

Research article

miR-375 INDUCES HUMAN DECIDUA BASALIS-DERIVED STROMAL CELLS TO BECOME INSULIN-PRODUCING CELLS

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Abstract: This paper focuses on the development of renewable sources of islet-replacement tissue for the treatment of type I diabetes mellitus. Placental tissue-derived mesenchymal stem cells (MSCs) are a promising source for regenerative

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Abbreviations used: α -MEM – alpha minimal essential medium, BMSCs – bone marrow stem cells, BSA – bovine serum albumin, CD34 – cluster of differentiation 34, CD44 – cluster of differentiation 44, CD90 – cluster of differentiation 90, CD105 – cluster of differentiation 105, CD133 – cluster of differentiation 133, cDNA – complementary DNA, Ct – cycle threshold, DMEM – Dulbecco's modified Eagle's medium, DMEM F12 – Dulbecco's modified Eagle's medium: nutrient mixture F-12, DTZ – diphenylthiocarbazone, ESC – embryonic stem cell, FACS – fluorescence-activated cell sorting, FAM – 6-carboxyfluorescein, GATA6 – transcription factor GATA-6, (GLP)-1 – glucagon-like peptide-1, GLUT2 – glucose transporter 2, GVHD – graft-versus-host disease, HBV – hepatitis B virus, HCV – hepatitis C virus, HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HIV – human immunodeficiency virus, Hnf1 β – hepatocyte nuclear factor1 β , HNF6 – hepatocyte nuclear factor 6, hPDMSCs – human placenta-derived mesenchymal stem cells, ICC – immunocytochemistry, IPCs – insulin-producing clusters, ITS – insulin–transferrin–selenium, KIR6. 2 – potassium inwardly-rectifying channel, KRH buffer – Krebs' ringer bicarbonate HEPES buffer, miR – micro ribonucleic acid, miRNAs – micro ribonucleic acid, MSCs – mesenchymal stem cells, NEUROD1 – neuronal differentiation 1, NGN3 – neurogenin 3, Nkx6. 1 – NK homeobox factor 6. 1, OCT4 – octamer binding transcription factor 4 (transcription factor expressed by embryonic stem cells), Opti-MEM – Opti-Minimum Essential Media, PAX4 – paired homeobox transcription factor 4, PAX6 – paired homeobox transcription factor 6, PBS – phosphate buffered saline, PDB-MSCs – human placental decidua basalismesenchymal stem cells, PDX-1 – pancreatic and duodenal homeobox 1, Sox17 – SRY-related HMG box 17, SVF – stromal-vascular cell fraction

medicine due to their plasticity and easy availability. They have the potential to differentiate into insulin-producing cells. miR-375 is a micro RNA that is expressed in the pancreas and involved in islet development. Human placental decidua basalis MSCs (PDB-MSCs) were cultured from full-term human placenta. The immunophenotype of the isolated cells was checked for CD90, CD105, CD44, CD133 and CD34 markers. The MSCs (P3) were chemically transfected with hsa-miR-375. Total RNA was extracted 4 and 6 days after transfection. The expressions of insulin, NGN3, GLUT2, PAX4, PAX6, KIR6.2, NKX6.1, PDX1, and glucagon genes were evaluated using real-time qPCR. On day 6, we tested the potency of the clusters in response to the high glucose challenge and assessed the presence of insulin and NGN3 proteins via immunocytochemistry. Flow cytometry analysis confirmed that more than 90% of the cells were positive for CD90, CD105 and CD44 and negative for CD133 and CD34. Morphological changes were followed from day 2. Cell clusters formed during day 6. Insulin-producing clusters showed a deep red color with DTZ. The expression of pancreatic-specific transcription factors increased remarkably during the four days after transfection and significantly increased on day 7. The clusters were positive for insulin and NGN3 proteins, and C-peptide and insulin secretion increased in response to changes in the glucose concentration (2.8 mM and 16.7 mM). In conclusion, the MSCs could be programmed into functional insulin-producing cells by transfection of miR-375.

Keywords: Pancreas, Beta cells, miR-375, Placenta, Mesenchymal stromal cells, Induced pluripotent stem cell, microRNA, Insulin, Differentiation, NGN3, GLUT2, PDX1

INTRODUCTION

The pancreatic islets have around a 70% beta cell content [1]. These cells secrete insulin, a hormone of great importance in regulating carbohydrate, fat and protein metabolism. Death and/or dysfunction of beta cells leads to an inadequate amount of insulin, and this in turn gives rise to diabetes mellitus [2]. One of the most common chronic diseases in children and adolescents is type 1 diabetes mellitus, which is an autoimmune destruction of insulin production. Sufferers become dependent on insulin injection, but an exogenous supply of insulin results in severe hypoglycemia-related complications [3, 4].

Whole pancreas transplantation restores endogenous insulin production, but perioperative morbidity due to the release of digestive enzymes from the exocrine pancreas during surgery and long-term immunosuppressive drug consumption are major issues [5–8].

