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In vitro generation of glucose-responsive insulin producing cells using lentiviral based pdx-1 gene transduction of mouse (C57BL/6) mesenchymal stem cells

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ABSTRACT

The objective of this study was to evaluate the potential of this type of recombinant lentivirus to generate glucose-responsive insulin producing cells in vitro. All steps of cloning were confirmed using restriction digests. After the transduction, mesenchymal stem cells gradually began to change their morphology and showed differentiation into islet like structures. RT-PCR results confirmed the expression of insulin1, insulin2 and pdx-1 in differentiated cells. Dithizone staining of mouse MSCs showed the concentration of glucose in islet like structures. ELISA analysis validated the insulin secretion of islet like structures which in the high-glucose medium (25 mmol/l) was 7.44 fold higher than that secreted in the low-glucose medium (5 mmol/l). Our results demonstrated that mouse mesenchymal stem cells can be differentiated into effective glucose-responsive insulin producing cells through our new recombinant lentiviral transduction of pdx-1 gene in vitro. This new lentiviral vector could be suggested as an effective candidate for using in gene therapy of type-1 diabetes.

1. Introduction

Type1 diabetes is a chronic disease characterized by severe insulin deficiency and hyperglycemia, due to autoimmune destruction of pancreatic islets of Langerhans [1]. Islet cell replacement has been considered as the potential cure for diabetes over the past 30 years. However, this treatment is limited by a shortage of pancreas donors and immune rejection against islets [2]. In recent years great interests has been generated in MSCs because of their potential use in regenerative medicine. Recent studies demonstrate that Mesenchymal Stem cells (MSCs) have the ability to differentiate into functional insulin-producing cells (IPCs) could become a promising source of islet cells [3]. It has been demonstrated that mesenchymal stem cells can be differentiated into insulin-producing cells by exposure to environmental inducers or direct delivery of some specified genes [4]. One of the most important factors which is involved in pancreas development and transcription of insulin gene is pancreatic & duodenal homeobox 1 (pdx-1) transcription factor. During embryonic development, a cascade of transcription factors might be activated to initiate the development of the pancreas [5]. The expression of this factor can switch on the differentiation and development of stem cells to pancreatic buds, and induce further differentiation [6]. Among different gene delivery methods, lentiviral vectors have significant advantages over other vector systems. Lentiviral vectors are at the forefront of gene delivery systems for research and clinical applications. These vectors have the ability to efficiently transduce nondividing and dividing cells, to insert large genetic segment in the host chromatin, and to sustain stable long-term transgene expression [7]. In this study, in order to generate functional insulin producing cells, we used a lentiviral vector which had been optimized for transduction of mesenchymal stem cells [8] to achieve long time and efficient expression of pdx-1 gene in mouse (C57BL/6) bone marrow derived mesenchymal stem cells. Using this vector system, we show that pdx-1-expressing mouse (C57BL/6) MSCs can differentiate into functional and glucose-responsive insulin producing cells. The function of these cells was confirmed by measuring insulin production and release upon different doses of glucose.

2. Materials and methods

2.1. Plasmids

pcDNA3.1-pdx-1 containing pdx-1 gene had constructed in our lab previously. pSINTREMSEAPHPGKRTTA2S_M2 (We will briefly mention it pSINTREM) was a kind gift from Dr. Isabelle Barde, University of UCL in London, UK [8]. pIRES2-EGFP, pMD2.G, psPAX2 and *Escherichia coli* TOP 10 were purchased from Invitrogen, USA. pTG19-T vector was purchased from Vivantis, Canada.

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2.2. Cell culture

HEK293T (Human Embryonic Kidney 293 cells) and mouse (C57BL/6) bone marrow derived mesenchymal stem cells were obtained from the National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran. HEK293T cells and mouse (C57BL/6) MSCs were maintained in DMEM medium (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA), 100 U/ml penicillin, 100 U/ml streptomycin (Invitrogen, USA), incubated at 37 °C in a humidified atmosphere containing 5% CO_2 / 95% air.

