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Sonic hedgehog pathway suppression and reactivation accelerates differentiation of rat adipose-derived mesenchymal stromal cells toward insulin-producing cells

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Abstract

Background aims. Sonic hedgehog (Shh) is an intercellular signaling molecule that regulates pancreas development in mammals. Manipulation of Shh signaling pathway can be used as reliable approach to improve the generation of functional insulin-producing cells (IPCs) from mesenchymal stromal cells (MSCs). *Methods.* In the present study, a novel differentiation protocol was used to produce IPCs from adipose tissue–derived MSCs (ATDMSCs) based on sequential inhibition and reactivation of Shh pathway. ATDMSCs were differentiated into IPCs via a 14-day basic protocol using 1% insulin transferrin selenium (ITS) and 1% nicotinamide in Dulbecco's Modified Eagle's Medium medium. A mixture of 0.25 μmol/L cyclopamine + 64 ng/mL basic fibroblast growth factor at day 3 of differentiation and 150 ng/mL recombinant Shh at day 11 of differentiation were used, respectively, to promote sequential inhibition and reactivation of Shh pathway. Insulin granule formation, glucose-stimulated insulin secretion and gene expression pattern related to the pancreatic endocrine development and function were analyzed in manipulated and unmanipulated IPCs. *Results.* IPCs obtained after Shh manipulation secreted higher amounts of insulin *in vitro*. This phenotype was accompanied by increased expression of both genes critical for β-cell function and transcription factors associated with their mature phenotype including *Pdx1*, *MafA*, *Nkx2.2*, *Nkx6.1*, *Ngn3*, *Isl1* and insulin at day 14 of differentiation. *Conclusions.* Our findings indicated that the early inhibition and late reactivation of Shh signaling pathway during the differentiation of ATDMSCs improved the functional properties of IPCs, a novel method that could be considered as an alternative approach for cell-based therapy for type 1 diabetes.

Key Words: *adipose tissue–derived mesenchymal stromal cells, insulin-producing cells, sonic hedgehog manipulation*

Introduction

In patients with type 1 diabetes mellitus (T1DM), beta-cell dysfunction results in hyperglycemia and metabolic disorders [1]. One of the most attractive interests of research is to achieve a permanent, noninvasive, non-immunosuppressive method treat T1DM. Cell-based therapy using differentiated embryonic or mesenchymal stromal cells (MSCs) seems to be a reliable approach for the treatment of T1DM [2,3]. Stem cell therapy protocols have limitations, however, such as insufficient insulin secretion *in vitro*, destruction of grafted cells with autoimmune system, tumor formation and ethical concerns in the case of embryonic stem cells [2]. Reassessment of some protocols yielded controversial data, indicating uptake of insulin from the medium or its expression by neuronal progenitors known to frequently appear in embryonic stem cells cultures [4,5]. Until recently, there was no protocol for straightforward *in vitro* differentiation of functional beta cells from stem cells. Therefore, much effort has been directed toward finding novel stem cell sources and improving differentiation protocols to develop a promising therapeutic method for treating T1DM.

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Hedgehogs (Hhs) are intercellular s
molecules that regulate tissue patterning in
lian development. Mammalian Hhs inclu
hedgehog (Shh), Indian hedgehog (Ihh) a
hedgehog (Dhh). Shh has a critical role i
morphogenesis of the human embryo through regulation of various basal procedures in embryonic evolution, such as controlling the fate, planning, regeneration, differentiation and survival of the cells in a concentration-dependent manner [6]. In adults, the Shh signaling pathway remains active and mediates stability, proliferation and reconstruction of tissues. Although the absence of Shh expression is required for the early development of the endocrine and exocrine pancreas [7], many discrepancies have been observed about the action of Shh in adult β cells. *In vivo* evidence showed that increased Shh signaling in adult β cells results in glucose intolerance due to impaired glucose-stimulated insulin secretion, likely resulting from a combined reduction in the expression of genes required for glucose sensing, insulin production and secretion [8]. In contrast, other reports have shown that Shh pathway is active at low levels in adult β cells, and pathway inhibition throughout pancreas development results in impaired β-cell function during adulthood. Previous research has also shown that endocrine activities of the pancreas, such as insulin and glucagon expression and secretion, depend on reactivation of Shh signaling pathway [9].

Given these findings, it has been concluded that time-dependent inactivation and reactivation of Shh signaling is required for normal expression of critical transcription factors and various components of the insulin sensitivity and insulin secretion machinery of mature β cells. Although previous work has shown that early inhibition of Shh signaling improves differentiation of embryonic stem cells to insulin-producing cells (IPCs) [10], whether early suppression and late reactivation of Shh signaling have beneficial effects on *in vitro* differentiation of MSCs to β cells has not been investigated.

Adipose tissue–derived MSCs (ATDMSCs) are self-renewing stromal cells that are isolated from visceral adipose tissues and are an abundant source of stromal progenitor cells. These cells can be obtained through noninvasive methods, and in rats there has been no evidence of immune system rejection after transplantation. ATDMSCs can differentiate into various cell lineages, and, compared with MSCs from other sources, ATDMSCs have the highest proliferation capacity and retain their pluripotency after multiple passages [11].