Transplantation of functional pancreatic islet cells is a subject of considerable interest as an alternative to whole pancreas transplantation. In this method, an infusion of fresh donor islets is followed by long-term non-steroidal immunosuppressive drug consumption. The obstacles are the worldwide shortage of insulin-producing islets, immune rejection of islets, and the

increased risk of malignancies and opportunistic infections [9, 10]. It is essential to find renewable islet replacement tissue sources that do not elicit ethical concerns that impede their clinical application.

Postnatal-derived mesenchymal stem cells (MSCs), such as those from adipose tissue, present fewer ethical concerns and are considered possible sources of transplantable insulin-producing cells [5, 6, 9, 11, 12]. Due to its plasticity and easy availability, placental tissue is very promising in regenerative medicine. Human placenta-derived mesenchymal stem cells (hPDMSCs) have broad developmental potential, and could be induced to differentiate into insulin-producing cells.

In recent years, one of the most fascinating discoveries in biology was the identification of the family of small, non-coding RNAs known as microRNAs (miRNAs). Initially described in worms [13], microRNAs (miRNAs) regulate the expression of genes through post-transcriptional mechanisms [14]. Recent evidence has revealed that miRNAs play a key role in the control of biological activities such as embryonic development, cell proliferation, metabolic homeostasis and apoptosis [15]. MicroRNAs [16, 17] play a direct role in pancreatic islet development [18], beta cell differentiation [19], insulin secretion [20], and the control of glucose homeostasis [21, 22]. miRNAs are also involved in secondary complications of diabetes [23, 24].

Kloosterman et al. reported that miR-375 plays an important role in pancreatic islet development in zebra fish [18]. A reduction in miR-375 expression was found to cause defects in pancreatic islet morphology. In situ hybridization studies revealed that miR-375 is expressed in endodermal endocrine progenitor cells [25]. The expression of miR-375 is regulated by transcription factors that are important in the development and function of the pancreas, such as hepatocyte nuclear factor 6 (HNF6), neurogenin 3 (Ngn3), neuronal differentiation 1 (NEUROD1), and pancreatic and duodenal homeobox 1 (PDX-1) [26].

Overexpression of miR-375 inhibits insulin secretion. Therefore, miR-375 regulates glucose-stimulated insulin secretion in a negative manner. In the same way, insulin secretion is enhanced by inhibition of endogenous miR-375 using antagomiRs [20]. These data suggest that miR-375 has a dual function of mediating pancreatic islet development and insulin secretion. The objective of this study was to transfect hPDMSCs with miR-375 in order to obtain effective insulin-producing clusters (IPCs) in a short period of time.

MATERIALS AND METHODS

Generation of MSCs from human placental decidua basalis (PDB-MSCs)

Full term (gestation ≥ 37 weeks) normal human placentas ($n = 2$) from cesarean section deliveries were obtained from the obstetrics department of Hafez Hospital, which is affiliated with Shiraz University of Medical Sciences. Informed consent was obtained from the mothers, and the ethical committee of

Shiraz University of Medical Sciences approved the study. Infectious pathology was excluded using HIV, HCV and HBV tests. The samples were transported in cold PBS.

Initially, a small fragment of the PDB-MSCs was separated, mechanically fragmented, and the resulting pieces were washed with PBS supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Then, the tissue was minced into small fragments (1–2 mm³). The tissue pieces were transferred to 10-cm² plates containing DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO₂. The explants were left undisturbed for 10 days to allow the migration of cells from the margins of the explants.

Upon reaching 70 to 80% confluence, adherent PDB-MSCs were harvested with 0.05% trypsin-EDTA (Gibco), and single-cell suspensions were used for subsequent experiments. Real-time qPCR was used to study the presence of transcription factors that regulate the maintenance of the pluripotent state in ESC (OCT4, Nanog).

Immunophenotyping of PDB-MSCs

PDB-MSCs were stained with a panel of monoclonal antibodies: CD90, CD44, CD105, CD34 and CD133 (BioLegend). Stained cells were re-suspended in PBS, and analyzed using a FACS Calibur flow cytometer (Becton Dickinson).

Optimization of chemical transfection

The PDB-MSCs were transfected with FAM-Labeled Pre-miR (Ambion) using siPORT *NeoFX* Transfection Agent (Invitrogen) as recommended in the manufacturer's protocol (Tables 1 and 2). Various cell numbers and FAM concentrations were used. The cells were cultured in DMEM F12, 10% FBS for 24 h and incubated at 37°C in a humidified atmosphere containing 5% CO₂, and then 24 and 48 h after transfection, the percentage of fluorescently labeled cells were calculated by flow cytometry (Becton Dickinson).

In another experiment, to improve the protocol outcome, cells from the previous experiment were cultured for 72 h and then transfected again. The percentage of fluorescently labeled cells was calculated again by flow cytometry after 24 h. In the final experiment, the percentage of FAM-labeled cells was measured after 96 h and then compared with the results of previous experiments.