2.3. Construction of recombinant lentiviral vector

To obtain the coding region of pdx-1, primers were designed using DNASIS software (Table 1). Sequences underlined are restriction sites of sacII and BamHI, respectively. The cycle parameters were 95 °C for 5 min, 35 cycles of 95 °C for 20 s followed by 60 °C for 45 s and finally 72 °C for 5 min. The PCR product was cloned into pTG19-T vector according to the manufacturer's protocol. To produce the pIRES2–EGFP-pdx-1 construct, the pdx-1 gene was cut out from the pTG19-T-pdx-1 by sacII and BamHI and cloned into linear pIRES2–EGFP by ligation carried out byT4 DNA ligase. Similarly, to creation of the pTRE-CMV-pdx-1-IRES-EGFP construct TRE-CMV fragment was cut out from pSINTREM vector by XhoI and sacII and cloned into linear pSINTREM. Finally, to produce the final recombinant construct, EGFP–IRES–pdx–1–TRE–CMV was cut out from the pTRE–CMV–pdx–1–IRES–EGFP construct by XhoI and HPaI and cloned into linear pSINTREM by T4 DNA ligation process. All constructs were confirmed by restriction enzyme mapping. Final recombinant lentiviral construct also was confirmed by DNA sequencing analysis. All restriction enzymes were purchased from Fermentas, USA. Restriction digests were performed according to the manufacturer's recommendations.

2.4. Flow cytometric analysis

To confirm pdx-1 gene expression, HEK293T cells were transfected with final recombinant construct, by lipofectamine 2000 and incubated for 48 h. Briefly, after trypsinization, the cells were fixed with 4% paraformaldehyde for 10 min, washed with cold PBS and centrifuged. Then SAP buffer and anti-PDX-1 antibody added to the cells and incubated for 30 min in the dark. After being washed with SAP buffer and centrifuged, the pellets were resuspended in PBS and determined by flow cytometer (FACsort, BD). Non-transfected cells were used as negative control cells.

2.5. Fluorescence microscopy

24 h after transfection some plates (Tet+) were exposed to 1 μg/ml tetracycline, whereas the other plates (Tet−) did not receive tetracycline. Non-transfected cells were used as negative control cells. 48 h after transfection, the transfected HEK293T cells were directly observed for GFP expression using a fluorescence microscope (Olympus Corporation, Japan).

Table 1
Primer sequences used for RT-PCR.

Gene	Primer sequence	Size
pdx-1	Forward: 5′-CCGCGGCCACCATGAACAGTGAGGAG-3′ Reverse: 5′- GGATCCGCTCACCCTCAGACTGCTG -3′	900 bp
Insulin1	Forward: 5′-CTATAAAGCTGGTGGGCATCC-3′ Reverse: 5′-AACGCCAAGGTCTGAAGGTC-3′	327 bp
Insulin2	Forward: 5′-AGCCTATCTTCCAGGTTATTGTTTC-3′ Reverse: 5′-GGTGGGTCTAGTTGCAGTAGTTCTC-3′	368 bp

2.6. Production and concentration of Lentiviral particles

HEK293T cells were seeded at a density of 3.5×10^6 per 100 mm tissue culture dish and transiently co-transfected with lentiviral recombinant construct and two viral packaging vectors (pMD2.G and psPAX2) using lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. The supernatants containing the viral particles were collected 48 and 72 h after transfection and were cleared of cellular debris by low-speed centrifugation (1500 rpm, 5 min) followed by filtration using a 0.45 μm filter. The supernatant was concentrated by ultracentrifugation at 60,000 *g* for 150 min. The concentrated lentivirus was aliquoted and stored at −80 °C for further use. Viral titers (TU/ml) were determined by transfection of HEK293T cells with serial dilutions of the viral supernatant and FACS analysis of the percentage of GFP-positive cells.

2.7. Transduction of mouse MSCs

The mouse MSCs at passage 3 were transduced with the medium containing viral particles ($1–2 \times 10^8$ transducing units per ml) at a 24-well plate and incubated at 37 °C, 5% CO_2 for 72 h. Polybrene (5 μg/ml) (Millipore, USA), and 1 μl/ml lipofectamine 2000 (Invitrogen, USA) were applied during the transduction reaction to enhance the transduction procedure [9].

2.8. Production of functional islet-like structures from transduced MSCs

To production of islet-like structures, transduced cells were cultured in a density of 1×10^5/well cells into 12 well culture plates in low glucose DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin for 8 days. Culture medium was changed at 3-days intervals.