In this study, we aimed to determine the effect of time-dependent suppression and reactivation of Shh signaling on the expression of β-cell molecular markers d insulin secretion capacity of IPCs derived from fferentiation of ATDMSCs.

lethods

reparation of rat ATMSCs

The experiment was performed with normal Sprague-Dawley male rats aged 2–3 months. All animals were cared for according to the guide for the care and use of laboratory animals by the U.S. National Academy of Sciences (National Institutes of Health Publication No. 86-23). Animals were euthanized with a combination of ketamine and xylazine (100 mg/kg of ketamine and 10 mg/kg of xylazine), and splanchnic adipose tissue was obtained under sterile conditions and preserved in phosphate-buffered saline (PBS, pH = 7) 4°C. The tissues were washed three times with PBS and sliced into small pieces. Then one volume of obtained tissues was immersed in three volumes of Dulbecco's Modified Eagle's Medium (DMEM; Bioidea) contained 1 mg/mL collagenase type 1 (Alfa Aesar), and the samples were centrifuged at 1500 RPM at 37°C for 25 min. Collagenase was neutralized by adding DMEM + 10% fetal bovine serum (FBS) (Gibco) and centrifuged at 1200 RPM for 7 min. The supernatant was removed, and precipitated cells were resuspended in DMEM + 10% FBS + 1% penicillin/streptomycin (Gibco) and seeded in 25 cm^2 and incubated at 37°C with 5% Co_2. The medium was changed every 2–3 days. After cells reached the third passage, differentiation was performed.

Characterization of ATDMSCs

Flow cytometry: Availability of cell surface biomarkers known as clusters of differentiation (CD), including CD34, CD45, CD90 and CD105, was examined using flow cytometry. At the third passage, after cell confluency reached 90% in each flask, cells were detached using 0.05% trypsin treatment (Gibco), PBS was added and the samples were centrifuged at 2000 RPM for 5 min. Supernatant was discarded and the remaining pellet was resuspended in PBS contained 5% FBS. Specific antibodies labeled with FITC (eBioscience) were added and incubated in the dark at 4°C for 30 min. PBS contained 5% FBS was added to each sample and after 5 min centrifugation at 120 RPM, fluorescence activity of samples was measured with Galaxy flow cytometer (Dako). The results were analyzed with FlowJo 8.8.7 software. Two negative controls including isotype control and stainless control were provided for each sample.

Adipogenic differentiation: ATDMSCs at third passage were cultured for 14 days in adipogenic differentiation medium containing DMEM/F12 (Bioidea), 100 nmol/L dexamethasone, 50 μg/mL indomethacin

and 50 μg/mL ascorbic-2-phosphate. The differentiation medium was replaced every 3 days. Typical adipocyte morphology was detected by oil red O staining.

Osteogenic differentiation: ATDMSCs at third passage were cultured for 21 days in osteogenic differentiation medium containing low-glucose DMEM, 50 μg/mL ascorbic-2-phosphate, 10 nmol/L dexamethasone and 10 nmol/L β-glycerol phosphate. The differentiation medium was replaced every 3 days. Typical osteocyte morphology was detected by alizarin red staining.

In vitro differentiation procedure

ATDMSCs were differentiated to IPCs in two separate experiments via a three-stage basic protocol as described previously [12]. In experiment 1, the cells were expanded in high-glucose DMEM contained glucose 25 mmol/L, 10% FBS and 1% penicillin/streptomycin until cells reached 80% confluency (stage 1). Culture medium was then replaced with 1:1 DMEM/F12 medium (1:1) containing 1% insulin transferrin selenium (ITS) (Gibco), 2% FBS and 1% penicillin/streptomycin for 7 days (stage 2). Finally, a mixture of low-glucose DMEM containing 5.5 mmol/L glucose, 10% FBS, 1% ITS, 1% nicotinamide (Sigma Aldrich) and 1% penicillin/streptomycin was used for 7 days (stage 3).

Experiment 2 was performed to promote sequential inactivation and reactivation of Shh pathway. The basic protocol was performed, and a mixture of 0.25 μmol/L cyclopamine (Sigma Aldrich) and 64 ng/mL basic fibroblast growth factor (bFGF) (Sigma Aldrich) was added to the medium at day 3 of differentiation to inhibit the Shh pathway. At day 11 of differentiation, Shh pathway reactivation was performed by adding 150 ng/mL recombinant Shh (Sigma Aldrich).

Dithizone staining

A working solution of 100 ng/mL of dithizone (DTZ; Sigma Aldrich) was prepared in dimethyl sulfoxide (Sigma Aldrich) and filtered through a 0.2-μm filter. At the final stage of differentiation, 3 mL of working solution was added to each 25-cm^2 flask and incubated at 37°C for 30 min. Cells were washed three times with PBS, and crimson red–stained clusters were observed using a phase contrast microscope (Olympus IX71).