Table 1. General transfection starting points for FAM-Labeled Pre-miR.

Plate format	24 wells
Transfection Agent (FAM-Labeled Pre-miR)	0.5–2.5 µl
FAM-Labeled Pre-miR concentration	10–100 pmol
Cell density	40,000 cells/well
siPORT <i>NeoFX</i> Transfection Agent	0.5–2.5 µl
Final volume per well	0.5 ml

Table 2. First stage: Dilute siPORT *NeoFX* Transfection Agent in Opti-MEM I medium and incubate for 10 min at room temperature. Second stage: Dilute RNA in Opti-MEM I medium. Mix diluted RNA and diluted siPORT *NeoFX* Transfection Agent. Incubate for 10 min at room temperature, and dispense into a culture plate.

First stage: 10 min	siPORT <i>NeoFX</i> Transfection Agent	1 μ l
	Opti-MEM medium	25 μ l
Second stage: 10 min	Opti-MEM medium	25 μ l
	miRNA	1.5 μ l

hsa-miR-375 transfection

hsa-miR-375 (accession number MIMAT0000728 in the miRBase database) was purchased from Ambion. The mature miR-375 sequence is UUUGUUCGUUCGGCUCGCGUGA.

The PDB-MSCs were transfected with hsa-miR-375 (final concentration, 80 pmol) using siPORT *NeoFX* Transfection Agent (Invitrogen; Table 3). Then the cells were cultured in 24-well plates containing DMEM/F12 supplemented with 10% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 μ g/ml and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 72 h, the cells were transfected again and cultured.

FAM-Labeled Pre-miR Negative Control (Ambion) is a non-targeting negative control. It was used at a final concentration of 80 pmol. In this study it was used as a negative control in order to monitor transfection efficiency during the optimization of transfection conditions.

Table 3. Transfection starting points for hsa-miR-375.

Plate format	24 wells
Transfection Agent (hsa-miR-375)	1.5 μ l
hsa-miR-375 concentration	80 pmol
Cell density	40,000 cells/well
siPORT <i>NeoFX</i> Transfection Agent	1 μ l
Final volume per well	0.5 ml

Real-time qPCR for hsa-miR-375 quantification

Total RNA was extracted from PDB-MSCs before and 24 h after transfection with hsa-miR-375 using Trizol (Gibco). The cDNA was synthesized with a Micro RNA cDNA Synthesis Kit (PARS GENOME). Amplification ran on an ABI Prism 7500 Step One Plus (Applied Biosystems). The PCR amplifications were carried out using SYBR Green (PARS Genome) following the manufacturer's instructions. The reaction conditions included pre-heating at 95°C for 5 min; followed by 40 cycles of denaturation at 95°C for 5 sec; annealing at 55°C for 20 sec; and extension at 72°C for 30 sec. A final stage was run to generate a melting curve for verification of amplification product

specificity. The U6 gene served as a control and the relative expression levels of miR-375 were calculated according to the formula: 2^{-DDCt} .

Real-time qPCR or quantification of pancreas-specific transcription factors

Total RNA was extracted from transfected PDB-MSCs (4 and 7 days after transfection) using Mini-RNease RNA extraction kit (Cinnagen). The amount of extracted RNA was measured by OD_{260/280} measurement. The cDNA was synthesized using MMULV reverse transcriptase (Cinnagen) from 1 µg of total RNA. The synthesized cDNA was used to examine changes in the levels of pancreas-specific transcription factors from differentiated cells. PCR amplifications were carried out using SYBR Green (PARS Genome) following the manufacturer's instructions.

The cDNA synthesized for pancreas-specific transcription factors were normalized to beta-actin. Amplification was as follows: denaturing at 95°C for 5 min; followed by 40 cycles of denaturation at 95°C for 1 min; and annealing for 1 min. Any amplification of more than 35 cycles (Ct) was considered negative.

The final stage was run to generate a melting curve for verification of the amplification product specificity. The relative expression levels of the genes were calculated according to the formula: 2^{-DDCt} .

After cDNA synthesis, serial dilution (1/10) was prepared and real-time qPCR was done for both the internal control (beta-actin) and the gene of interest (insulin). The efficiency was about 95%, with a slope of -3.43. The primers, conditions and product sizes are summarized in Table 4.

Table 4. Real-time qPCR primers, sequences and product sizes.