2.9. RT-PCR analysis

8 days after transduction, total cellular RNA was isolated using TRIzol extraction kit (Invitrogen, USA) and quantified by OD at 260 nm. Synthesis of cDNA was performed using AMV reverse transcriptase (Takara, Japan) following the manufacturer's instruction. PCR was performed using EX Taq polymerase (TaKaRa, Japan) according to the manufacturer's protocol. PCR Cycle parameters for insulin gene were 94 °C for 10 min for initial denaturation followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s and extension at 72 °C for 30 s. Finally the PCR products were confirmed on a 1.5% agarose gel and visualized under the UV light after Ethidium bromide staining. The primer sequences were listed in Table 1.

2.10. Dithizone staining

8 days after transduction, the cells were collected and washed in PBS. Then, 0.1 ml 0.05% (mg/l) Dithizone (Sigma, USA) was added and incubated at 37 °C for 20 min. Finally, cells were examined under a microscope.

2.11. Determination of insulin secretion

For insulin secretion assays, non-transduced and transduced mouse MSCs (islet-like structures) were pre-incubated for 1 h in glucose-free Krebs–Ringer bicarbonate (KRB), followed by incubation for 60 min in KRB containing 5.5, 12, 16.7, 25 mmol/l glucose concentration, respectively. The KRB media were collected and frozen at −70 °C until assay. Insulin assay was performed by

enzyme-immunoassay (Human Insulin ELISA kit, Linco Research, USA) according to the manufacturer's instruction.

2.12. Statistical analysis

The data quantitated are expressed as mean ± SEM. The results were analyzed by one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Construction of recombinant lentiviral vector

To construct final recombinant lentiviral vector (pSINTREM-TRE-CMV-PDX-1-IRES2-EGFP), the coding region of pdx-1 was amplified (900 bp) using pcDNA3.1-pdx-1 vector as template and specific primers for pdx-1 gene (Table 1) and cloned into pTG19-T vector to produce pTG19-T-PDX-1 (Fig. 1A and B). To create pPDX-1-IRES2–EGFP construct (6200 bp), pTG19-T-PDX-1 was digested by sacII and BamHI and then pdx-1 gene was cloned into pIRES2–EGFP (Fig. 1C). As shown in Fig. 1D, TRE-CMV fragment (441 bp) was cut out from pSINTREM by XhoI and sacII digestion and was cloned into pPDX-1-IRES2–EGFP to produce pTRE-CMV-PDX-1-IRES2-EGFP construct. Finally TRE-CMV-PDX-1-IRES2-EGFP fragment (2776 bp) was cut out from pTRE-CMV-PDX-1-IRES2-EGFP construct by XhoI and HpaI double digestion and was cloned into pSINTREM to produce final recombinant lentiviral vector pSINTREM- TRE-CMV-PDX-1-IRES2-EGFP (Fig. 1E).

3.2. Analysis of Pdx-1 gene expression in transfected HEK293T cells

To determine the expression of pdx-1, transfected HEK293T cells were analyzed by flow cytometry. Fig. 2A shows transfected HEK293T cells whereas Fig. 2B illustrates non-transfected cells. FL1 and FL2 Channels indicate GFP and pdx-1 expressions, respectively.

3.3. GFP expression under Tet on inducible system control

48 h after transfection, Tet + and Tet – HEK293T cells were observed under a reverse fluorescence microscope. As shown in Fig. 2C Tet + HEK293T cells illustrate GFP expression at high level. In contrast, Tet – HEK293T cells indicate GFP expression at background level (Fig. 2D). Non-transfected cells were used as negative control cells (Fig. 2E).

3.4. Morphological changes of transduced mouse MSCs

To determine the morphological changes which transduced mouse BM-MSCs undergo during transduction, the cells were monitored by an inverted microscope. Fig. 3A and B shows MSCs before and 8 days after transduction. Non-transduced MSCs were typical of adherent spindle and fibroblast-like cells as seen in Fig. 3A. However, after transduction, the cells began to form a ball-like appearance or three dimensional spherical or grape-like clusters (Fig. 3B).

3.5. Histochemical staining of islet-like structures

Dithizone staining was used for confirmation of insulin expression in islet-like structures (Figs. 3C and D). As shown in Fig. 3D, most of the cells were positive for ditizone staining.