Gene expression analysis by reverse transcription polymerase chain reaction during differentiation of ATDMSCs

To determine the gene expression pattern of IPCs at various stages of differentiation, conventional reverse transcriptase polymerase chain reaction (RT-PCR) analysis was performed. Cells were taken at each stage of differentiation, and total RNA was extracted using the RNX isolation reagent according to the manufacturer's procedure (CinaClon). Concentration of extracted RNA was calculated at a wavelength of 260 nm using NanoDrop spectrophotometry (Thermo Scientific NanoDrop). To detect the purity of the RNA, its optical density absorption ratio at 260/280 nm was determined, and samples with a ratio greater than 1.8 were used for cDNA synthesis. Reverse transcription was carried out with the CycleScript cDNA synthesis kit (CycleScript RT PreMix Bioneer) using 1 μg of RNA and oligo dT based on manufacturer's protocol (Bioneer Corporation).

The list of genes and characteristics of their primers that were used in the present study are shown in Table I. PCR was performed in a 20-μL reaction containing 10 μL Taq DNA polymerase master mix red (Amplicon), 0.5 μL of each primer (10 μmol/L) and 3 μL cDNA template (~100 ng) and 6 μL DNase free DW. PCR cycling included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 45 s and extension at 72°C for 60 s. This was followed by a final extension at 72°C for 5 min. PCR products were electrophoresed in 1.5% agarose (SinaClon) in tris-acetate-EDTA buffer, stained with Green Safestain (SinaClon, Iran) and visualized under ultraviolet light.

Effect of Shh pathway manipulation on gene expression pattern of IPCs

The gene expression pattern between differentiated IPCs with Shh manipulation and without Shh manipulation was compared by real-time PCR analysis, performed using Ampliqon RealQ Plus Master kit for SYBR Green I (Ampliqon) on a Lightcycler Detection System (Roche). The list of genes and characteristics of their primers that were used in the present study are shown in Table I. Reactions were prepared in a 12.5-μL mixture containing 6.25 μL of master mix kit, 0.25 μL of each primer (200 nmol/L), 3 μL cDNA (100 ng) and 2.75 μL nuclease. The PCR protocol used consisted of a 5-min denaturation at 94°C followed by 45 cycles of 94°C for 15 s, 60°C for 30 s. Two separate reactions without cDNA or with RNA were performed in parallel as controls. Relative quantification was performed according to the comparative $2^{-\Delta\Delta Ct}$ method and using Lightcycler 96 software. Assay validation to ensure that the primer for the target genes and *GAPDH* had similar amplification efficiencies was performed as described previously [13]. All quantitative PCR analysis was performed according to the Minimum Information for

Table I. Characteristics of primers used in the present study.

Gene name	Sequence (5′″ 3′)	Length (bp)	Accession number
GAPDH	TG GTATCGTGGAAGGACTC CCTGCTTCACCACCTTCTTG	290	NM_017008.4
Fox A2	AATGGACCTCAAGGCCTACGAAA AGTTCATAATGGGCCGGGAGTAA	170	NM_012743.1
Sox 17	TGGACCGCACGGAATTTGAACA TTGCAGTAATATACCGCGGAGCG	157	NM_001107902.1
Oct4	TGTCCTTTCACGATGCTCTG TCTTCTGCTTCAGGAGCTTG	131	EU419996.1
Pdx1	GGAGGGTTTGGAAAACCAGT ACAAACATAACCCGAGCACA	131	NM_022852.3
Nkx2.2	AAACCGTCCCAGCGTTAAT TGCTTTAGAAGACGGCTGAC	126	NM_001191904.1
Nkx6.1	ACACACGAGACCCACTTTTT TTCTGGAACCAGACCTTGAC	147	NNM_031737.1
Isl-1	GCTTTTCAGCAACTGGTCA AATAGGACTGGCTACCATGC	123	NM_017339.3
Insulin	ATCTTCAGACCTTGGCACTG ATCTTCAGACCTTGGCACTG	141	NM_019129.3
MafA	CTGCTGTCCTACTATGCTCA TGTATTTCCCCAGGAGTTACAG	137	XM_006241903.2
Ngn-3	CTATTCTTTTGCGCCGGTAC CTGACGGTCACTTGGCAG	128	NM_021700.1

Publication of Quantitative Real-Time PCR Experiments guideline [14].

Insulin secretion assay

To determine the insulin secretion potency of the IPCs, an insulin secretion assay was performed on fully differentiated cells. Kerebs Ringer bicarbonate (KRB) buffer was prepared using 120 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 10 mmol/L HEPES, 1.1 mmol/L $NaHCO_3$ in sterile deionized water. KRB buffer was filtered using 22-μm filter and 0.5% bovine serum albumin (Sigma Aldrich) was added to the filtered buffer. Undifferentiated ATDMSCs were used as a negative control. The differentiated and control cells were washed three times with KRB buffer. Cells were pre-incubated with KRB buffer containing 5 mmol/L glucose at 37°C for 1 h. The buffer was removed and replaced with 1 mL of fresh KRB buffer with 25 mmol/L glucose/30 mmol/L KCl, then incubated for 30 min at 37°C. The supernatant was obtained, and insulin concentrations were determined using a rat-specific insulin enzyme-linked immunoassay kit (Monobind) based on the protocol recommended by the manufacturer. Insulin concentration was reported as μU/L.

Statistical analyses

Data analyses were done using the SPSS 18.0 software package (SPSS Inc.). One-way analysis of variance followed by *Tukey* post hoc analyses were used to test differences between various means. All experimental data were presented as the mean ± SEM. The level of significance for all tests was set at $P < 0.05$.

