Pdxl and its role in activating Ngn3 and Pax6 to induce differentiation of iPSCs into islet β cells

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ABSTRACT. The pancreatic and duodenal homeobox factor 1 (Pdx1) protein is the most pivotal transcription factor in the development of islet β cells. This study investigated the role of Pdx1 and its mechanism in differentiating induced pluripotent stem cells (iPSCs) into islet β cells. iPSCs derived from human skin fibroblasts were cultured *in vitro* and directionally induced to differentiate for 20 days. The expression of insulin-related genes was then detected by RT-PCR, and the expression of several differentiation-related transcription factors was assessed both before and after the differentiation process. Lastly, the specific promoter regions where Pdx1 binds were detected by ChIP. The insulin-related genes, *MafA*, *insulin*, *Glut2*, *Nkx6.1*, *GCK*, and *Tcf1*, showed increased expression during differentiation, and nearly peaked on the 20th day. Similarly, the expression of transcription factors, Pdx1, Ngn3, and Pax6 showed enhanced expression during differentiation as compared with that of the control group. ChIP experiments confirmed

that Pdx1 activates the expression of the downstream transcription factors, Ngn3 and Pax6, by combined with the promoter regions of *insulin* (Insulin-P), *Ngn3* (Ngn3-P), and *Pax6* (Pax6-P). In conclusion, Pdx1 activates downstream transcription factors Ngn3 and Pax6, and may be one of the mechanisms that promote differentiation of iPSCs into islet β cells.

Key words: Induced pluripotent stem cell; islet β cells; Insulin; Pancreatic and duodenal homeobox factor 1; Neurogenin 3; Paired box gene 6

INTRODUCTION

Diabetes is a common disease that is associated with many serious complications. Pancreas transplantation is one way to treat diabetes, but a shortage in donors and immunological rejection of the transplanted tissue limit its use (Hecht et al., 2009). Islet β cells are the only cells responsible for the transport, synthesis, and release of insulin. Insulin is the hormone that acts to lower blood sugar, and regulate peripheral blood sugar levels.

The development and differentiation of islet β cells in the embryo are regulated by multiple transcription factors, and the molecular mechanisms behind this developmental process are essential for diabetes research. Given this fact, we attempted to follow the molecular changes during the differentiation of induced pluripotent stem cells (iPSCs) into pancreatic islet β cells. Specifically, RT-PCR was used to detect the expression of pancreatic and duodenal homeobox factor 1 (Pdx1), neurogenin 3 (Ngn3), paired box gene 6 (Pax6), and other important transcription factors both before and after the differentiation process. Moreover, Pdx1 binding sites on DNA promoter regions were detected by chromatin immunoprecipitation (ChIP). These experiments were aimed at determining the specific role and mechanism of Pdx1 in the differentiation of iPSCs into islet β cells.

MATERIAL AND METHODS

Culture of iPSCs derived from human skin fibroblasts

iPSCs derived from human skin fibroblasts (purchased from Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences) were cultured in the TeSR-E8 medium (STEMCELL Technologies Inc., Canada) for human embryonic stem cells (ES cells) containing Matrigel Matrix (BD Company, America) without a feeder layer, and placed in a 5% CO_2 incubator at 37°C. Cells were typically grown to 90% or more, after which they were treated with Dispase II (Gibco, Life Technologies, Thermo Fisher Scientific Inc.) to passage the cells. Cultured iPSCs were observed by inverted microscope and photographed.

iPSC differentiation

When iPSCs were grown to more than 90% or more, they were digested using 200 U/mL Collagenase IV for 4 min at 37°C. Cells were then separated into single cells, and undif-