3.6. RT-PCR analysis of transduced mouse MSCs

To determine whether the transduced mouse MSCs had undergone pancreatic differentiation, gene expression profiles of insulin 1, insulin 2 and pdx-1 genes were evaluated using RT-PCR. As illustrated in Fig. 3E, the mentioned genes are highly expressed in transduced mouse MSCs after 8 days of transduction.

3.7. Insulin release in response to glucose stimulation

Islet-like structures released increasing amounts of insulin in a glucose-concentration-dependent fashion (Fig. 4). The mean insulin secretion was 0.92 ± 0.09 ng/ml in response to 5.5 mM glucose, 2.86 ± 0.36 ng/ml is response to 12 mM glucose, 5.01 ± 0.10 ng/ml in response to 16.7 mM glucose and 6.85 ± 0.53 ng/ml when the 25 mM concentration was used. These results represent the mean of 5 experiments.

4. Discussion

In recent years, cell transplantation has become a research hotspot concerning surgical methods for the treatment of diabetes. The β islet cells are unique in their ability to produce, process,

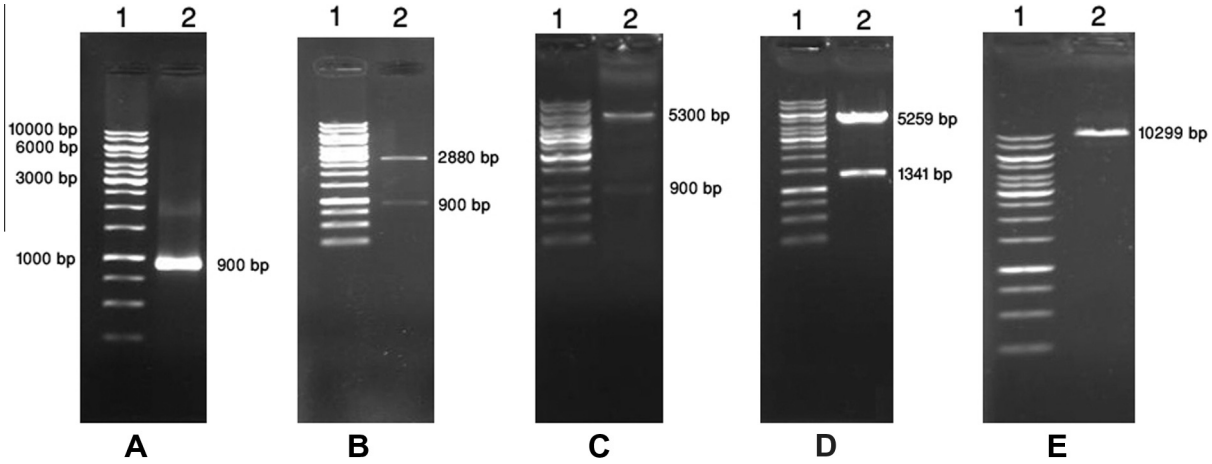

Fig. 1. Agarose gel electrophoresis of PCR product and constructs. (A) Lane 1, DNA ladder 1 kb. Lane 2, PCR product of pdx-1 gene; (B) Lane 1, DNA ladder 1 kb. Lane 2, Double digestion of pTG19-T-pdx-1(3780 bp) with sacII and BamHI; (C) Lane 1, DNA ladder 1 kb. Lane 2, Double digestion of pPDX-1-IRES2–EGFP construct (6200 bp) by sacII and BamHI; (D) Lane 1, DNA ladder 1 kb. Lane 2, Double digestion of pTRE-CMV-PDX-1-IRES2-EGFP construct (6600 bp) using BamHI, XhoI; (E) Lane 1, DNA ladder 1 kb. Lane 2, Digestion of pSINTREM- TRE-CMV-PDX-1-IRES2-EGFP construct (10299 bp) by XhoI.

