1:100; Neomarkers, USA) at 4°C for 18 hs. Paraffin sections were washed in PBS and incubated with goat anti-mouse IgG (1:100; Neomarkers, USA) at room temperature for 0.5-1 h. After washing with PBS, paraffin sections were visualized by the presence of 3,3'-diaminobenzidine.

Statistical analyses

All statistical analyses were done with SPSS 13.0, and the significance level was set at p<0.05. The results of mRNA levels of INS,
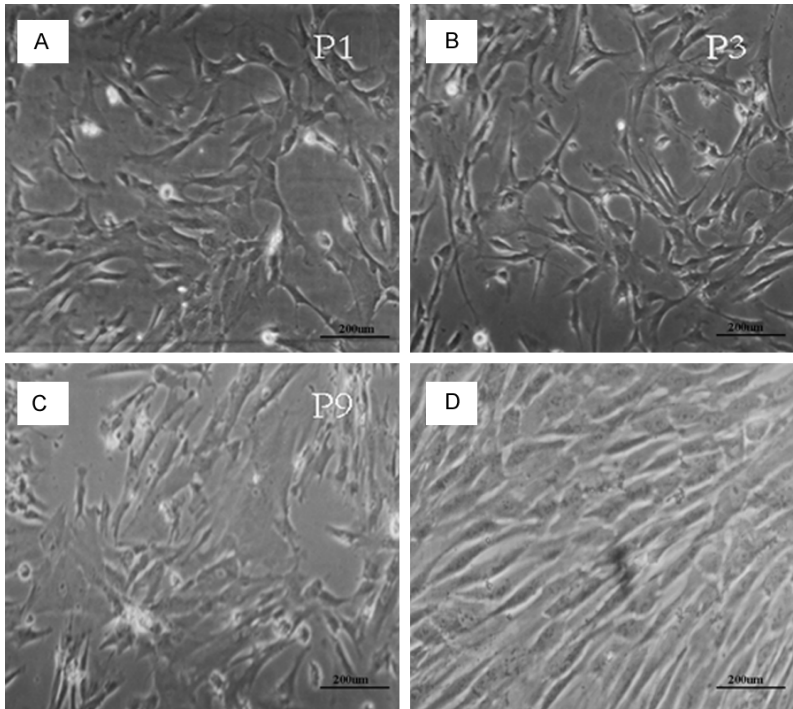
Figure 1. Morphology of HUMSCs. HUMSCs were freshly isolated from the umbilical cords of patients delivering full-term infants. (A) HUMSCs migrated from fragments of Wharton's jelly at 5 d of culture (first passage, P1); (B) HUMSCs passaged in HUMSC growth medium three times (P3); (C) HUMSCs passaged in HUMSC growth medium nine times (P9). Cells began to grow slowly. (D) The cell morphology became wider and flatter when passage more than nine times. (A-D, Bar=200 μm).
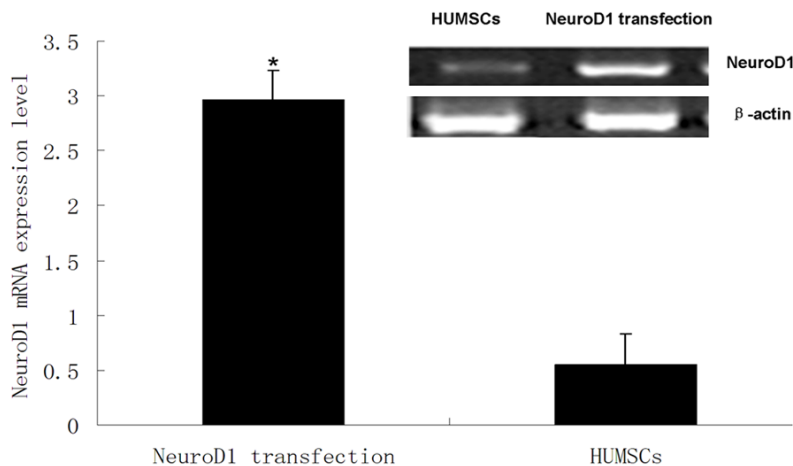
ed cells were compared to un-differentiated HUMSCs with the Student's t-test. Arbitary units of islet area in STZ-rat receiving differentiated cells were compared to STZ-rat receiving PBS by Student's t-test. Blood glucose level, body weight, survival ratios, and insulin levels of were compared between STZ-rats with or without differentiated HUMSCs by one-way ANOVA.

