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Original Article

PAX4 promotes PDX1-induced differentiation of mesenchymal stem cells into insulin-secreting cells

Lifa Xu[1*], Congjing Xu[2*], Shuping Zhou[3*], Xueke Liu[1], Jian Wang[1], Xinkuang Liu[3], Suping Qian[2], Yingru Xin[2], Yi Gao[3], Yongqiang Zhu[4], Xiaolong Tang[1]

[1]Stem Cell Engineering Research Center, School of Medical, Anhui University of Science & Technology, Huainan 232001, P.R. China; [2]Department of Respiration, Tumour Hospital of Affiliated Huainan Oriental Hospital Group, Anhui University of Science and Technology, Huainan 232035, P.R. China; [3]Huainan First People's Hospital and First Affiliated Hospital of Medical College, Anhui University of Science & Technology, Huainan 232001, P.R. China; [4]Department of Medical Genetics, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, P.R. China. [*]Equal contributors.

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Abstract: A shortage of postmortem pancreatic tissue for islet isolation impedes the application of cell replacement therapy in patients with diabetes. As an alternative for islet cell transplantation, transcription factors, including PDX1, PAX4, and neurogenin-3, that aid in the formation of insulin-producing β cells during development have been investigated. The present study evaluated the effects of PAX4 and PDX1 on the differentiation of mesenchymal stem cells (MSCs) into insulin-producing β-like cells *in vitro* using recombinant adenoviruses carrying PDX1 or PDX1 plus PAX4. RT-PCR, Western blot, and immunofluorescence assays were used to detect the expression levels of relevant genes and proteins, and enzyme-linked immunosorbent assays were used to determine the amount of insulin and C-peptide secreted by the virus-infected cells following stimulation with high glucose. The results showed that PAX4 markedly enhanced the propensity of PDX1-positive MSCs to form mature islet-like clusters and functional insulin-producing β-like cells. Our findings provide a novel foundation for generating β-like cells from MSCs with PAX4 and PDX1 for future clinical application.

Keywords: PAX4, PDX1, MSC, differentiation, pancreatic β cell

Introduction

Pancreatic β cells defects cause diabetes and persistent hyperglycemia, leading to long-term health complications [1, 2]. An unfortunate shortage of postmortem pancreatic tissue for isolation of islets is a major obstacle for the broad use of cell replacement therapy in diabetes. Thus, new sources of β cells or insulin-producing cells need to be identified. Several groups have reported evidence for the presence of cells resembling β cells among differentiated derivatives formed in embryoid bodies of human embryonic stem cells [3, 4]. Other insulin-producing cells generated from non-β cells through gene induction or genetic engineering methods have also been investigated [5, 6]. As some of these techniques have proved unreliable and difficult to replicate [7-9], attention has switched to testing whether specific transcription factor cascades aiding in the formation of β cells during development can be applied to mesenchymal stem cells (MSCs) *in vitro*.

During embryonic development, the pancreatic primordium arises from the posterior foregut region, and this development is dependent on the transcription factor pancreatic and duodenal homeobox-1 (PDX1) [10, 11]. The switch gene PDX1 expressed in pancreatic endocrine and exocrine precursor cells is important for the developing pancreas and for maintaining the function of mature β cells [12, 13]. Although overexpression of PDX1 enhances pancreatic endocrine cell differentiation, it fails to induce mature β-cell formation *in vitro*. We speculated that activation of the transcription factor cascade may be partly involved in the formation of mature β cells.

Although the precise developmental mechanisms of pancreatic secretary cells remain uncertain, β-cell generation is regulated by many transcription factors. Transcription factor PAX4, a transcriptional repressor of insulin, glucagon, and somatostatin promoters [14, 15], is thought to be positioned upstream of those late-stage transcription factors that promote pancreatic endocrine β-cell differentiation, replication, maturation, and survival in human islets by modulating apoptosis through Bcl-xL expression and proliferation via c-myc [16]. These transcription factors are essential for proper endocrine cell development [17, 18]. However, no studies have examined whether overexpression of PAX4 enhances the expression of β-cell related or specific genes, such as insulin, or promotes pancreatic endocrine β-cell differentiation or maturation in PDX1-positive ($^+$) MSCs.