Name	Forward (F) 5'-3'	Reverse (R) 5'-3'	Tm (°C)	Cycle number	Size (bp)
Insulin	GCAGCCTTTGTGAACCAACA	TTCCCCGCACACTAGGTAGAGA	64	40	69
PDX1	GGATGAAGTCTACCAAAGCTCACGCCAGATCTTGATGTGTCTCTCGGTC		63	40	230
NGN3	CAATCGAATGCACAACCTCA	GGGAGACTGGGGAGTAGAGG	64	40	254
Glucagon	AGGCAGACCCACTCAGTGA	AACAATGGCGACCTTCTCTG	60.1	40	308
PAX4	GTGGGCAGTATCCTGATTCAGT	TGTCACCTCAGACACCTTCTCTGG	64	40	308
NKX6.1	GTTCTCTCCTCCTCTCTCCTC	AAGATCTGCTGTCCGGAAAAAG	66	40	381
KIR6.2	CGCTGGTGGACCTCAAGTGGC	CCTCGGGGCTGGTGGTCTTGCG	71	40	497
GLUT2	AGGACTTCTGTGGACCTTATGTG	GTTCATGTCAAAAAGCAGGG	64	40	231
OCT4	CAGTGCCCGAAACCCACAC	GGAGACCCAGCAGCCTCAAA	64	40	161
β-actin	GATCGGCGGCTCCATCCTG	GACTCGTCATACTCCTGCTTGC	64	40	74

Immunocytochemistry (ICC)

Six days after transfection, the cells were collected, washed with PBS, fixed with acetone for 5 min, and incubated with anti-neurogenin 3 antibody (3B5; Abcam; dilution 1:1000) and anti-insulin (DAKO) overnight. After washing in PBS, the

Envision Detection System (DAKO) was used. The slides were washed with PBS for 10 min, visualized with diaminobenzidine (DAB; DAKO) and counterstained with hematoxylin (Sigma-Aldrich). The morphology of the cells was observed under a light microscope. FAM-Labeled Pre-miR Negative Control with no morphological change was used in parallel, in which no staining was observed in ICC.

DTZ staining

Diphenylthiocarbazone (Dithizone, DTZ; Sigma) is a zinc-chelating chemical substance that is used for the identification of IPCs. The dye selectively stains the live pancreatic beta-cells as bright crimson red.

C-peptide and insulin secretion

The adherent clusters produced through this procedure were rinsed twice in Krebs-Ringer HEPES (KRH) buffer containing 125 mM NaCl, 4.74 mM KCL, 1 mM CaCl_2 , 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 5 mM NaHCO_3 , 25 mM HEPES, 1 mM, 0.1% BSA (pH 7.4) and 2.8 mM glucose (Sigma-Aldrich) for the low glucose concentration experiments. After 1 h incubation, the medium was collected and the cells were stimulated with KRH buffer containing 16.7 mM glucose for 1 h (high glucose concentration). The insulin and C-peptide levels were determined using a radioimmunoassay kit (Immunotech) and the results were compared with those for the control group (untransfected MSC cells).

Statistical analysis

The data were presented as means \pm SD. Each experiment was repeated 3 times. Data from related assays were assessed by one-way ANOVA followed by Tukey's test for pair wise comparison. $p < 0.05$ was considered statistically significant. The statistical analyses and design of the graph were performed using Graph Pad Prism 5 software.

RESULTS

Morphological changes before and after transfection with miR-375 in PDB-MSCs

PDB-MSCs formed a homogenous monolayer of adherent and spindle-shaped fibroblast-like cells. In passage 3 (P3), the cells were transfected with has-miR375. After three days, the islet-like cluster was partially formed and MSCs and IPCs appeared on day 6 (Fig. 1A–D). The induced MSCs shrank after three days and became round in shape. After 6 days, cell clusters were formed. On day 7, the clusters gradually detached from the bottom of the plate and floated in the medium. The PDB-MSCs that were transfected with FAM-Labeled Pre-miR (negative control) did not show any morphological change after 7 days.