Results

Characteristics of proliferating HUMSCs

We freshly isolated HUMSCs from the umbilical cords of patients delivering full-term infants by caesarean section. Proliferating HUMSCs were spindle-shaped and grew at a doubling time of 3 to 5 days (**Figure 1**). At the ninth passage, HUMSCs was flat, indicating that cells grew slowly. HUMSCs at passage 3 were positive for stem cell markers CD29 and CD59, but negative for the immune response-related surface antigens CD80, CD86, CD40, and CD40L (data not shown). Cells at passage 3 with these characteristics were used for differentiation study.

Induce differentiation of HUMSCs to insulin-producing cells

HUMSCs were induced to differentiate toward pancreatic β cells using a two-step method that combined genetic and chemical

Figure 2. Expression of NEUROD1 in HUMSCs. HUMSCs were transfected with plasmid DNA (NEUROD1, under the control of a cytomegalovirus promoter). Expression of NEUROD1 was measured by Q-PCR. The mRNA levels of NEUROD1 in transfected cells compared with those of proliferating HUMSCs show statistical differences (P=0.0242).

NKX2-2, GCG, PDX1 and the insulin level in response to glucose stimulation in differentiat-

approaches. First, NEUROD1 gene was introduced into HUMSCs by transfecting cells with
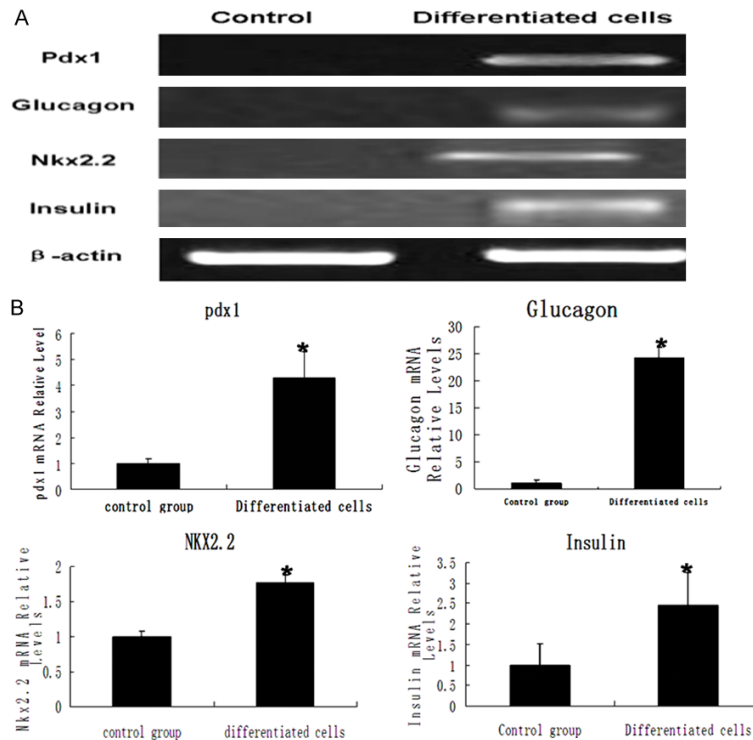
Figure 3. Expression of pancreatic β cell-specific genes in differentiated cells. HUMSCs were induced to differentiate into insulin-producing cells using two-step method. Total RNA was extracted from differentiated cells and reverse transcribed to cDNA for PCR (A) and real-time PCR (B) analysis for pancreatic β cell-specific genes, INS, NKX2-2, GCG and PDX1. For (A), RNA extracted from proliferating HUMSCs was used as negative control. PCR for β-actin was used as RNA quality control. For (B), the relative levels of mRNA of INS, NKX2-2, GCG and PDX1 in differentiated cells compared with proliferating HUMSCs is statistical differences ([INS]P=0.0345, [NKX2-2]P=0.0064, [GCG]P<0.0001, [PDX1]P=0.0222).
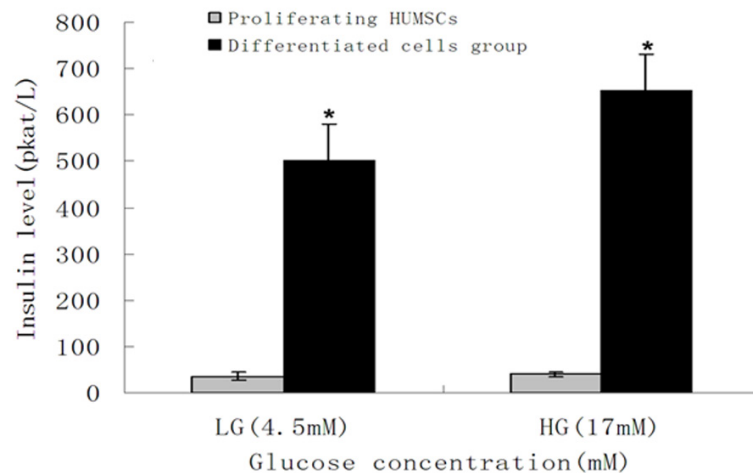