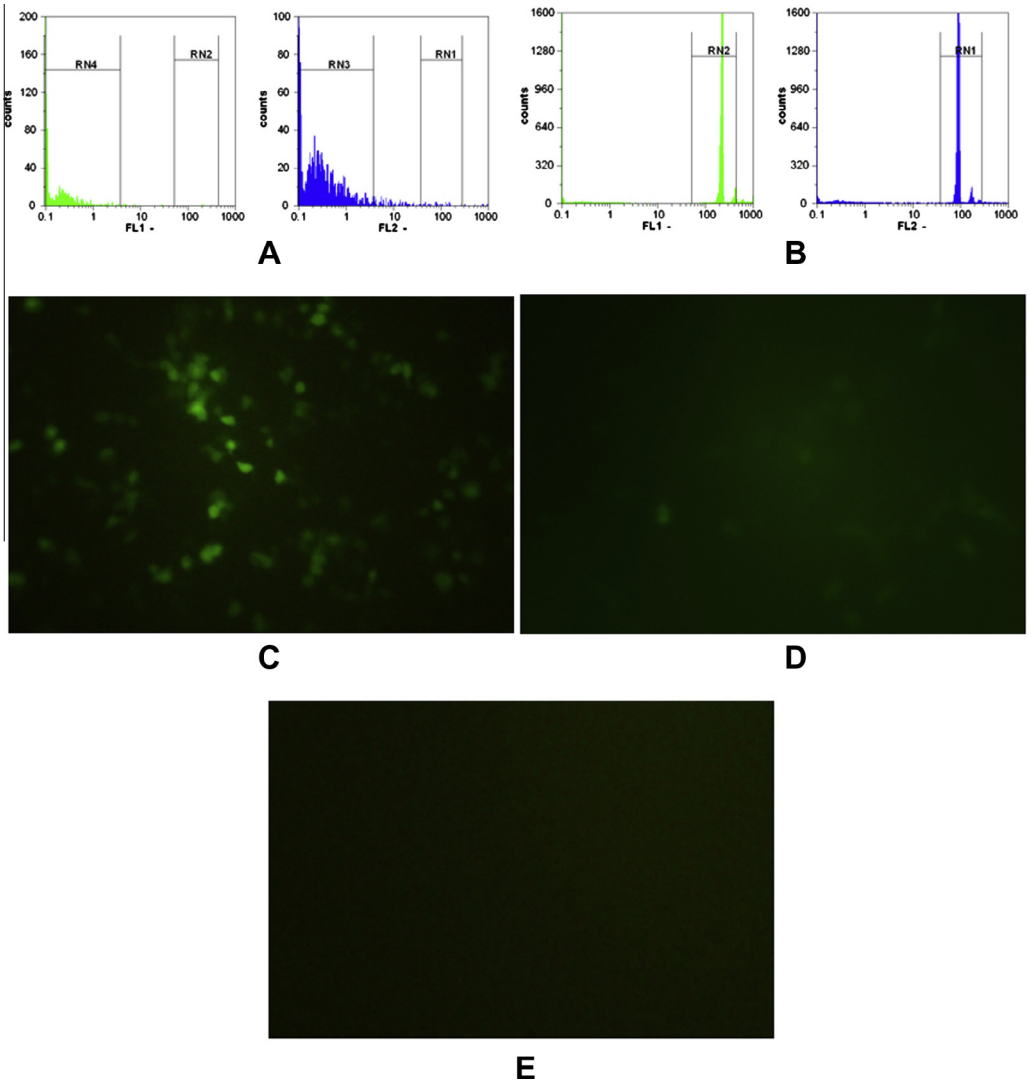

Fig. 2. pdx-1 and GFP expressions in HEK293T using Flow cytometry analysis and fluorescence microscope. (A) Non-transfected HEK 293T cells as negative control cells; (B) HEK 293T cells transfected by final recombinant lentiviral construct; (C) Tet (+) HEK 293T cells; (D) Tet (−) HEK 293T cells; (E) Negative control HEK 293T cells. FL1 and FL2 represent GFP and pdx-1 genes expression, respectively. All magnifications are 250×.

and secrete significant amounts of insulin [10,11]. These cells not only produced insulin but also could secret insulin in response to different concentration of glucose stimulation in a regulated manner [12]. The ability of mesenchymal stem cells to self-renewal at a high proliferative rate makes the cell an excellent target for gene therapy [13].