In the present study, PDX1- and PAX4-carrying recombinant adenoviruses were used to induce MSC differentiation *in vitro*. We found that PAX4 regulated the expression of Nkx6.1, MafA, insulin, and GLUT2 in PDX1$^+$ MSCs to promote β-cell fate. Under high glucose conditions, the cells infected with combined PDX1 and PAX4 adenoviruses expressed β-cell markers and showed normal β-cell fate development. Thus, our findings indicated that PAX4 promoted the differentiation of PDX1$^+$ MSCs to β-cell fate.

Materials and methods

Materials and reagents

The adenovirus pAdxsi vector system was provided by the Gene Research Center of Beijing. Plasmids pEGFP-N1-PDXI and pEGFP-N1-PAX4, *Escherichia coli* DH5α, and HEK293 cells were preserved in our laboratory. Plasmid extraction, DNA gel recycling, and DNA purification kits were obtained from Omegar. RT-PCR kits, T4 DNA ligase, restriction endonucleases *Xba*I, *BamH*I, *Pme*I, *Sal*I, *Nhe*I, *Xho*I, *Bgl*II, and *EcoR*I as well as Klenow were purchased from New England Biolabs. Calf intestinal alkaline phosphatase was purchased from Promega. PCR primers were purchased from Shanghai Biotechnology Engineering Company, which also performed the sequencing. Mouse anti-PDXI, anti-insulin, anti-PAX4, and anti-GLUT2 antibodies were purchased from Creative Biomart (USA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG, HRP-conjugated anti-rabbit IgG, Cy5-labeled anti-mouse IgG, FITC-labeled anti-mouse IgG, and FITC-labeled anti-rabbit IgG were purchased from Bethyl Laboratories. Dulbecco's Modified Eagle's Medium/nutrient mixture F-12 and B27 were purchased from Gibco. Fetal bovine serum was purchased from Hyclone. The original generation of umbilical cord MSCs was purchased from Beijing YiKeLiHao Biotechnology Co., Ltd.

Construction of adenovirus plasmid vectors

The pEGFP-N1-PDX1 vector was linearized by *Sal*I and then digested with Klenow, followed by digestion with *Nhe*I. The released 0.66-kb human *PDX1* gene coding sequence fragment was recovered. To remove EGFP, the parental pShuttle-EGFP-CMV vector was digested with *Nhe*I and *Pme*I. The linearized pShuttle-CMV vector was recovered with DNA gel recycling and purification kits. The *PDX1* gene coding sequence fragment was ligated to the pShuttle-CMV vector with T4 ligase, generating a pShuttle-CMV-PDX1 vector. The *PAX4* target gene fragment was obtained from the pEGFP-N1-PAX4 vector with *BamH*I and *Sal*I double digestion and subcloned into the pShuttle-CMV-PDX1 vector, generating the pShuttle-CMV-PDX1/CMV-PAX4 shuttle plasmid vector. Then, the target CMV-PDX1/CMV-PAX4 fragment was subcloned into the backbone of a pAdxsi vector with an adenovirus pAdxsi vector system to construct the pAdxsi-CMV-PDX1/CMV-PAX4 expression plasmid. The products were sequenced and transformed into DH5α cells. The pAdxsi-CMV-PDX1/CMV-PAX4 plasmid was amplified. A similar method was employed to construct the pAdxsi -CMV-PDX1 plasmid and the pAdxsi-CMV-EGFP plasmid.