Results

Morphological characteristics of ATDMSCs in cell culture

Rat adipose MSCs appeared small, non-adherent and round immediately after isolation (Figure 1A). After attachment of cells to the flask, some nonspecific cells were present among the main group of MSCs. The main group of cells were spindle-shaped and with a fibroblast-like appearance; however, nonspecific cells showed different morphologies (Figure 1B). Nonspecific cells were isolated from the main cells by passaging. By passage 3, there were no nonspecific cells, and all remaining cells were MSCs with a fibroblast-like shape (Figure 1C).

In vitro differentiation of ATDMSCs toward adipocytes and osteocytes

To establish multipotent activity of ATDMSCs, cells in the third passage were differentiated toward adipocytes and osteocytes. Osteogenic differentiation was confirmed by alizarin red staining of calcium vacuole deposits in differentiated cells. Results confirmed that cells could successfully differentiate into adipocytes and osteocytes (Figure 2A). Adipogenic differentiation was identified by neutral lipid vacuole formation (stained with oil red O) in cultures (Figure 2B).

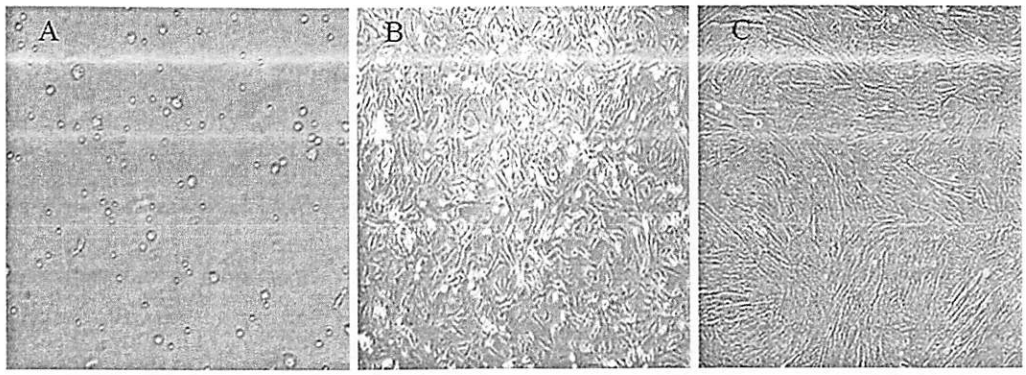

Figure 1. Changes in cells morphology during the expansion of ATMSCs in culture medium. (A) Newly isolated cells from rat adipose tissue with round morphology. (B) Spindle-like specific cells with some nonspecific cells during early days of expansion. (C) Specific ATMSCs with a fibroblast-like shape at passage 3.

Flow cytometry and RT-PCR analysis of stem cell markers

Analysis of specific surface cell markers of multipotent MSCs, revealed that ATDMSCs were positive for CD90 (99%) (Figure 2E) and CD105 (98%) (Figure 2F). The cells showed negative expression of hematopoietic marker (CD34) (Figure 2C) and leukocyte common antigen (CD45) (Figure 2D). They maintained their phenotype in the following passages. ATDMSCs also expressed proliferation and differentiation gene markers including *Oct4* and *Nanog* (Figure 2G). The expression of *Oct4* was reduced in ATDMSCs in a time-dependent manner after induction of differentiation (Figure 2H).