Figure 4. Insulin secretion in differentiated cells in response to glucose stimulation. Differentiated HUMSCs were cultured in medium with low (4.5 mM) and high (17 mM) concentrations of glucose, respectively, for 2 hours. Insulin levels in culture medium were measured by radioimmunoassay. Proliferating HUMSCs were used as control. Data were collected from 7 experiments. The insulin level of differentiated cells in response to glucose stimulation is higher than that of proliferating HUMSCs (P=0.0437).

plasmid DNA bearing NEU-ROD1 gene under the control of a cytomegalovirus promoter. Two days later, these cells were further incubated for 6 days in medium containing a high-glucose concentration (25 mM) supplemented with the β-cell promoting factor nicotinamide and retinoic acid. The expression of NEUROD1 gene in the transfected cells was detected by Q-PCR at 2 days after transfection. The mRNA levels of NEUROD1 in transfected cells was statistically higher than that of untreated HUMSCs (P=0.0242) (**Figure 2**). The success of differentiation was first evaluated immediately after full induction procedure by measuring the expression of the pancreatic β cell-specific marker genes, INS, PDX1, GCG, and NKX2-2. All these genes were expressed in the differentiated cells, but not in proliferating HUMSCs (**Figure 3A**). The relative mRNA levels of INS, NKX2-2, GCG and PDX1 in differentiated cells compared with proliferating HUMSCs showed statistical differences ([INS]P=0.0345, [NKX2-2]P=0.0064, [GCG]P<0.0001, [PDX1]P=0.0222) (**Figure 3B**).