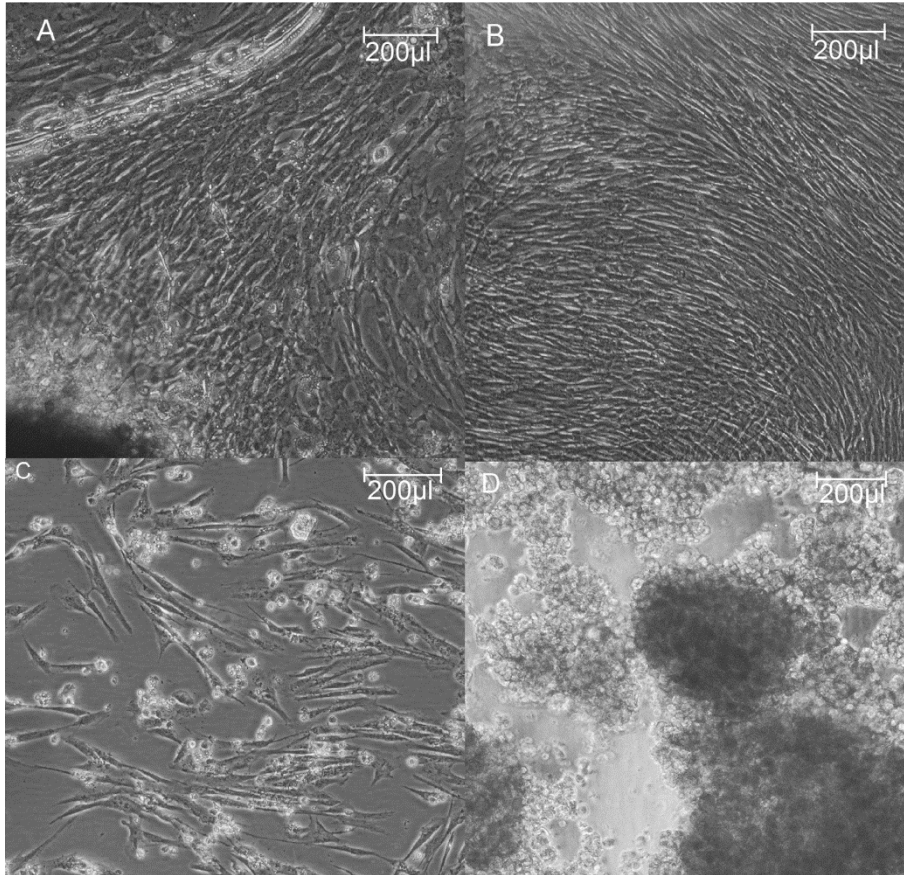


Fig. 1. Morphological changes before and after transfection with miR-375 ($\times 200$). A – Fibroblast-like cells migrated from human placental tissue. B – Homogenous population of small spindle-shape cells in passage 3 before transfection. C – After three days, an islet-like cluster had partially formed. D – IPCs (dark stain) appeared on day 6.

Flow cytometry analyses of PDB-MSCs

Flow cytometry analyses showed that the PDB-MSCs were a homogeneous cell population in which more than 90% of the cells were positive for mesenchymal markers (CD90, CD44, CD105), and negative for CD34 and CD133 (Fig. 2), which is in line with the internationally recognized criteria for MSCs [27, 28]. Real-time qPCR showed that PDB-MSCs express the embryonic stem cell (ESC) transcriptional factors Nanog and OCT4.

Optimization of chemical transfection

PDB-MSCs were transfected with FAM-Labeled Pre-miR. The highest percentage of FAM-labeled cells (49.16%) occurred when 4×10^4 cells were transfected two times for three consecutive days with 80 pmol of FAM-Labeled Pre-miR (final concentration; Fig. 3). After 96 h, no morphological change was observed in the MSCs.

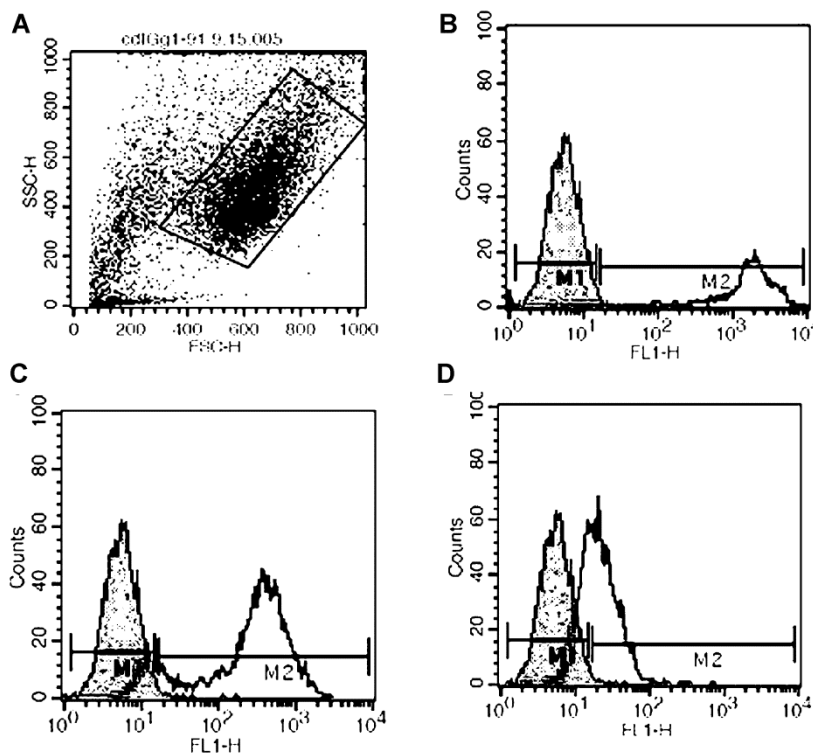


Fig. 2. Immunophenotyping of PDB-MSCs with a panel of monoclonal antibodies (CD44, CD105, and CD90) by flow cytometry. A – PDB-MSCs gated with FSC-SSC. B – The cells were stained with FITC-label CD44 antibody and about 98.63 % of cells were positive. C – The cells were stained with FITC-label CD90 antibody and 90.09 % of cells were CD90 positive. D – The cells were stained with FITC-label CD105 antibody and about 57.69% of cells were positive. Each experiment was performed 3 times. Data showed these cells were a pluripotent homogenous population.