In this study, we succeeded in constructing a single Tet-on inducible lentiviral vector (pSINTREM- TRE-CMV-PDX-1-IRES2-EGFP) with efficient potency in transduction of stem cells. Transduction of mouse (C57BL/6) bone marrow derived mesenchymal stem cells with this recombinant lentiviral vector led to production of islet-like structures in vitro. Our data indicated that direct transfer of pdx-1 gene by this vector forced the cells to differentiate towards glucose-responsive insulin producing cells.

In order to obtain surrogate β-cells, the target cells were trans-differentiated, dedifferentiated, or differentiated to surrogate β-cells in the usual by expressing some key transcription factors involved in the pancreas development and β-cell gene expression [10,14]. Generally there are two methods for differentiation of MSCs into insulin producing cells. Some of them based on differentiation using chemical inducers (indirect differentiation) and the others rely on gene transfer (direct differentiation). Different protocols have been tried to induce MSCs to differentiate into insulin-producing cells using chemical inducers. Tang et al., demonstrated that two steps in cell culture conditions, high-glucose concentration and nicotinamide, were important for inducing differentiation of MSCs into insulin-producing cells [15]. Wang et al., devised a protocol for efficiently reprogramming MSCs into IPCs within 12 days. They induced MSCs into functional IPCs under specific in vitro conditions based on a combination of matrigel, high concentrations of glucose, activin A, RA (rheumatoid arthritis) and exendin 4. They found that Matrigel could increase the efficiency of IPCs and the size of cell clusters similar to pancreatic islets [16].

There is increasing evidence that transcription factors act synergistically to achieve normal pancreatic development and function. Rapid progress has been made elucidating regulatory mechanisms of key β-cell genes by transcription factors [17]. Traditional transfection methods have shown little success in delivering exogenous DNA into primary MSCs [18] due to inherent stem cell resistance to transfection.

A variety of studies using different viruses have attempted to transduce hMSCs, Zhang et al., have shown that lentiviral vectors can efficiently transduce hMSCs and possess the ability for long-term transgene expression [19]. Lentivirus vectors are a valuable