We then determined whether the differentiated cells could secrete insulin in response to glucose stimulation. The result showed that differentiated cells, but not proliferating HUMSCs, secreted insulin when stimulated with low or high concentrations of glucose and the insulin level were 0.2 fold higher when cells were treated with high concentration of glucose than with low concentration of glucose (P=0.0437) (**Figure 4**). These results together suggested that differentiated cells induced by the
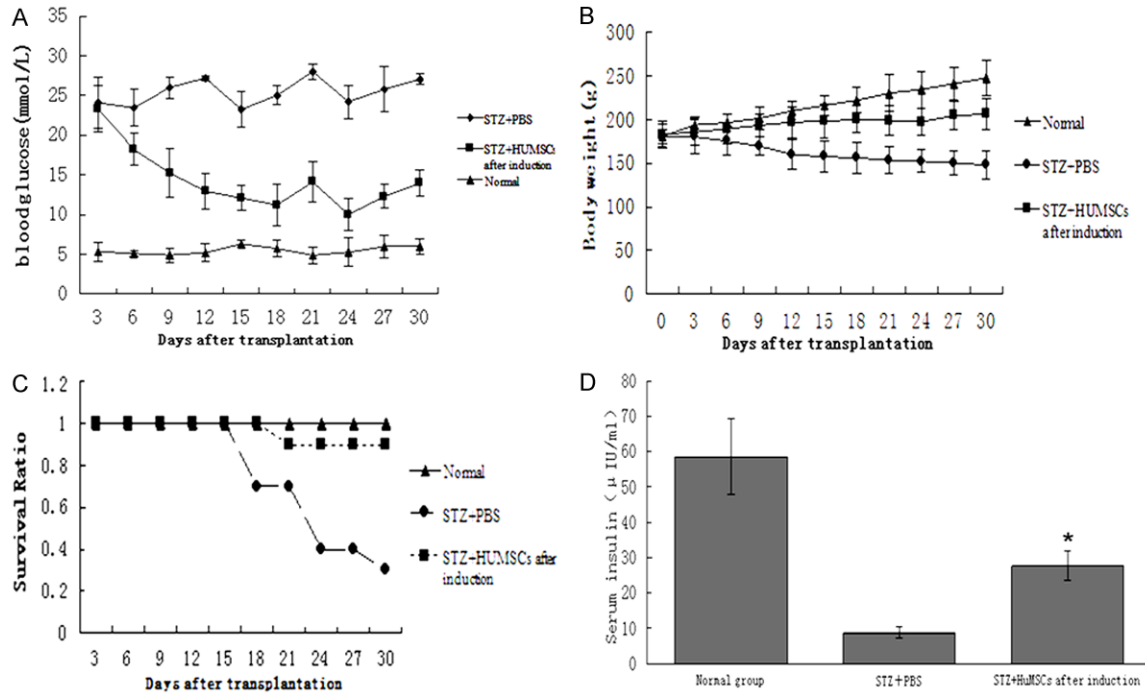
Figure 5. Therapeutic effects of differentiated cells in STZ-induced diabetic rats. Five million differentiated cells were injected into each STZ-induced diabetic rat (STZ-rat) via tail vein. The blood glucose levels (A), body weight (B), survival ratios (C), and insulin levels (D) in these rats were monitored and recorded throughout the course of the 30 d. Normal rats and STZ-rats+PBS were used as controls, respectively. Data showed that blood glucose level (P=0.001), body weight (P=0.047), survival ratios (P=0.028), and insulin levels (P=0.013) of differentiated cells treated STZ-rats were statistical different compared with those of the controls.

two-step method were partially functional pancreatic β cell-like cells.

Therapeutic effects of differentiated cells on diabetic rat model

To evaluate the therapeutic effects of differentiated cells on diabetes, we used a diabetic rat model induced with STZ (designated as the STZ-rat) for the transplantation study. Each STZ-rat received five million differentiated cells or PBS (vehicle control) via tail vein injection. Blood glucose levels, body weight, survival rates, and serum insulin levels were monitored for 30 days.

STZ-rats receiving PBS showed consistently high blood glucose levels, gradually decreased body weight, low survival rates, and decreased serum insulin levels when compared with normal rats (**Figure 5**), suggesting that STZ-induced damage on the pancreatic glands were retained. This was further confirmed by histological analysis of the pancreatic glands in these rats, which showed that islets was significantly

reduced in size and contained few cells (**Figure 6D** and **6E**). STZ-rats receiving differentiated cells showed a marked decrease in blood glucose from post-transplantation day 6 to 9, and this level remained stable until day 30, the last day of this experiment (**Figure 5A**, P=0.001). The body weight of the differentiated cell-treated STZ-rats was steady or slightly increased (**Figure 5B**, P=0.047), and their survival rate was 90 percent, which was much higher than the PBS-treated STZ-rats (**Figure 5C**, P=0.028). Serum insulin in differentiated cell-treated STZ-rats was significantly higher than that of PBS-treated STZ-rats at day 30 post-transplantation (**Figure 5D**, P=0.013).

Taken together, these results suggested that the HUMSCs induced by two-step method functioned in STZ-induced diabetic rat after systematic transplantation.