Expression of has-miR-375 in PDB-MSCs

Real-time qPCR was used to determine the expression of has-miR-375. The level of miR-375 increased 3981.23-fold in PDB-MSCs 24 h after chemical transfection.

The effect of has-miR-375 on PDB-MSCs-derived IPCs

Pancreatic islets have high levels of zinc compared with other tissues, so DTZ stains them crimson red [29]. On day 7, the clusters appeared red, suggesting the presence of IPCs (Fig. 4). We examined the expression of pancreatic-specific transcription factors in IPCs using real-time qPCR on days 4 and 7 after chemical transfection (Fig. 5). The character of the differentiated cells was similar to that of pancreatic islet cell development *in vivo*, but all of the changes happened in a short period.

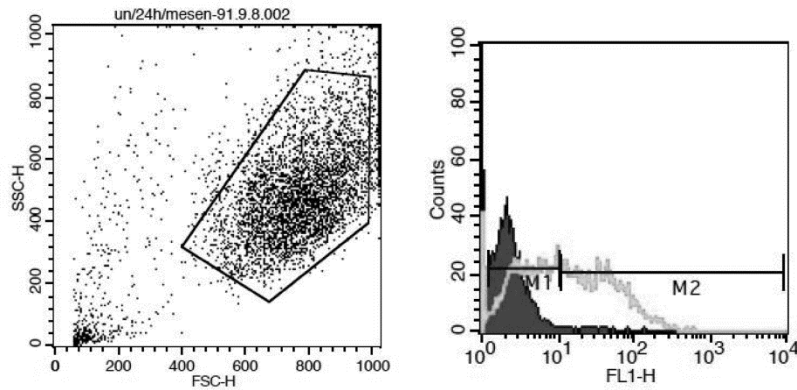


Fig. 3. Optimization of chemical transfection of PDB-MSCs with FAM by flow cytometry. Adherent PDB-MSCs were transfected with FAM-Labeled Pre-miR using siPORT *NeoFX* Transfection Agent. A – Transfected PDB-MSCs gated with FAM. B – Cells were stained with FAM and about 49.16% of the cells were positive.

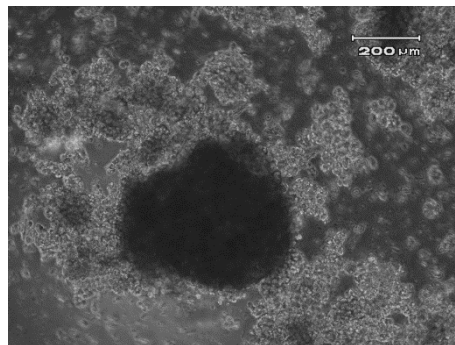


Fig. 4. The differentiated clusters stained with DTZ ($\times 200$).

The expressions of PDX1 and NKX6.1 (specific markers of pancreatic progenitors) increased on day 4 and peaked on day 7, when the expression level of OCT4 had rapidly reduced (Diagram 1). At the same time, the expression levels of NKX6.1, KIR6.2 and PAX4 had increased.

The upregulated gene expression of NGN3, PDX1 and GLUT2 was in favor of pancreatic specialization. The expression of insulin increased on day 7, indicating that the progenitors had differentiated towards insulin-producing cells. This protocol also induced the expression of glucagon. The clusters were positive for neurogenin 3 (nuclear and cytoplasmic; Fig. 6A) and insulin (cytoplasmic) protein (Fig. 6B).