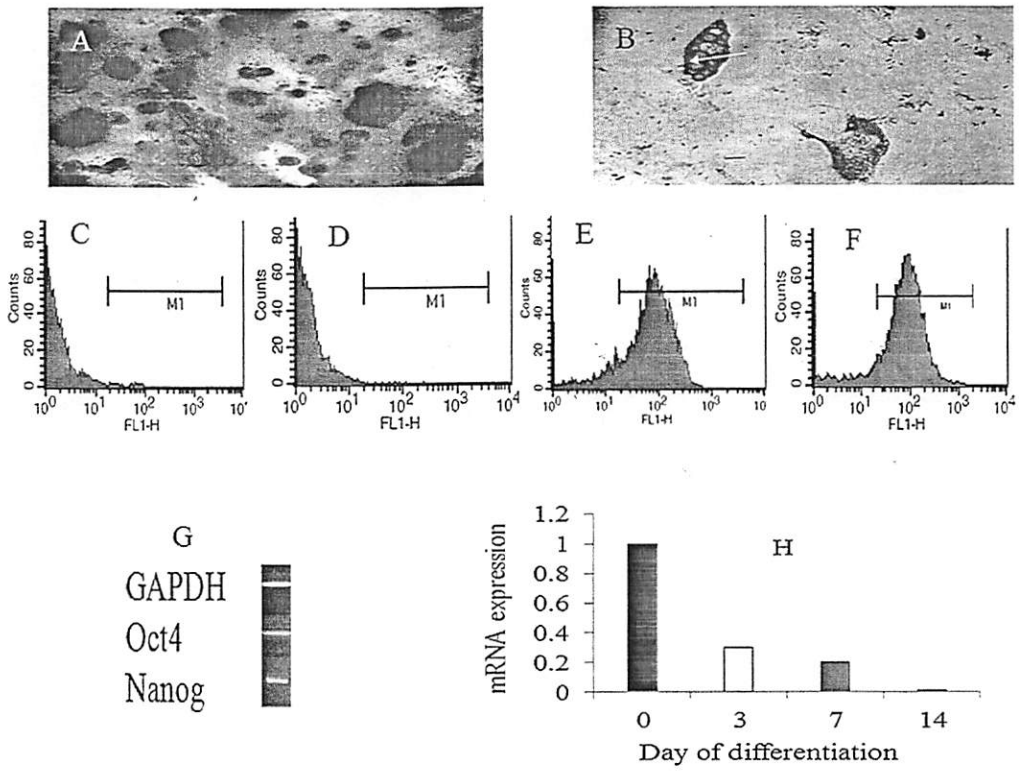

Figure 2. Characteristics of ATDMSCs. Osteogenic differentiation capacity of ATDMSCs was confirmed by detection of alizarin red–stained calcium deposits (A). Adipogenic differentiation potential of ATDMSCs was determined by oil red O–stained oil droplets (B). Flow cytometry results showed that undifferentiated ATDMSCs were negative for hematopoietic and leukocyte common antigens including CD34 (C) and CD45 (D) and positive for MSC-specific surface marker proteins including CD90 (E) and CD105 (F). RT-PCR analysis revealed that undifferentiated ATDMSCs expressed *Oct4* and *Nanog*, demonstrating the proliferation and differentiation capacity of ATDMSCs (G). Real-time PCR analysis showed reduction of Oct4 mRNA expression throughout differentiation stages (H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

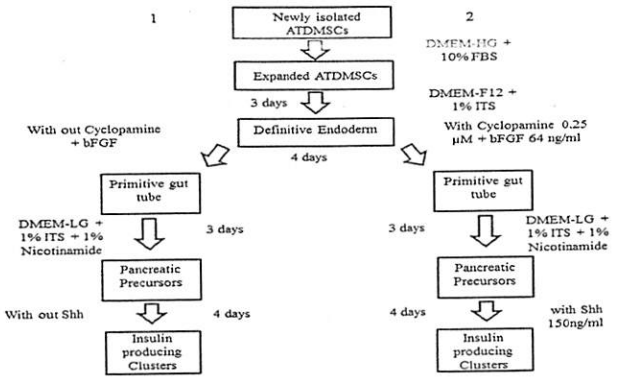

Figure 3. Three-stage protocol for differentiation of adipose tissue–derived MSCs into functional IPCs. Cells in experiment 2 were treated with 0.25 µmol/L cyclopamine + 64 ng/mL bFGF at day 3 of differentiation for suppression of Shh pathway and were subsequently treated with recombinant Shh at day 11 of differentiation for reactivation of Shh pathway.

Characteristics of ATDMSCs during the differentiation to IPCs

Differentiation protocol of ATDMSCs into IPCs with or without Shh manipulation is summarized in Figure 3. ATDMSCs were expanded rapidly in HG-DMEM + 10% FBS culture medium in stage 1 of differentiation. Cells displayed fibroblast-like, spindle-shaped morphology during the early days of incubation (Figure 1C). Furthermore, expression of *Oct4* and *Nanog* genes indicated that ATDMSCs in passage 3 were undifferentiated cells with high proliferation and differentiation potential (Figure 2G). Real-time PCR results emphasized an obvious decrease in Oct4 mRNA expression after differentiation of ATDMSCs (Figure 2H).

During the stage 2 of differentiation, cell shape changed to round, similar to epithelial cells (Figure 4A,B). Reverse transcription PCR analysis showed that cells expressed definitive endoderm-specific genes such as *Sox17* and *FoxA2* on day 3 of stage 2 (Figure 4A). On day 7 of stage 2, expression of *Pdx1* (marker of pancreatic development) was also determined (Figure 4B). ATDMSCs were exposed to LG-DMEM with 1% ITS and 1% nicotinamide at stage 3 of differentiation. At the end of this stage, cells displayed the phenotype of round adult cells. The morphology of cells was spheroid with an islet-like structure. At day 10 of this stage, some aggregations of round cells were formed (Figure 4C). IPCs showed expression of endocrine- and pancreas-specific genes, such as *Nkx2.2*, *Nkx6.1*, *Pdx1* and *Ngn3*, on day 10 of differentiation, demonstrating the pancreatic progenitor formation (Figure 4C). At the end of stage 3, specific makers of functional islet-like cell creation, such as *Pdx1*, *MafA*, *Isl1* and *Insulin*, were observed (Figure 4D). As shown in Figure 4D, cells that were distinctly stained crimson red by DTZ became apparent at final step of differentiation on day 14.