ferentiated cells were pelleted, seeded on a Petri dish, and covered with Matrigel Matrix. Cells were grown until 60%, and then cultured for 4 days in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium containing 0.2% bovine serum albumin (BSA), N2 (1:50), B27 (1:50), 100 ng/mL activin A, and 1 μM wortmannin. These cells were then cultured for an additional 4 days in F12/Iscove's Modified Dubecco's Medium containing 0.5% BSA, 0.5% Insulin Transferrin Selenium (ITS), B27 (1:50), 2 μM retinoic acid, 20 ng/mL fibroblast growth factor 7, and 50 ng/mL Noggin, subsequently, these cells were cultured for 5 days in DMEM high glucose medium containing 0.5% BSA, 1% ITS, N2 (1:100), and 50 ng/mL epidermal growth factor. Finally, these cells were cultured for another 7 days in DMEM/F12 medium containing 1% ITS, 10 ng/mL basic fibroblast growth factor, 10 mM nicotinamide, 50 ng/mL exendin-4, and 10 ng/mL Bone morphogenetic protein 4, to promote cell maturation.

Primer design

The primers used in this study were: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (F: 5'-ATCTTCCAGGAGTGAGCGAGA-3', R: 5'-ACTGACACGTTGGCAGTG G-3'; produces a 504-bp product), Pdx1 (F: 5'-CAAAGCTCACGCGTGGAAAG-3', R: 5'-TTTTTCCACTTCATGCGGCG-3'; produces a 234-bp product), NGN3 (F: 5'-CTCAGGA AATCCCTGCGGTC-3', R: 5'-CATCCTTTCTACCGGCGCAA-3'; produces a 216-bp product), Pax6 (F: 5'-GTCGGTAAAGCTAGCTGGTT-3', R: 5'-CGTTTAGGCAACCCGGCTTA-3'; produces a 318-bp product), insulin-promoter (F: 5'-GACTATAAAGCCAGCGGGGG -3', R: 5'-CCTGCTTGATGGCCTCTTCT-3'; produces a 76-bp product, Ngn3-promoter (F: 5'-TGCCACTATCTTCCTGTGCG-3', R: 5'-CAGCAACTCTCGGTTCCTCA-3'; produces a 225-bp product), GAPDH-promoter (F: 5'-TACTAGCGGTTTTCGGGG-3', R: 5'-TCGAACA GGAGCAGAGAGCG-3'; produces a 166-bp product), Pax6-promoter (F: 5'-GCTAATTTAG GCATCACGGA-3', R: 5'-CTGAGCGGATCAGCATAGCC-3'; produces a 221-bp product).

RT-PCR

RNA extraction

In this experiment, control cells, which were those iPSCs before the induction of differentiation, are termed group A, while the experimental group, which are those iPSCs that had been differentiated into islet β cells, were termed group B. In either case, 1 x 10^6 cells from each group were removed from medium, washed twice in cold PBS, and added to 1 mL TRIzol. These samples were placed at room temperature for 5-10 min, after which 0.2 volumes of chloroform were added. Subsequently, these samples were centrifuged at 12,000 *g* for 15 min at 4°C.

Following centrifugation, the aqueous layer was removed and placed in a clean test tube. We then added 0.5 volumes of isopropanol, mixed, and incubated the samples at room temperature for 15 min. These samples were then centrifuged at 12,000 *g* for 10 min at 2°-8°C. Following centrifugation the upper layer was removed and the RNA precipitate was washed with 1 mL 75% ethanol, mixed on the shaker, and centrifuged at 7500 *g* for 5 min at 2°-8°C, The supernatant was then discarded, and the RNA precipitate was air dried. The pellet was then resolubilized in RNase-free water.

cDNA synthesis 20-µL system

To synthesize cDNA, we prepared a 100 µL Eppendorf tube, and successively added 1 µL RNA, 1 µL oligo primer, and deionized water to a total volume of 12 µL on ice. This sample was mixed gently, spun-down, and incubated for 5 min at 65°C. After the incubation we successively added 4 mL 5X reaction buffer, 1 µL 20 U/µL RNA inhibitor, 2 µL 10 mmol/µL dNTP, and 1 µL 200 U/µL reverse transcriptase RevertAid M-MuLV to the sample. This sample was then gently mixed, spun-down, and incubated for 60 min at 42°C. The sample was then incubated for 10 min at 70°C, after which the reaction was terminated and the cDNA was obtained.