We further analyzed the damaged pancreatic glands in these differentiated cell-treated STZ-rats using histology and immunohistochemis-
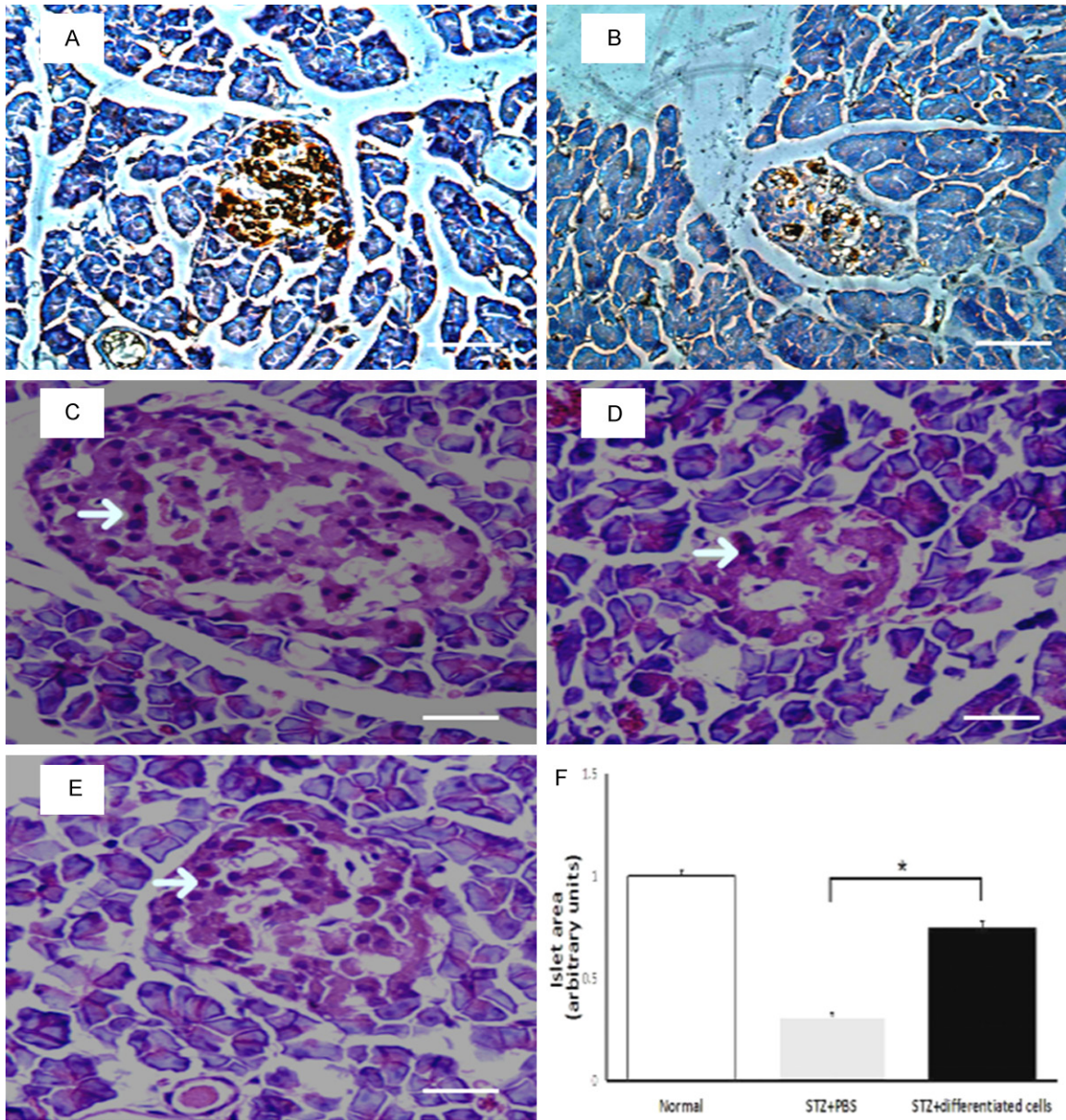
Figure 6. Homing and insulin secretion of differentiation cells in STZ-induced diabetic rats. Five million differentiated cells were injected into each STZ-induced diabetic rat (STZ-rat) via the tail vein. Immunohistochemical (A, B) and Histopathological (H&E) staining (C-E) analysis was performed in pancreatic tissues from these STZ-induced diabetic rats at 30 d post-cell transplantation. (A) Pancreatic tissue section from STZ-rat receiving differentiated cells shows colonized cells expressing human insulin. (B) Pancreatic tissue section from STZ-rat receiving PBS was negative for staining against human insulin. (C) Normal morphology of a pancreatic island from a normal rat. (D) Damaged pancreatic island in the pancreatic tissue from STZ-rat receiving PBS was small with fewer cells in it. (E) Partially repaired pancreatic island in pancreatic tissue from STZ-rat receiving differentiated cells (A-E Bar=100 µm). (F) Arbitary units of islet area in STZ-rat receiving differentiated cells was superior to STZ-rat receiving PBS group (P=0.026).