Effect of Shh pathway manipulation on gene expression pattern of IPCs

To determine the effect of manipulation of Shh pathway on differentiation potential of ATDMSCs to IPCs, Shh was first inhibited by cyclopamine + basic fibroblast growth factor at day 3 of differentiation and

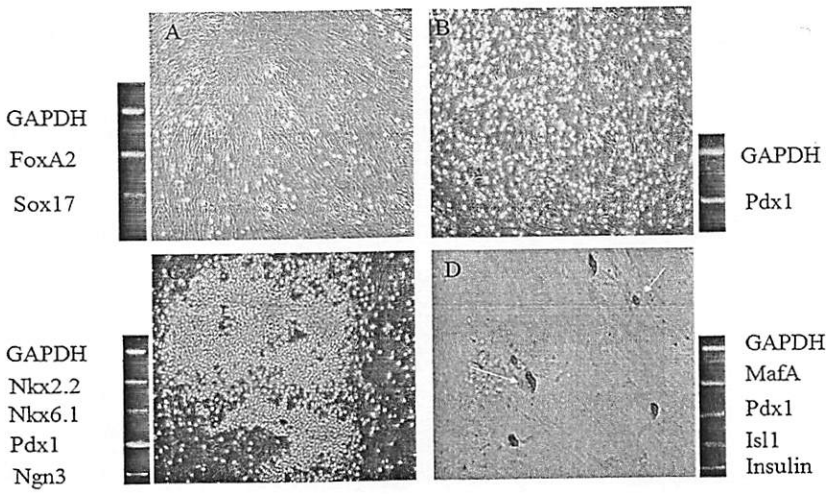

Figure 4. Morphological and molecular characteristics of ATDMSCs during differentiation into IPCs. The number of cells with an epithelial-like shape increased, consistent with differentiation progress (A, B). At day 3 of differentiation, cells expressed definitive endoderm-specific markers, such as Sox17 and FoxA2, and showed expression of Pdx1, a significant marker of pancreatic evolution, at day 7 of differentiation. Clusters of round IPCs at day 10 (C) exhibited expression of Pdx1, Nkx2.2, Nkx6.1 and ngn3, consistent with pancreatic precursor formation. Insulin-producing clusters stained with DTZ (D) showed expression of Pdx1, MafA, Isl1 and insulin, demonstrating the formation of functional IPCs.

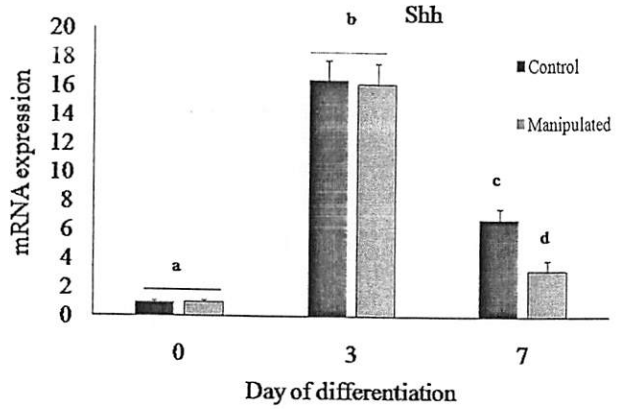

Figure 5. Relative mRNA expression of Shh in manipulated and control ATDMSCs at days 3 and 7 of differentiation. Shh pathway was inhibited in manipulated cells by treatment of cells with a combination of bFGF and cyclopamine at day 3 of differentiation. Data are mean ± SD. Bars with different letters differ significantly ($P < 0.05$).

subsequently was reactivated by recombinant Shh at day 11 of differentiation. Combination of cyclopamine and bFGF could reduce the expression of Shh in ATDMSCs at day 3 of differentiation when compared with un-manipulated cells at similar day (Figure 5). To determine the effect of Shh pathway manipulation on expression of beta cells related genes Real time PCR analysis was carried out at the end of experiment (day 14). Results revealed that the expression of genes related to the pancreatic endocrine development and function including *Pdx1*, *MafA*, *Nkx2.2*, *Nkx6.1*, *Ngn3*, *Isl1* significantly were up-regulated when Shh pathway was manipulated during the differentiation of ATDMSCs to IPCs (Figure 6A–G). As shown in Figure 6G, *Insulin* mRNA expression was significantly higher in Shh-manipulated cells compared with untreated cells.

Insulin secretion assay

To investigate whether insulin secretion from ATDMSC-derived IPCs could be regulated by glucose, cells were treated with KRB buffer containing either a low or high concentration of glucose, and insulin was then measured in the culture medium by enzyme-linked immunoassay. Differentiated IPCs (stage 3), but not undifferentiated ATDMSCs (stage 1), secreted insulin in a glucose-dependent manner, and insulin release in the high-glucose medium was higher than that in the low-glucose medium. The cells treated with Shh secreted significant higher amounts of insulin in glucose concentration–dependent manner (Figure 7).

Discussion

Previous studies have shown that manipulation of Shh signaling can affect the *in vivo* or *in vitro* insulin secretion potential of IPCs [15,16]. In the present study, we developed a novel differentiation protocol for production of IPCs from ATDMSCs based on sequential inactivation and reactivation of Shh signaling.