PCR

The components for the PCRs were: 2 µL template cDNA, 1 µL Primer A, 1 µL Primer B, 0.5 µL Taq enzyme, 5 µL 10X buffer, 1 µL 10 mM dNTP mix, 25 mM $MgCl_2$, and ddH_2O added to a total volume of 50 µL. Once complete, this solution was placed in a PCR machine under the following program: 92°C for 2 min, 92°C for 30 s, 59°C for 30 s, and 72°C for 45 s, for a total of 30 cycles. Following the last cycle, the sample was subjected to a 2-min incubation at 72°C. Samples were then preserved at 4°C. A 2% agarose gel was used to assess the amplified product.

ChIP of iPSCs

iPSCs were grown to 80%, removed from medium, and washed once with 10 mL 1X PBS. These cells were then washed with 10 mL 1X PBS plus 1% formaldehyde and rotated for 20 min at room temperature. After this time, cross-linking was terminated with 0.125 M glycine, and the cells were rotated for 10 min at 4°C. Following this incubation, the PBS solution was discarded and cells were washed twice with ice-cold PBS, and collected with a cell scraper into 15 mL centrifuge tubes. These cells were then centrifuged at 2000 g for 5 min to collect cells.

The supernatant was removed and SDS Lysis Buffer (containing 50 mM Tris, pH 8.1, 1% SDS, sodium pyrophosphate, β-glycerophosphate, sodium orthovanadate, sodium fluoride, EDTA, leupeptin) plus protease inhibitors was added to the cell pellet to a final cell concentration of 1 x 10^6 cells/100 µL. Cells were then disrupted on ice using ultrasonic energy (30% of the maximum output, 10 s each time for a total of 20 times, an interval of 1 min each time). Samples were then centrifuged at 12,000 g for 10 min at 4°C.

One hundred microliters of whole-cell extract was added 900 µL ChIP dilution buffer (50 mM Tris-HCL, pH 8.0, 1 mM EDTA, 1% SDS, 50 mM $NaHCO_3$) and 60 µL Protein G agarose, and gently mixed for 60 min at 4°C. The sample was then centrifuged for 1 min at 4°C at 6000 g. The supernatant was removed divided into three 1.5-mL centrifuge tubes (labeled as negative control, positive control, input group), where 5 µg rabbit IgG and anti-Pdx1 rabbit antibody were added, respectively. These samples were then rotated and incubated at 4°C overnight.

Following the incubation, 60 µL protein G Sepharose was added to the solutions and incubated for 60 min at 4°C, after which samples were centrifuged at 7000 g for 1 min at 4°C. The resulting supernatants were discarded.

Pellets were washed with 1 mL low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1%

Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), and LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% SDC, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Lastly, the samples were washed twice with TE wash buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and centrifuged at 7000 g for 1 min at 4°C. The resulting supernatant was discarded.

One hundred microliters of freshly prepared ChIP extracting solution (1% SDS, 0.1 M $NaHCO_3$) was added to the precipitate and incubated for 15 min at room temperature. Subsequently, the samples were centrifuged at 7000 g for 1 min at 4°C. The resulting supernatant was removed and placed in another centrifuge tube. This procedure was repeated one more time and the supernatants were combined.

To this solution, we added 8 μL 5 M NaCl and incubated the solution at 65°C for 5 h. One microliter of 10 g/L RNase A solution was then added and the samples were then incubated at 37°C for 30 min. The resulting solution was then treated with 4 μL 0.5 M EDTA, 8 μL 1 M Tris-HCl, pH 6.5, and 1 μL 10 mg/mL proteinase K for 2 h at 45°C. Following this incubation, 1 mL binding buffer (4.5 M guanidine isothiocyanate, 0.5 M potassium acetate, pH 5.0) was added to the samples. A DNA combination column was then used to isolate the DNA, and samples were centrifuged through the column at 12,000 g for 30 s. The column was then washed with washing buffer (10 mM potassium acetate, pH 5.0, 80% ethanol, 16.7 μM EDTA, pH 8.0) twice, and DNA was eluted with 50 μL elution buffer (10 mM Tris-Cl, pH 8.5).