Recombinant adenovirus packaging, amplifying, and titer testing

The adenoviral plasmid was linearized by *Pac*I and transfected into HEK293 cells using Lipofectamine 2000. Plaques appeared after 5 days. After 8 days, a cytopathic effect was observed in approximately 80% of the cells. The cells were collected and placed in liquid

Table 1. Primer and amplification information for the target genes

Name	Forward (F) 5'-3'	Reverse (R) 5'-3'	Tm (°C)	Cycle number	Size (bp)
PDX1	TGGCACATCTTCACCATCA	CAGTCCTGCTCAGGCTCT	61	28	446
PAX4	TCCCAGTGTCTCCTCCATC	ACCTTTCCGGTGCTGTTGC	61	28	515
GLUT2	AGGACTTCTGTGGACCTTATGTG	GTTCATGTCAAAAAGCAGGG	63	35	231
Insulin	AGCCTITGTGAACCAACACC	GCTGGTAGAGGGAGCAGATG	65	30	246
β-actin	GATCGGCGGCTCCATCCTG	GACTCGTCATACTCCTGCTTGC	62	35	74

nitrogen. Then, the frozen cells were subjected to three cycles of freeze-thawing (at room temperature). The supernatant was collected by centrifugation at 12,000 rpm and 4°C. The recombinant adenovirus was packaged and amplified in HEK293 cells. A TCID50 assay was used to determine Adxsi-CMV-PDX1/CM-PAX4, Adxsi-CMV-PDX1, and Adxsi-CMV-EGFP virus titers.

Transcription detection of MSCs infected with recombinant virus

The MSCs seeded in 6-well plates at 2.0 × 10^5 cells/well were grown until the cell density reached 80%. The harvested cells were infected with an adenovirus at 100 pfu/cell and divided into three groups, namely, the Adxsi-CMV-PDX1, Adxsi-CMV-PDX1/CMV-PAX4, and Adxsi-CMV-GFP groups. Infected cells in each group were collected at appropriate stages for RT-PCR of PAX4, PDX1, insulin, and GLUT2. The primer sequences and PCR conditions of the respective genes were presented in **Table 1**.

Immunofluorescence staining and Western blot analysis

After being fixed in PBS with 4% paraformaldehyde for 20 min, the cells were washed three times in PBS and incubated for 10 min in PBS with 0.1% Triton X-100. After being blocked with blocking solution (PBS with 0.1% Triton-X and 1% sheep serum) for 30 min, the cells were incubated overnight at 4°C with one of the following primary antibodies: anti-PAX4 (1:200), anti-insulin (1:100), anti-Ngn3 (1:100), or anti-GLUT2 (1:100). Cells were then washed in blocking solution and incubated with Cy3 goat anti-mouse IgG (1:500; Sigma, Dorset, UK) or FITC-conjugated goat anti-rabbit IgG (1:500; Stratech Scientific Ltd., Newmarket, UK) for 1 h at room temperature, followed by three washes with blocking solution. Immunofluorescence with the specific antibodies was compared with that from negative control antibodies or rabbit IgG FITC-conjugated secondary antibody (1:100; Abcam, Cambridge, MA, USA) to indicate specificity. Cell nuclei were counterstained with DAPI contained in the mounting reagent (Sigma-Aldrich). Fluorescence images were captured with an Olympus CKX41 microscope and Nikon Coolscope DS-5M digital camera at 5MP resolution.

For Western blot analysis, cells were harvested into 50 μL of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% [w/v] Triton X-100, 0.1% [w/v] SDS) supplemented with a cocktail of protease inhibitors (Roche, UK). Total protein (20 μg) of the collected lysate was loaded on each lane, separated by SDS-PAGE, and transferred to PVDF membranes (Roche Applied Science). After being blocked with a 5% milk solution for 30 min, the membranes were incubated overnight at 4°C with an antibody against human NeuroD1 (1:1,000; RayBiotech, Inc.), Ngn3, Nkx6.1, MafA, MafB, or β-actin, followed by incubation for 1 h with a secondary anti-rabbit antibody conjugated to HRP (Santa Cruz, distributor Autogen Bioclear, Calne, UK). Membranes were developed using an ECL Western blotting detection system according to the manufacturer's protocol (Amersham Biosciences, Cardiff, UK).