In our study, proliferative ATDMSCs with a spindle-like phenotype had adipogenic and osteogenic differentiation capacities. Characterization of initial ATDMSCs with flow cytometry detected positive expression of CD90 and CD105 and negative expression of CD34 and CD45. Expression of *Oct4* and *Nanog* genes by initial ATDMSCs confirmed the undifferentiated status and multipotent ability of

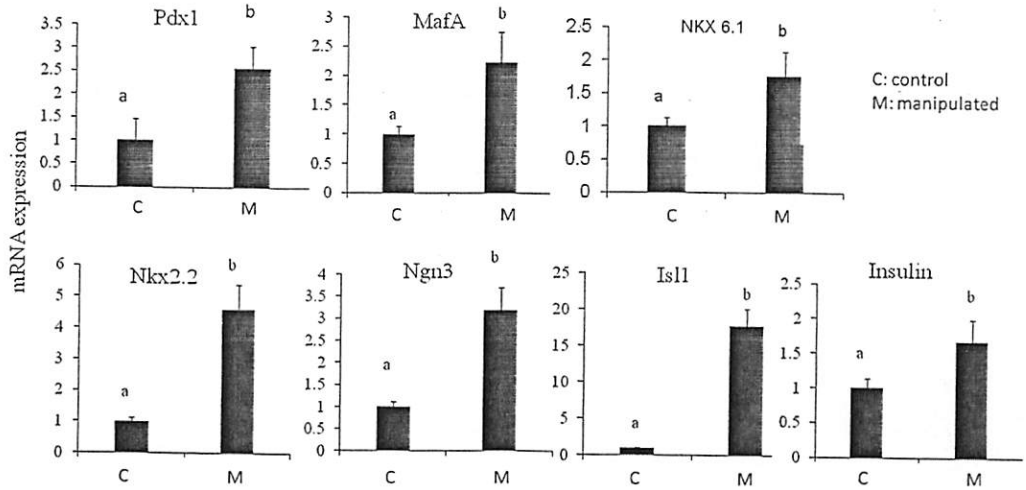

Figure 6. Comparison of relative mRNA expression of pancreas-related genes (*Pdx1*, *MafA*, *Ngn3*, *Isl*, *Nkx2.2* and *Nkx6.1*) between control and manipulated IPCs. Manipulation consisted of Shh suppression with a combination of bFGF and cyclopamine at day 3 of differentiation and subsequent Shh reactivation by treatment of cells with recombinant Shh at day 11 of differentiation. Data are means ± SD. Bars with different letters differ significantly ($P < 0.05$).

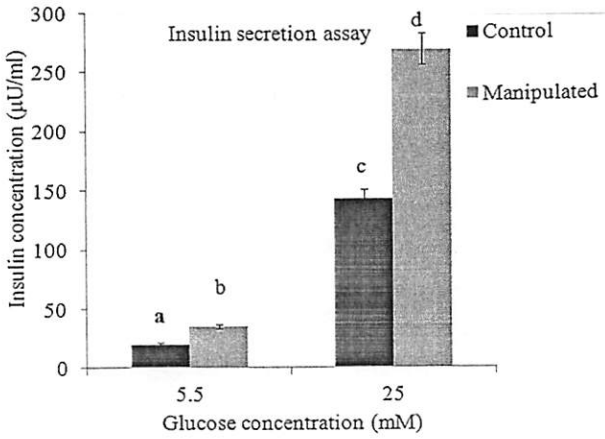

Figure 7. Comparison of insulin secretion capacity between control and manipulated **IPCs**. Manipulation consisted of Shh suppression with a combination of bFGF and cyclopamine at day 3 of differentiation and subsequently Shh reactivation by treatment of cells with recombinant Shh at day 11 of differentiation. Glucose-stimulated insulin secretion assay was performed by exposure of IPCs with glucose at concentrations of 5 mmol/L and 25 mmol/L. Data are mean ± SD. Bars with different letters differ significantly ($P < 0.05$).

ATDMSCs. These findings indicate that the initial ATDMSCs were multipotent MSCs.

In the present study, IPCs were successfully obtained from ATDMSCs using a three-step protocol. First, rat ATDMSCs were isolated and used for differentiation to IPCs at passage 3. In the second step, cells were incubated with ITS in low-glucose DMEM for 7 days. In the final step, in order to induce the differentiation, the cells were treated with nicotinamide and ITS in high-glucose DMEM for 7 days.

There is currently little known about the differentiation of ATDMSCs into insulin-secreting cells, particularly with regard to the optimal differentiation protocol. On the basis of previous studies, DMEM/F12 is a suitable medium to lead cells toward pancreas formation [17]. FBS elimination diminishes the chance of cell differentiation toward cells other than pancreatic progenitor cells, but FBS-free medium reduces cell growth. ITS administration can compensate for the lack of FBS [17]. ITS also induced MSC insulin-like growth factor-I expression, which facilitated IPC maturation and insulin production [18]. Previous studies have also shown that nicotinamide can induce the differentiation and maturation of human fetal pancreatic islet cells [19]. Also, duct tissues treated with nicotinamide can be made to differentiate into glucose-responsive islet tissue *in vitro* [20]. Thus, in our differentiation procedure, ITS and nicotinamide were added to the media to promote differentiation and maturation of precursor cells into IPCs.

In agreement with definitive endoderm formation, our investigation exhibited maximal expression of *FoxA2* and *Sox17* genes at day 7 of differentiation, confirming definitive endoderm creation as described previously [21,22]. Our RT-PCR analyses showed that IPCs expressed transcriptions factors related to pancreatic endocrine development and function including *Pdx-1*, *Nkx 2.2*, *Nkx 6.1*, *Isl-1*, *MafA* and *Ngn3*. Furthermore, cluster formation and insulin secretion by IPCs, similar to the pancreatic islet phenotype, were recognized at day 14 of differentiation. These results indicate that ATDMSCs can be induced to differentiate into functional IPCs, suggesting that they have secretory characteristics similar to those of pancreatic β cells. Taken together, we found that the combination of ITS and nicotinamide effectively promoted the differentiation of ATDMSCs into IPCs with insulin secretion potency in the presence of glucose.