Statistical analysis

Data were analyzed by SPSS 17.0. Measurement data are reported as means ± standard deviation. Comparisons were performed using the Student t-test in two groups and were performed using ANOVA among multiple groups. $P < 0.05$ was considered to be statistically significant.

RESULTS
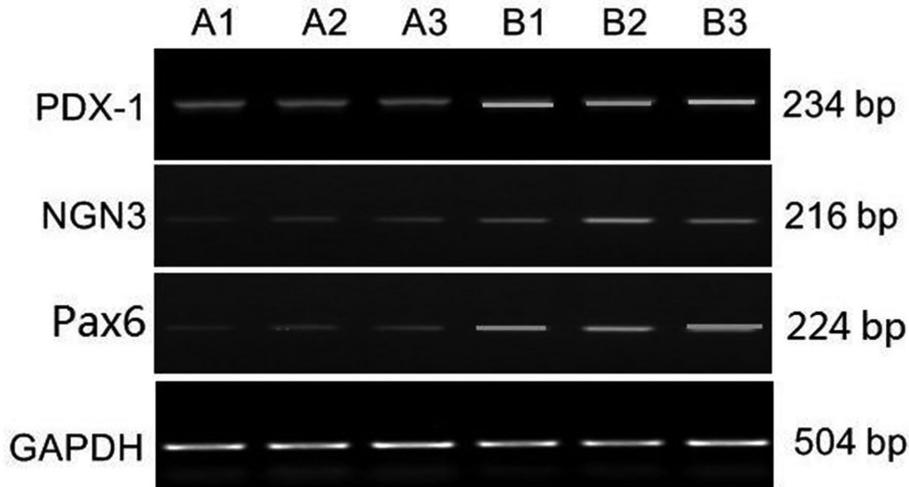
Differentiation of iPSCs into islet β cells

iPSCs derived from human skin fibroblasts were cultured successfully *in vitro* (Figure 1A). After the induction of differentiation, these cells were observed under a microscope on day 4, 8, 13, and 20. We found that cell morphology and distribution changed (Figure 1B-E). During the induction of differentiation, the expression of insulin-related genes *MafA*, *insulin*, *Glut2*, *Nkx6.1*, *GCK*, and *Tcf* were measured every 2 days. We found that the expression of all of these genes increased and peaked on the 20th day after the initiation of differentiation (Figure 1F), indicating that mature islet β cells have been formed.