Dithizone staining

Dithizone (DTZ; 50 mg) was dissolved in 5 mL of DMSO as a stock solution. The DTZ stock solution (0.1 mL) mixed with 10 mL PBS was filtered for use as the DTZ staining solution. The cells of each experimental group were washed with PBS, and the staining solution was added to the cells. The cells were incubated at 37°C for 15 min, followed by three washes with PBS for detection under a microscope.

Statistical analysis

The statistical significance of the differences between two groups was examined using *t* tests with SPSS 15.0 software. All data are expressed as the mean ± standard deviation.

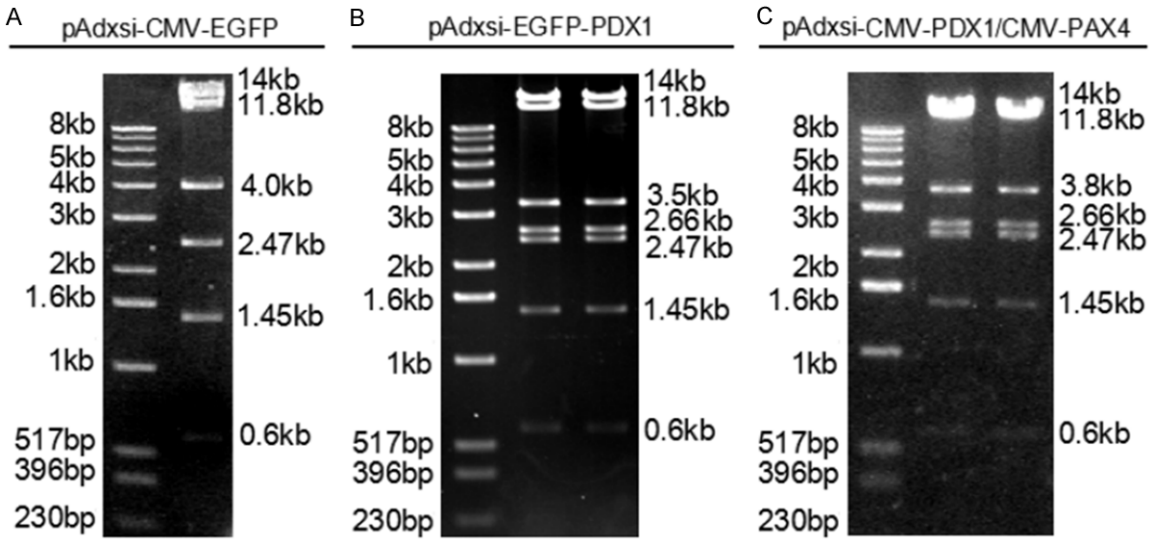

Figure 1. *Xho*I restriction enzyme digestion analysis. Markers represent 1-kb DNA ladders, as indicated.