try. In the pancreatic tissues of the transplanted STZ-rats, cells secreting human insulin was detected (**Figure 6A**) and arbitary units of islet area were significantly increased compared with the PBS-treated control STZ-rats (**Figure** 6D-F). These results indicated that systematically transplanted differentiated cells induced by the two-step method homing to pancreatic glands and partially repaired damaged islets in the STZ-rats.

Discussion

Obtaining enough low-immunogenic autologous adult stem cells for treating diabetic patients is still challenging. In this study, we investigated using HUMSCs as a source of differentiated into insulin-producing cells under special culture conditions, and evaluated the therapeutic potential for the differentiated cells to treat STZ-induced diabetic rats.

A novel two-step approach was used to induce HUMSCs to differentiate into insulin-producing cells. First, HUMSCs were transfect with plasmid DNA expressing NEUROD1, a potent activator of insulin gene transcription [23]. Second, transfected HUMSCs were cultured in DMEM containing a high-glucose concentration (25 mM), with further induction using the β-cell promoting factor nicotinamide and retinoic acid. Nicotinamide is a poly (ADP-ribose) synthetase inhibitor that has been shown to induce differentiation of cultured human fetal pancreatic cells [24], and protects β-cells against desensitization induced by prolonged exposure to large amounts of glucose [25]. Retinoic acid was shown to promote the generation of pancreatic endocrine progenitor cells and their further differentiation into β-cells [26]. Very importantly, this induction process only took about ten days, which could reduce the risk of cell transformation.

With this two-step method, HUMSCs were efficiently induced to differentiate toward fully functional pancreatic β cell-like cells. In vitro, the differentiated cells expressed the pancreatic β cell-specific marker genes, PDX1, NKX2-2, INS, and GCG (**Figure 3**) and secreted insulin in response to glucose stimulation (**Figure 4**). When systematically transplanted into STZ-induced diabetic rats, the differentiated cells homed to and partially repaired the damaged pancreatic glands of STZ-rats (**Figure 6**), leading to stable lower serum glucose levels, steady body weight, higher survival rate, and increased serum insulin levels (**Figure 5**).

The differentiated cells were transplanted into STZ-induced diabetic rat via the tail vein. This means of systematic delivery is relatively non-invasive and easily applied clinically. Other cell transplantation methods, including direct liver injection [15] and renal capsule injection [16], have been used to transplant chemically-induced HUMSCs into diabetic mice. Although in the present study we did not compare the efficiency of these transplantation methodsit is obvious that tail vein injection is more feasible and suitable than other transplantation avenues for the clinical application of stem cell transplantation in treating diabetes. This is particularly true when we consider that differentiated cells transplanted via tail vein home to and repair damaged islets (**Figure 6**).

In conclusion, we prepared a two-step method that combined genetic and chemical approaches to induce HUMSCs to differentiate into functional pancreatic β-like cells. These cells homed to and partially repaired the damaged pancreatic islets in STZ-induced diabetic rats via the vascular system. Therefore, MSCs harvested from umbilical cords could be an excellent candidate in β cell replacement therapy for diabetes. However, the underlying mechanism of HUMSC differentiation into insulin-producing pancreatic β-like cells needs further detailed investigation.

Disclosure of conflict of interest

None.

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