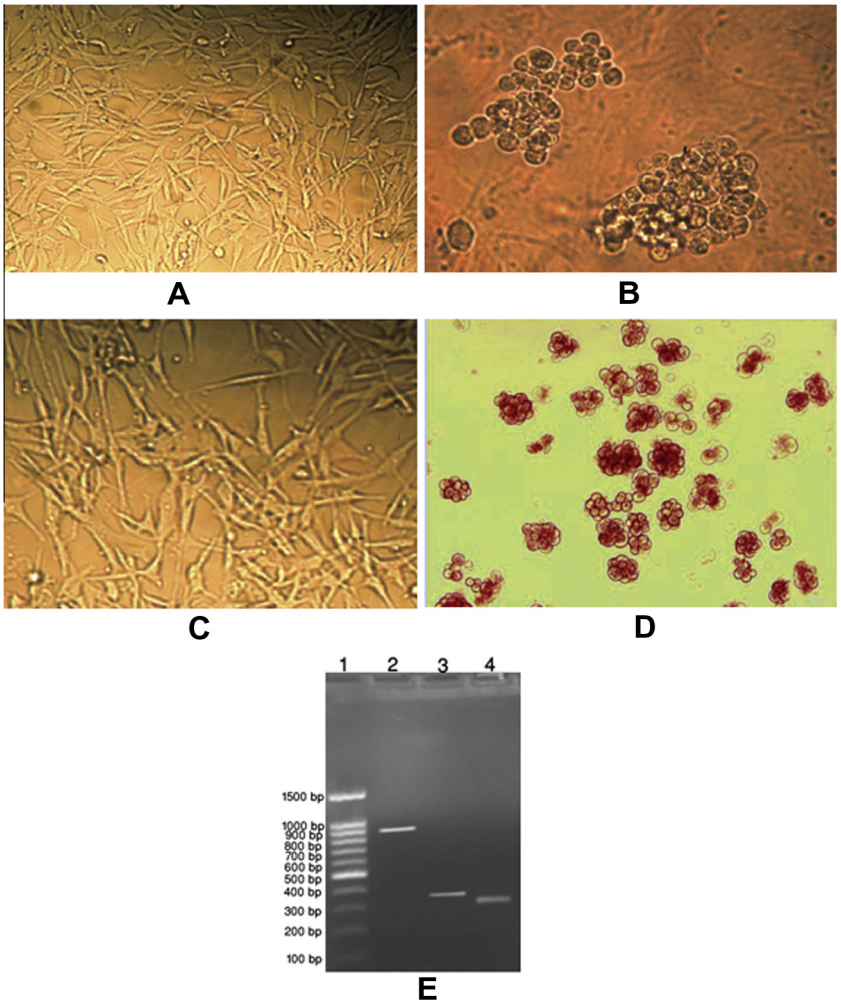

Fig. 3. Evaluation of transduced mouse MSCs. (A) Typical morphology of non-transduced MSCs; (B) Morphological shape of transduced Mscs 8 days after transduction; (C) Negative control (Non-transduced MSCs) of DTZ staining; (D) Transduced Mscs stained with DTZ 8 days after transduction. As shown in Fig. 3D, positive staining for insulin in the cytoplasm of mouse MSCs are presented in red(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.); (E) RT-PCR results of transduced mouse MSCs 8 days after transduction. Lane 1, DNA ladder 100 bp. Lane 2, 3 and 4 show the expression of pdx-1, insulin 1 and insulin 2 genes, respectively. Magnifications of A and D are × 250 and magnifications of B and C are × 400.

gene delivery tool and lentivirus-mediated genetic modification of MSCs has the potential to significantly augment the therapeutic benefit of MSC-based therapies [20].

Different factors such as pdx-1 (pancreatic & duodenal homeobox 1), Ngn3 (neurogenin 3), MafA (v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A), Pax4 (paired box protein 4), Pax6 (paired box protein 6), NeuroD1(Neurogenic differentiation factor 1), Hnf1α (Hepatic Nuclear Factor 1α) and Hnf-3β (Hepatic Nuclear Factor 3β) have been reported to bring about the transdifferentiation of stem cells to β-like cells in vitro or in vivo [21].

It is well known that pdx-1 is one of the key transcription factors in pancreatic β-cell differentiation and expressed throughout all development periods as well as broadly in the initial pancreatic precursor population, and in mature pancreas [22].

Chiou et al., overexpressed MafA in PDMSCs using a lentivector, and investigated the role of MafA in the reprogramming of pancreatic lineage cells and the enhancement of insulin-secreting function in placenta-derived multipotent stem cells. They demonstrated that transduction of a single, critical pancreatic transcriptional factor, MafA, can efficiently convert PDMSCs into functional pancreatic islet-like progenitors [23].

Guo et al., reported a procedure for delivery of combination of pdx-1, NeuroD1, and MafA into mouse MSCs on adenoviral vectors and their differentiation into IPCs in vitro and in vivo [24]. Using a recombinant adenovirus carrying the pdx-1 gene Heet al. indicated that MSCs from human umbilical cord could be induced to differentiate into mature islet-like cell clusters, which possess insulin-producing ability in vitro [25]. Kim et al., examined genetically altered hMSCs after lentiviral transduction of the pdx-1 gene to determine their ability to differentiate into insulin-producing cells in vitro. Their results showed that hMSCs can be differentiate into insulin-producing cells in vitro [22].

Recently, Barde et al., produced a range of lentiviral expression vectors using Tet on inducible system for stem cell transduction. They conclude that one of their new lentiviral vectors (pSINTREM) is the most efficient expression vector in the hematopoietic stem cells in vivo and in vitro in comparison with other vectors investigated [8]. Also, Loebinger et al., used this vector to express and transduction of TRAIL gene into MSCs in vivo and in vitro for the reduction and elimination of metastatic disease in a lung metastatic cancer model [26].

Based on these evidences, to reach an efficient expression of pdx-1 gene, we cloned this gene into pSINTREM vector as a single