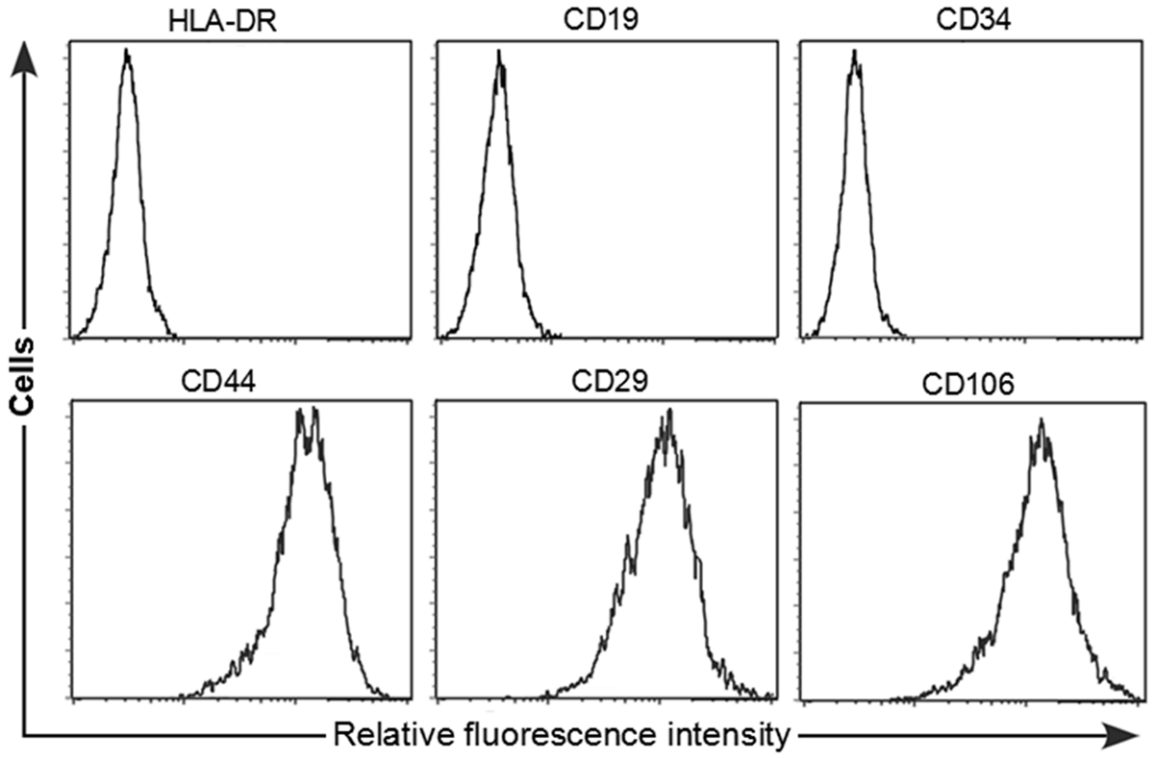

Figure 2. Characterization of undifferentiated MSC surface markers by flow cytometric analysis. MSCs were strongly positive for CD44, CD29, and CD106, but negative for HLA-DR, CD19, and CD34.

Results

Restriction enzyme digestion analysis

The pAdxsi-CMV-EGFP, pAdxsi-CMV-PDX1, and pAdxsi-CMV-PDX1/CMV-PAX4 plasmids were digested with *Xho*I, and the specific bands with the correct sizes are presented in **Figure 1**. All constructs were further confirmed by sequencing.

Morphological changes of MSCs infected with recombinant virus, and DTZ staining analysis

Analyzed by flow cytometric analysis, > 98% of the MSCs were positive for CD44, CD29, and