Expression of *Pdx1*, *Ngn3*, and *Pax6* in differentiated pancreatic β cells

RT-PCR showed that the expression of *Pdx1*, *Ngn3*, and *Pax6* in group B was significantly enhanced as compared to group A (Figure 2), suggesting enhanced expression of *Pdx1* activated downstream genes *Ngn3* and *Pax6*. The expression of *Ngn3* and *Pax6* increased after iPSCs differentiated into islet β cells.
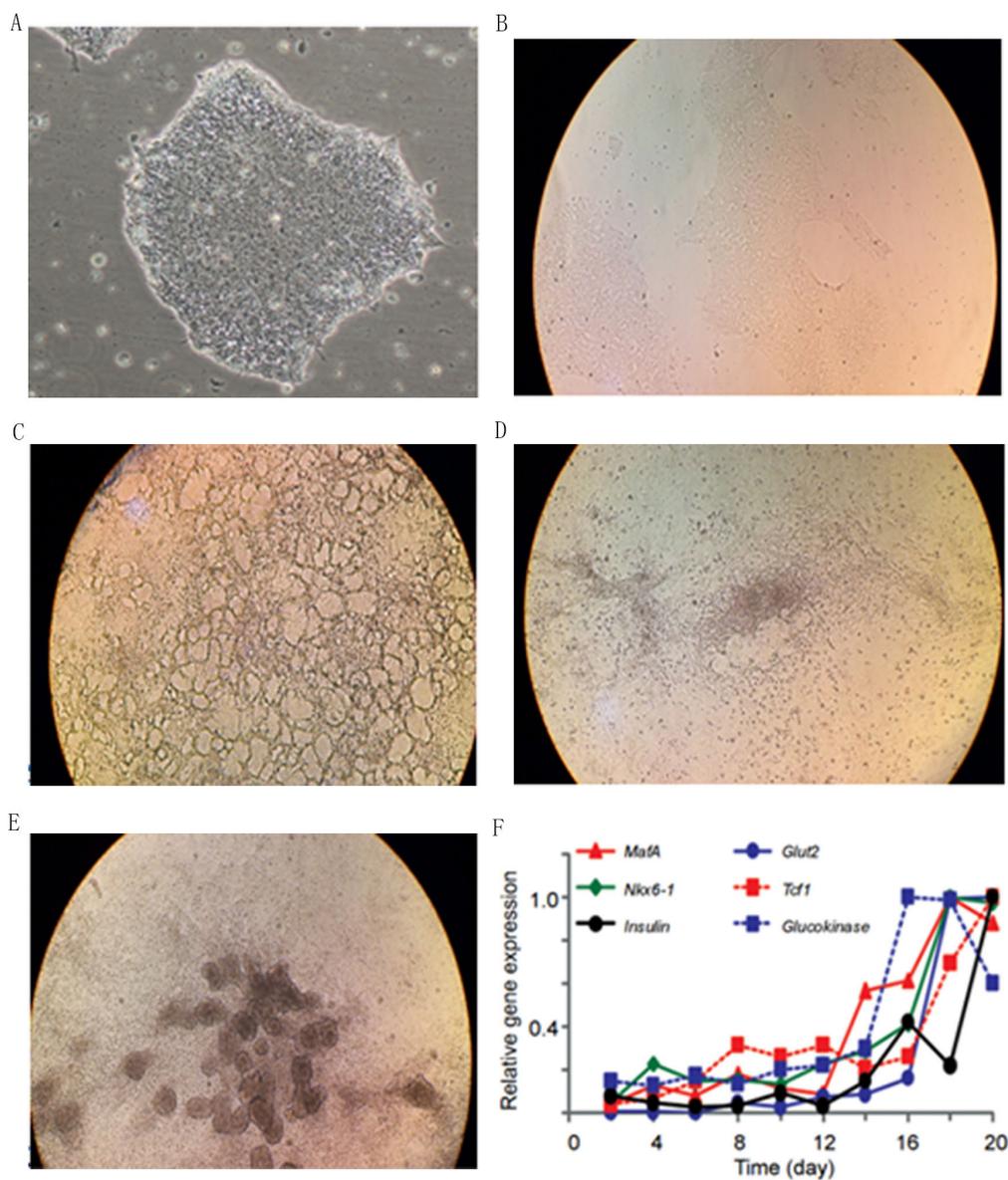
Figure 1. iPSCs induced to differentiate into islet β cells. **A.** Before induction, the growth of iPSCs is good and dense (20X magnification). **B.** Four days after differentiation was induced the cell shape changes and the differentiation into pancreatic cells begins (20X magnification). **C.** Eight days after differentiation was induced, the cells began to differentiate into progenitor cells (20X magnification). **D.** Thirteen days after differentiation was induced, the number of progenitor cells grew significantly (4X magnification). **E.** Twenty days after differentiation was induced, the progenitor cells differentiated into islet β-like cells (4X magnification). **F.** Expression of the insulin-related genes in differentiated pancreatic β cells.

Figure 2. Expression of insulin-related genes *Pdx1*, *Ngn3,* and *Pax6* in Groups A and B.