Increasing evidence indicates that the differentiation state of IPCs is not permanent, and they lose their identity and functionality in response to a variety of signals, including changes in the transcriptional profile normally present in mature β cells [23,24]. Manipulation of the pancreatic signaling pathway is a practical approach to increase the efficiency of differentiation of MSCs to IPCs [25]. However, additional studies are needed to evaluate the signaling pathway manipulation on pancreatic differentiation potency of ATDMSCs and to create a precise protocol for this aim as a potential tool for cell therapy of diabetes.

Results of the previous studies demonstrate that anti-Shh antibody, Hip, cyclopamine and b-FGF have Shh pathway inhibitory effects [9,26]. In the present study, we applied both b-FGF and cyclopamine to cooperatively inhibit Shh pathway at day 3 of differentiation. Real-time PCR analysis revealed obviously decreased Shh expression at day 7 compared with day 3, confirming the inhibition of Shh transcription as described previously [27]. According to our results, the tendency of ATDMSCs to differentiate into IPCs was accelerated when Shh signaling was sequentially inhibited and reactivated during the differentiation procedure. Treatment of ATDMSCs with cyclopamine + bFGF at day 3 and recombinant Shh at day 11 of differentiation resulted in higher insulin secretion to the medium compared with the unmanipulated cells. Increased insulin secretion, in parallel with the real-time PCR results that demonstrated higher expression of pancreatic formation genes such as *Pdx-1*, *Nkx 2.2*, *Nkx 6.1*, *Isl-1*, *MafA* and *Ngn3*, supported the fact that the manipulation of Shh signaling during the differentiation of ATDMSCs provides the higher differentiation efficiency. Although manipulation of Hedgehog signaling has profound effects on the developing endocrine cells within the pancreas, sequential inactivation and reactivation of Shh signaling during the differentiation of MSCs has not been analyzed. In accordance with our results, Ghanbari *et al.*

[10] have shown that inhibition of Shh during the differentiation of mouse embryonic stem cells upregulates the expression of some developmental and functional pancreas genes such as *Pdx1*, but the effect of this manipulation on the insulin secretion potential of differentiated cells has not been studied. Ectopic expression of Shh in cultured β-cell lines also has beneficial effects on insulin production and cell survival. These effects are mediated in part through the elevated expression of *Pdx1*, a transcription factor essential to maintaining the mature β-cell state [28]. Similarly, Thomas *et al.* [9] showed that cyclopamine diminishes insulin promoter activity and insulin secretion in INS-1 cell line, and over expression of Shh using Shh plasmid construct results to detectable increase of insulin production [9]. In our research, IPCs with Shh reactivation showed greater insulin secretion and higher expression of the transcription factors essential for maintaining mature β-cell function and normal response to glucose. Therefore, we conclude that the increased glucose-stimulated insulin secretion observed in Shh-pathway-manipulated cells likely resulted from higher expression of multiple transcription factors required for maintenance of the β-cell phenotype.

The data presented here are somewhat in conflict with previous work showing decreased insulin production by β-cell lines in response to elevated Shh signaling *in vitro*. Landsman *et al.* [8] demonstrated that transgenic β cells with increased Shh signaling can transiently de-differentiate toward a more progenitor-like state characterized by decreased *Pdx1*, *Glut2*, *MafA*, *NeuroD*, *Ngn3* and insulin expression levels. Importantly, similar changes in gene expression have been noted in human patients and in mice with impaired glucose-stimulated insulin secretion [8].

Several explanations could account for these divergent findings. Regulation of Shh signaling is achieved at multiple layers of the signaling pathway, and diverse approaches for Shh signaling manipulation may result in different outcomes. For example, Thomas *et al.* [9] ectopically expressed the Shh in cultured cells, and Landsman *et al.* [8] overexpressed GLI2, a transcriptional mediator of Shh signaling, whereas we treated ATDMSCs directly with Shh protein. On the basis of previous studies, a specific range of Shh signaling is required for optimal β-cell function [29]. Thus, the positive results obtained from our study with regard to increased insulin production in response to treatment of ATDMSCs with Shh may be due to elevation of Shh levels within this beneficial range. Furthermore, in previous works, Shh signaling activation has been performed on mature β cells, whereas Shh reactivation in our study was performed at day 11 of ATDMSC differentiation, when they did not display the fully differentiated state found in adult β cells *in vivo*.

In conclusion, in our study, ATDMSCs were successfully differentiated into IPCs with glucose-stimulated insulin secretion ability using a combination of ITS and nicotinamide. Our findings also indicate that early inactivation and late reactivation of the Shh signaling pathway during differentiation of ATDMSCs can beneficially affect the expression of functional and developmental pancreas-related genes and insulin secretion in differentiated cells. Because maintaining the Shh activity at a certain level in β cells is important, as demonstrated by our findings, and a high level of Shh activity in mature β cells causes impaired function, further *in vivo* study is required to determine whether Shh-manipulated IPCs are applicable for the treatment of T1D in diabetic animal model.

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