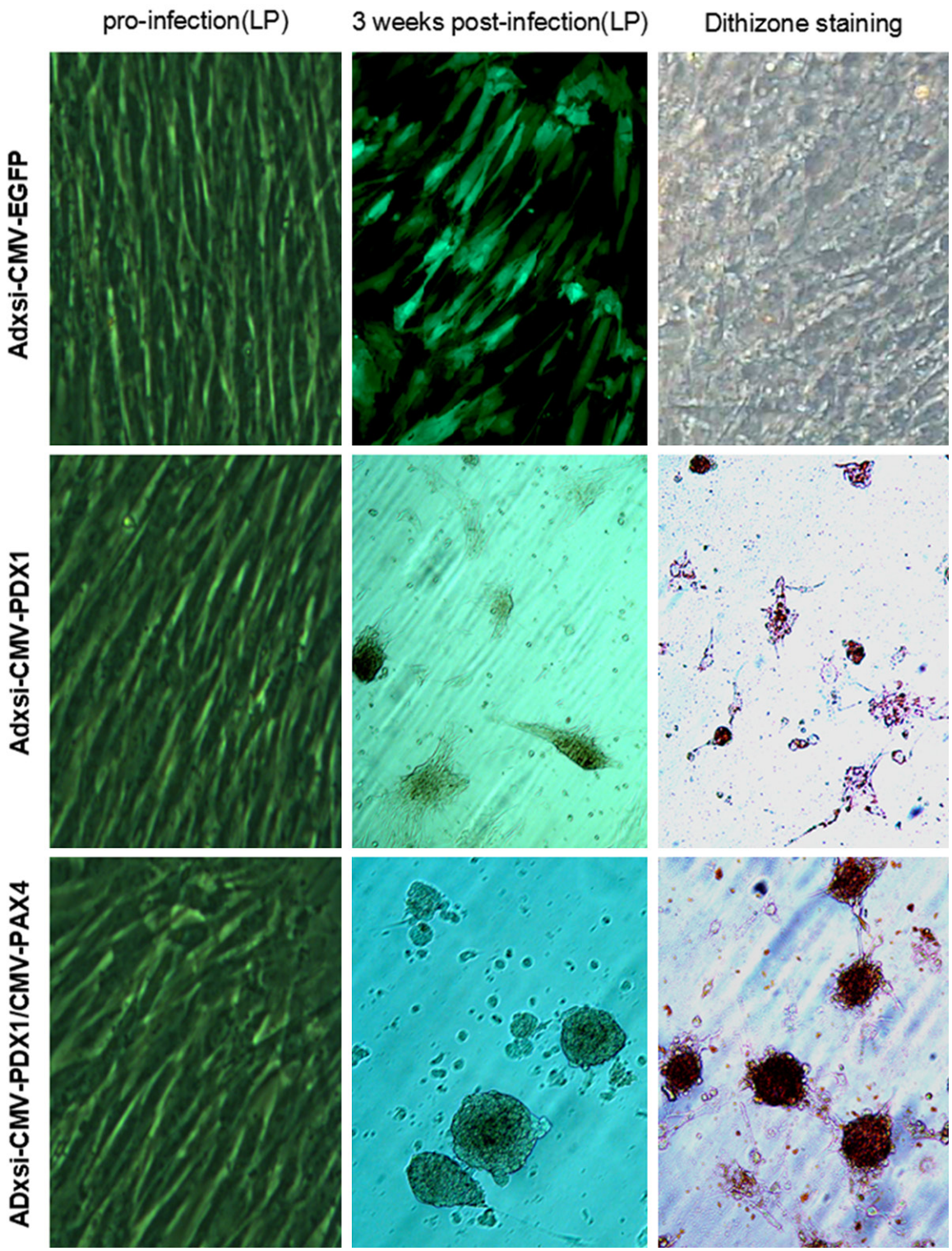

Figure 3. Morphological changes in MSCs infected with different Adxsi systems, and DTZ staining analysis. After 3 weeks of infection, cells in the Adxsi-CMV-PDX1 and Adxsi-CMV-PDX1/CMV-PAX4 groups become round and assemble together, whereas the round cells in the Adxsi-CMV-PDX1/CMV-PAX4 group aggregate into mature islet-like clusters. The mature islet-like clusters in both the Adxsi-CMV-PDX1/CMV-PAX4 and Adxsi-CMV-PDX1 groups are positive for DTZ staining. By contrast, the cells in the Adxsi-CMV-EGFP control group are negative for DTZ staining.