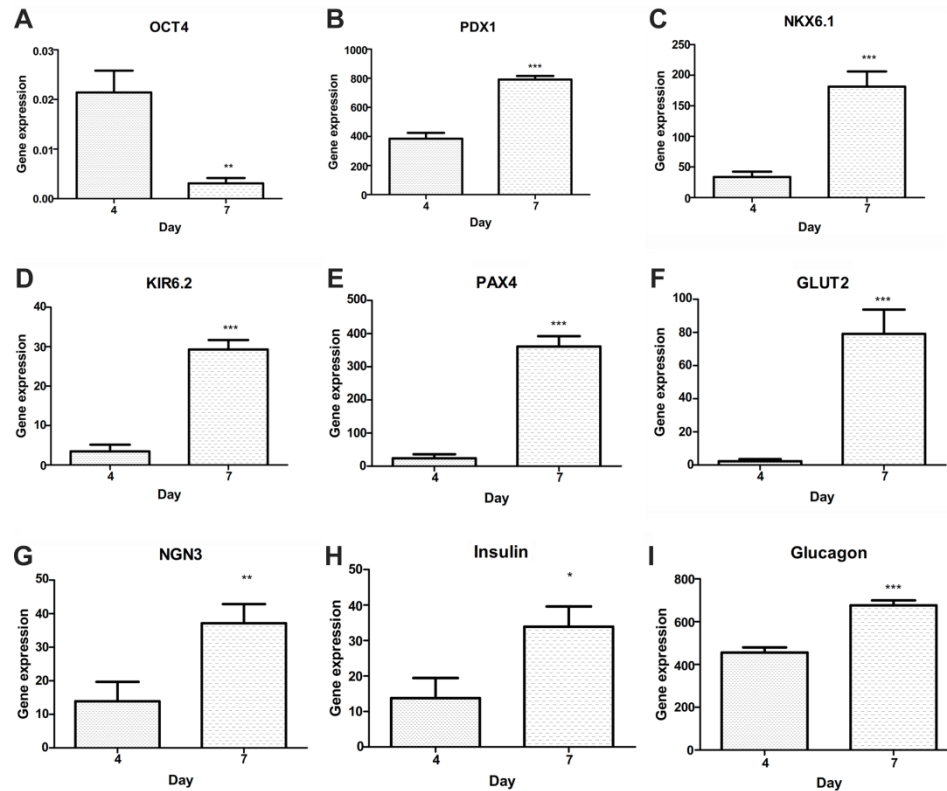


Fig. 5. Real-time qPCR data. A – The expression of the OCT4 gene decreased significantly on day 7. B, C, D, E, G, I – The expressions of PDX1, NKX6.1, KIR6.2, PAX4, GLUT2 and glucagon genes increased significantly on day 7. F, H – The expression NGN3 and insulin increased on day 7. These data showed that on day 7, clusters had formed and the cells had ability to express pancreatic-specific transcription factors. The data are shown as means \pm SD from three independent experiments. Analysis was performed by one-way ANOVA followed by Tukey's test.

The effects of miR-375 on insulin and C-peptide secretion

The clusters were treated with 2.8 mM glucose for 1 h, and subsequently with 16.7 mM glucose for 1 h. A significant increase in insulin secretion was recorded: from 1.48 ± 0.34 to 26.34 ± 0.45 μ Iu/ml (2.8 mM glucose) and 39.57 ± 0.51 μ Iu/ml (16.7 mM glucose; Fig. 7A). A significant increase in C-peptide secretion was also recorded: from 0.01 ± 0.03 to 0.3 ± 0.04 ng/ml (2.8 mM glucose) to 0.47 ± 0.05 ng/ml (16.7 mM glucose; Fig. 7B).

Target genes of miR-375 in the beta-cell differentiation

The target genes of miR-7 were investigated using Mir walk, miRanda (miRBase) and TargetScanS. MiR-375 has ten target genes, and some of them are critical for pancreas development, for example Hnf1 β , Sox17, GATA6 and Pax6 [30].

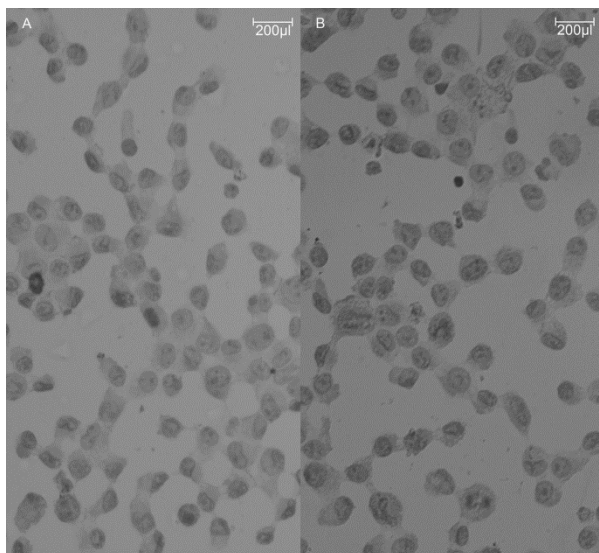


Fig. 6. Immunocytochemistry for Ngn3 and insulin. A – About 30% of the cells show cytoplasmic and nuclear staining for Ngn3. Immunocytochemistry $\times 200$. B – About 50% of cells show cytoplasmic insulin staining. Immunocytochemistry $\times 200$.