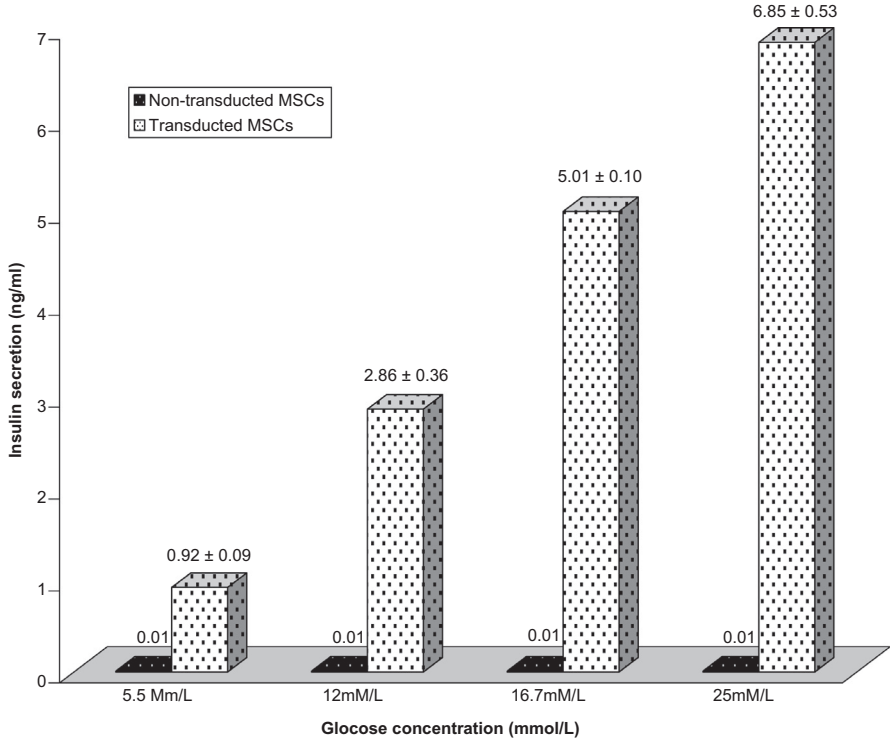

Fig. 4. Insulin release in response to glucose challenge. Different glucose concentrations (5.5, 12, 16.7, 25 mmol/L) was used for insulin secretion stimulation after 1 h incubation.

Tet-on inducible lentiviral vector backbone and used it to transduction of mouse (C57BL/6) bone marrow derived mesenchymal stem cells. In addition, the IRES-EGFP fragment was added into this lentiviral vector as a reporter gene.

Our data showed that the titer of this recombinant pdx-1lentivirus reached 1–2 × 108 TU/ml. The results of flow cytometry demonstrated that the recombinant lentiviral vector was capable of expressing pdx-1 efficiently in vitro. Furthermore, the results of fluorescence microscopy showed that Tet on inducible system in lentiviral vector (pSINTREM- TRE-CMV-PDX-1-IRES2-EGFP) was right in vitro.

Cellular monitoring after taransduction reveals that lentiviral based transduction of pdx-1 gene can lead to morphological changes towards islet-like structures.

RT-PCR analysis detected mRNA synthesis of insulin 1, insulin 2 and pdx-1 from the transduced mouse MSCs.

DTZ staining is a valuable to specifically identify IPCs [27,28]. DTZ is a binding substance and due to higher zinc contents of the pancreatic islets compared with other cells, they become crimson red after DTZ staining [29]. Zinc is required in pancreatic β-cells for packaging insulin [30] and we took advantage of zinc assembly to identify IPCs differentiated from MSCs. Since islet-like cells differentiated from stem cells can uptake insulin from culture medium [31], control cells were also stained with DTZ which were negative and proved that insulin has been produced by differentiated cells.

Furthermore, glucose-induced insulin secretion was used to investigate the function of transduced mouse MSCs. Insulin secretion after glucose stimulation was measured by ELISA analysis. In this study, the mouse MSCs were cultured in different concentrations of glucose (5.5, 12, 16.7, 25 mmol/l) medium. Glucose-stimulated insulin release increased 0.92 ± 0.09, 2.86 ± 0.36, 5.01 ± 0.10, 6.85 ± 0.53 at 5.5, 12, 16.7, 25 mmol/l glucose challenges, respectively 1 h later.

In conclusion, our results demonstrate that mouse (C57BL/6) bone marrow mesenchymal stem cells are capable of being reprogrammed in vitro to produce glucose-responsive insulin-producing cells. We exploited a novel system for direct differentiation of mouse bone marrow derived mesenchymal stem cells based on lentiviral vector system. Our results suggest that pdx-1-expressing mouse (C57BL/6) MSCs may be a useful source of insulin-producing cells.

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