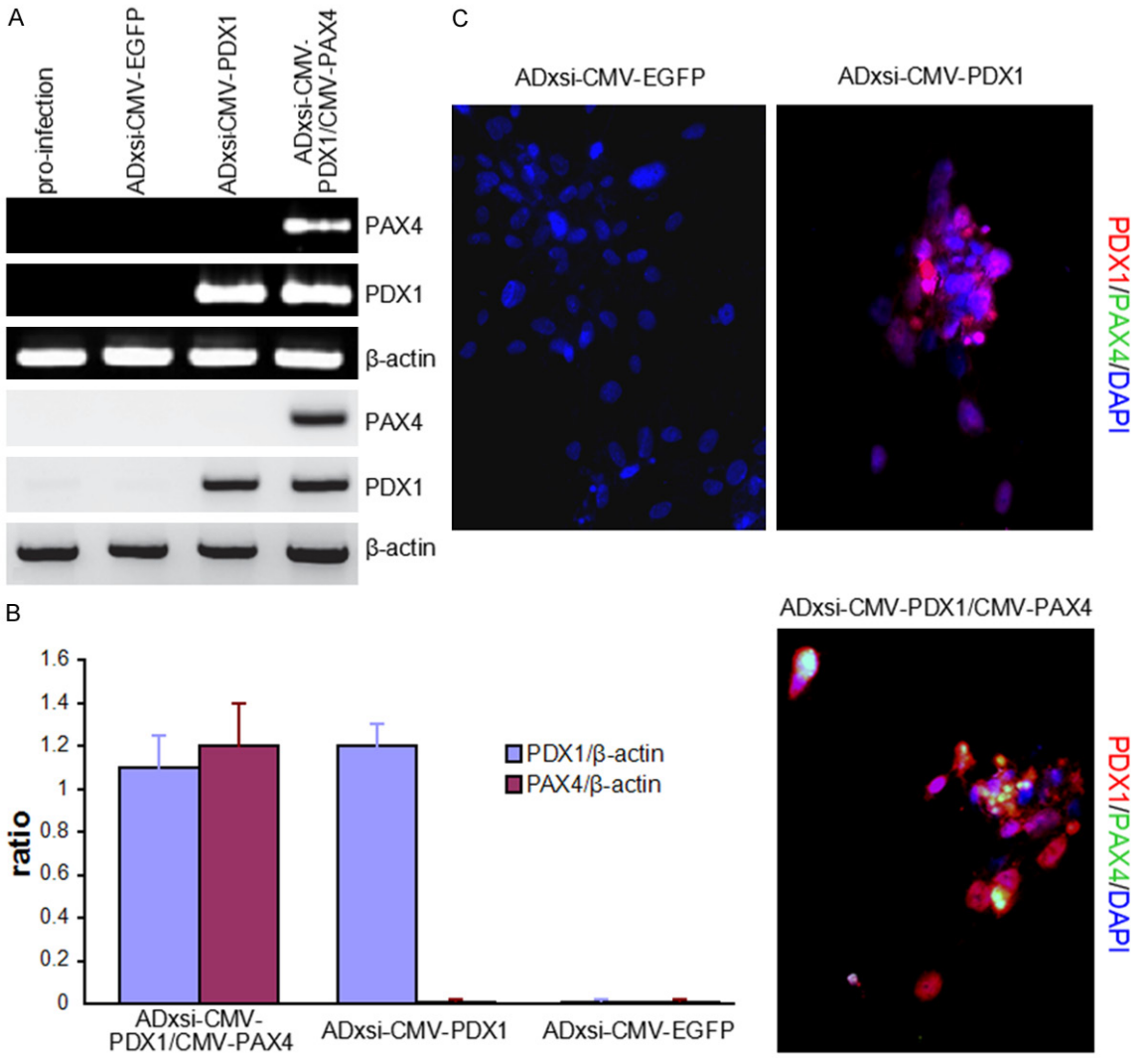

Figure 4. A. mRNA and protein levels of PDX1 and PAX4 after 15 days of recombinant virus infection. B. Protein level ratios of PDX1 or PAX4 to β-actin in the different groups. C. Immunofluorescence analysis demonstrates that the expressed PDX1 (Cy5, red) and PAX4 (FITC, green) are localized in the nucleus, which is stained with DAPI (blue).