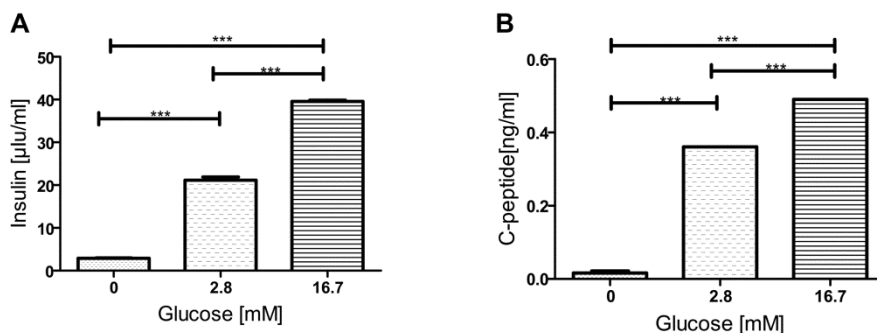


Fig. 7. Insulin and C-peptide secretion in response to different glucose concentrations. A – Insulin secretion in response to 2.8 and 16.7 mM glucose. On day 6, the clusters were incubated with 2.8 and 16.7 mM glucose for 60 min. The response to insulin secretion was evaluated and the results were compared with those for the negative control group (FAM-Labeled Pre-miR transfected cells). The bars represent means \pm SD for three independent experiments ($n = 3$). *** $P < 0.0001$ – significantly different from the negative control group and from each other. Analysis was performed by one-way ANOVA followed by Tukey's test. B – C-peptide release in response to 2.8 and 16.7 mM glucose. On day 6, the glucose-stimulated C-peptide release of the ILCs was measured after 60 min incubation in a different glucose concentration. The result was compared with the negative control group (FAM-Labeled Pre-miR transfected cells). The bars represent the means \pm SD for three independent experiments ($n = 3$). *** $P < 0.0001$ – significantly different from the negative control group and also from each other. Analysis was performed by one-way ANOVA followed by Tukey's test.

DISCUSSION

During the progression of diabetes, pancreatic beta cells are either destroyed or become dysfunctional [2]. Stem cell therapy is promising for the treatment of diabetic patients and PDB-MSCs are good candidates for cell therapy. These cells are abundant and are an available source with low immunological reactions. According to immune regulatory properties, the cells are also used for overcoming graft-versus-host disease (GVHD) in allogeneic transplantation [31, 32].

In the literature, there are many approaches that have been used to induce MSC differentiation into IPCs: various growth factors [33]; chemical substances [34, 35] and transfection with viral vectors [36]. Kadam et al. [34] studied the differentiation of human placenta-derived mesenchymal stem cells (hPDMSCs) into insulin-producing cells. Their method had 3 stages. The medium culture in stage 1 contained α -MEM, BSA Cohn fraction V, fatty acid-free insulin–transferrin–selenium (ITS). In stage 2, it contained α -MEM, BSA, ITS and taurine. In the final stage, it contained α -MEM, BSA, ITS, taurine, glucagonlike peptide (GLP)-1 and nicotinamide. Talebi et al. [36] studied the differentiation of MSCs from the bone marrow of Sprague–Dawley rats toward islet-like cells using PDX1-transducing lentiviruses. The cells were transplanted to diabetic rats and a reduction in blood glucose level from high to normal was revealed. However, many problems remain in the differentiation pathway. The differentiation efficiency was low and the differentiated cells were not functional for a long time [36]. Therefore, the main goal of our study was to produce functional IPCs in a short time using a new approach.

miRNAs are thought to have important roles in stem cell differentiation [37, 38]. One of the first miRNAs to be detected in the pancreas was miR-375 [18]. The expression level of miR-375 increases during pancreas organogenesis [18, 39–41]. Joglekar et al. revealed that the miRNA levels of miR-7, miR-9, miR-375 and miR-376 significantly increased during human pancreatic islet development [42].

Wei et al. induced hES cells into insulin-producing cells and the dynamic expression of microRNAs during the differentiation was evaluated. They found that miR-375 expression increased from day 4, the maximum expression was detected on day 8, and this then decreased until the end of the differentiation [30]. In the Wei et al. study, the maximum expression of miR-375 occurred at the same time that the expression levels of pancreatic hormone encoding genes began to rise [43].

To explore the direct effect of miR-375 on MSC differentiation into IPCs, PDB-MSCs were transfected with miR-375 in our project. Comparison between the increased expression of miR-375 and changes in crucial beta-cell transcription factors, such as insulin, glucagon, PAX4, NGN3, GLUT2, NKX6.1, KIR6.2, PDX1, and GLUT2, demonstrated that miR-375 could promote MSC differentiation toward IPCs. Overall, the expression dynamics during *in vitro* differentiation of IPCs from PDB-MSCs is similar to that of pancreatic beta-cells

development in vivo, but it occurs in a shorter time [44, 45]. On day 6, IPCs have the capacity of C-peptide and insulin secretion in response to glucose stimulation. However, our study has a few limitations. The presence of the insulin hormone was confirmed in ICC. This result should be confirmed by immunostaining with human c-peptide antibody. The qPCR results suggest a high induction of glucagon expression. It is necessary to verify if the expression of glucagon co-localizes with insulin, as reported for immature beta cells (polyhormonal cells). Making use of this approach, it is possible to generate functional IPCs in a shorter time than has been seen in other studies.

Although overexpression of miR-375 induces MSC differentiation into IPCs, cell differentiation has a very complex network, and microRNAs considered as regulatory factors take part in complex cell regulation systems. Therefore, clarifying the mechanism of MSC differentiation into IPCs could improve the induction efficiency. Further investigation is required.

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