Role of Pdx1 in the differentiation of iPSCs into islet β cells

The DNA obtained from the Pdx1 ChIP was analyzed by PCR using the primers specific to the promoter regions of *insulin* (Insulin-P), *Ngn3* (Ngn3-P), and *Pax6* (Pax6-P) genes. Results indicated that fragments obtained corresponded to the target regions with no nonspecific amplification (Figure 3). Described Pdx1 antibody precipitated DNA fragment contained Insulin, Ngn3 and Pax6 gene sub-region. It is suggested that Pdx1 promotes differention of iPSCs into islet β-like cells by activating insulin, Ngn3, and Pax6 expression.
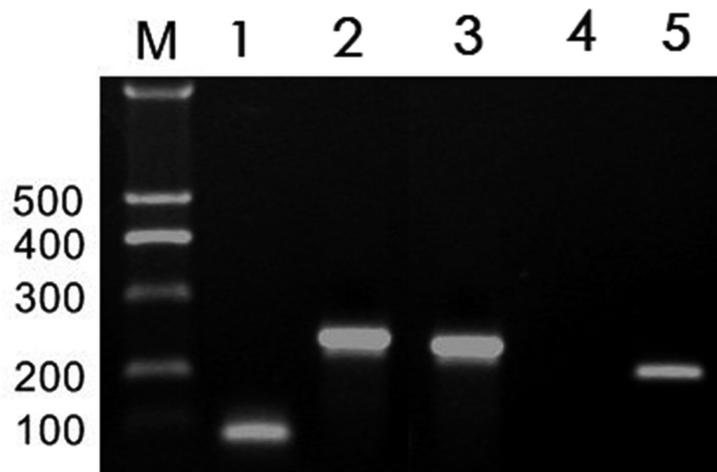
Figure 3. PCR amplification of the promoter regions for the *insulin*, *Ngn3*, and *Pax6* genes following Pdx1 ChIP. *Lane 1* = Insulin-P, *lane 2* = Ngn3-P, *lane 3* = Pax6-P, *lane 4* = negative control, and *lane 5* = internal control GAPDH; *lane M* = DNA ladder.

DISCUSSION

Studies have shown that Pdx1 is the most important transcription factor in the process of directional differentiation and maturation of the pancreas. It is necessary for pancreatic bud formation during the embryonic period and the subsequent differentiation of islet β cells (Guz et al., 1995). Pdx1 is known to directly bind to the insulin gene promoter region (Ashizawa et al., 2004), which is consistent with our results. In addition to Pdx1, epithelial cells that have further differentiated into pancreatic β cells require the interaction of a series of transcription factors such as Ngn3 and Pax6 to drive maturation into islet β cells (Serafimidis et al., 2008).

Ngn3 is not expressed in pancreatic progenitor cells, but becomes expressed in endocrine progenitor cells (Jiang and Morahan, 2012). It is speculated that *Ngn3* acts as a switch in regulating the pancreatic development process. Wang et al. (2009) confirmed that in rat embryonic development and adult pancreatic islet repair, the expression of Ngn3 decreased. Moreover, the expression of related genes (NeuroD1, Nkx2.2, and Pax6), which are located at downstream of *Ngn3* and regulate islet cell differentiation and maturation, will also be reduced, and eventually lead to islet endocrine dysfunction.

Pancreatic differentiation of the endoderm requires Pax6 participation, which together with the other key transcription factors plays an important role in the processes of pancreatic morphopoiesis and regulating pancreatic islet β cell development (Laakso, 2009). St-Onge et al. (1997) have shown that *Pax6* is expressed during early pancreatic development and maturation of endocrine cells. Moreover, if *Pax6* is deleted then pancreatic endocrine cell development will be subject to developmental dysfunction.

Yuan et al. (2010) have shown that transcription factors Pdx1 and Ngn3 can activate other downstream transcription factors and have a synergistic effect in the process of pancreatic endocrine cell development. Oliver-Krasinski et al. (2009) indicated that Pdx1 could activate Ngn3 and the crossover network of downstream transcription factors, making ES cells or pancreatic progenitor cells differentiate into pancreatic islet β cells.

The experiments conducted in this study confirmed that *in vitro* iPSCs were capable of differentiating into islet β-like cells, and that the expression of the transcription factors Pdx1, Ngn3, and Pax6 were significantly enhanced in this process. Furthermore, ChIP experiments indicated that Pdx1 activates *Ngn3* and *Pax6* expression by binding to their respective promoter regions, which further activates downstream gene networks and promotes iPSCs to differentiate into islet β cells. Accordingly, we speculate that Pdx1 activates Ngn3 and starts the expression of downstream transcription factor Pax6, thereby promoting iPSCs to differentiate into islet β cells. However, numerous questions remain, such as whether Pdx1 activates these genes simultaneously, inhibits other transcription factors after Pdx1 activates Ngn3, or plays the role in multiple signaling pathways.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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