CD106, whereas < 2% were positive for HLA-DR, CDI6, CD34, CD45, and CDI9 (**Figure 2**). These results indicated high purity MSCs. After the recombinant Adxsi adenovirus infection, the MSCs in the Adxsi-CMV-PDX1/CMV-PAX4 group with a fusiform shape became round. The rounded cells gathered into a ball and formed an islet-like structure (**Figure 3**). By contrast, a considerable portion of the MSCs in the Adxsi-CMV-PDX1 group remained spindle shaped and affixed to the wall of the flask (**Figure 3**). Three weeks after infection, the cells in each experimental group were subjected to DTZ staining for 15 min. The MSCs in the Adxsi-CMV-PDX1/CMV-PAX4 group were still round and arranged in islet-like structures and were stained red, whereas most of the Adxsi-CMV-PDX1-infected MSCs continued to be spindle shaped and affixed to the sides of the flask and few were stained red. The cells in the Adxsi-CMV-EGFP control group retained their spindle arrangement, and no morphological changes were observed in this control group (**Figure 3**).

Analysis of recombinant virus infection

After 15 days of recombinant virus infection, the mRNA and protein levels of target genes were detected by RT-PCR and Western blotting. As shown in **Figure 4A** and **4B**, both PDX1 and PAX4 were detected in the Adxsi-CMV-PDX1/CMV-PAX4 group, while only PAX4 was detected

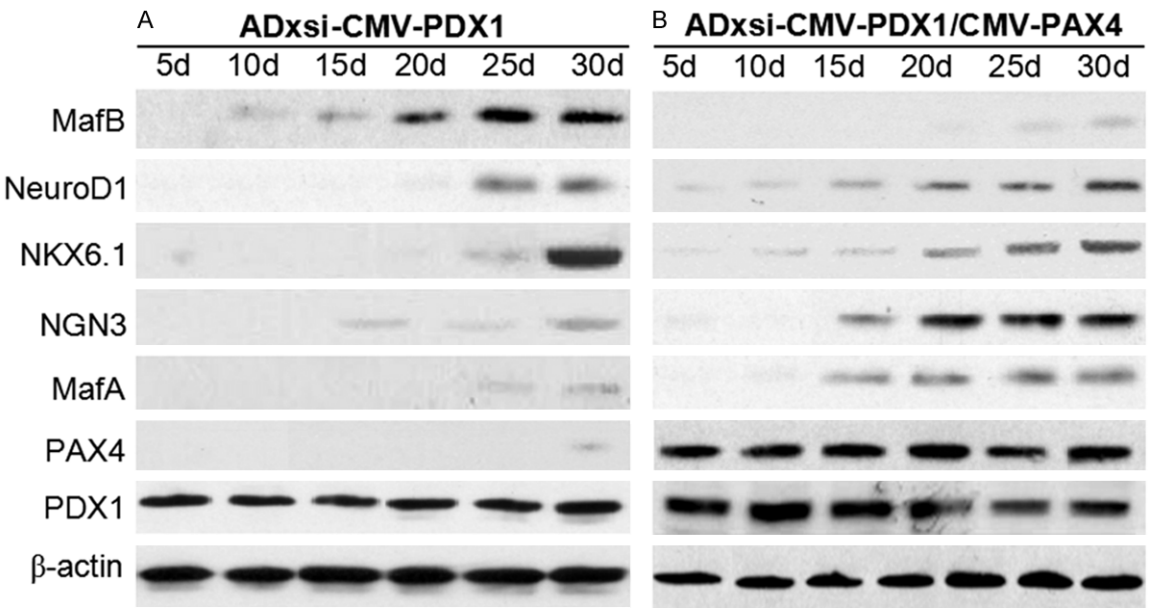

Figure 5. Western blot analysis of transcription factors related to islet β-cell function after recombinant virus infection at 5, 10, 15, 25 and 30 days.

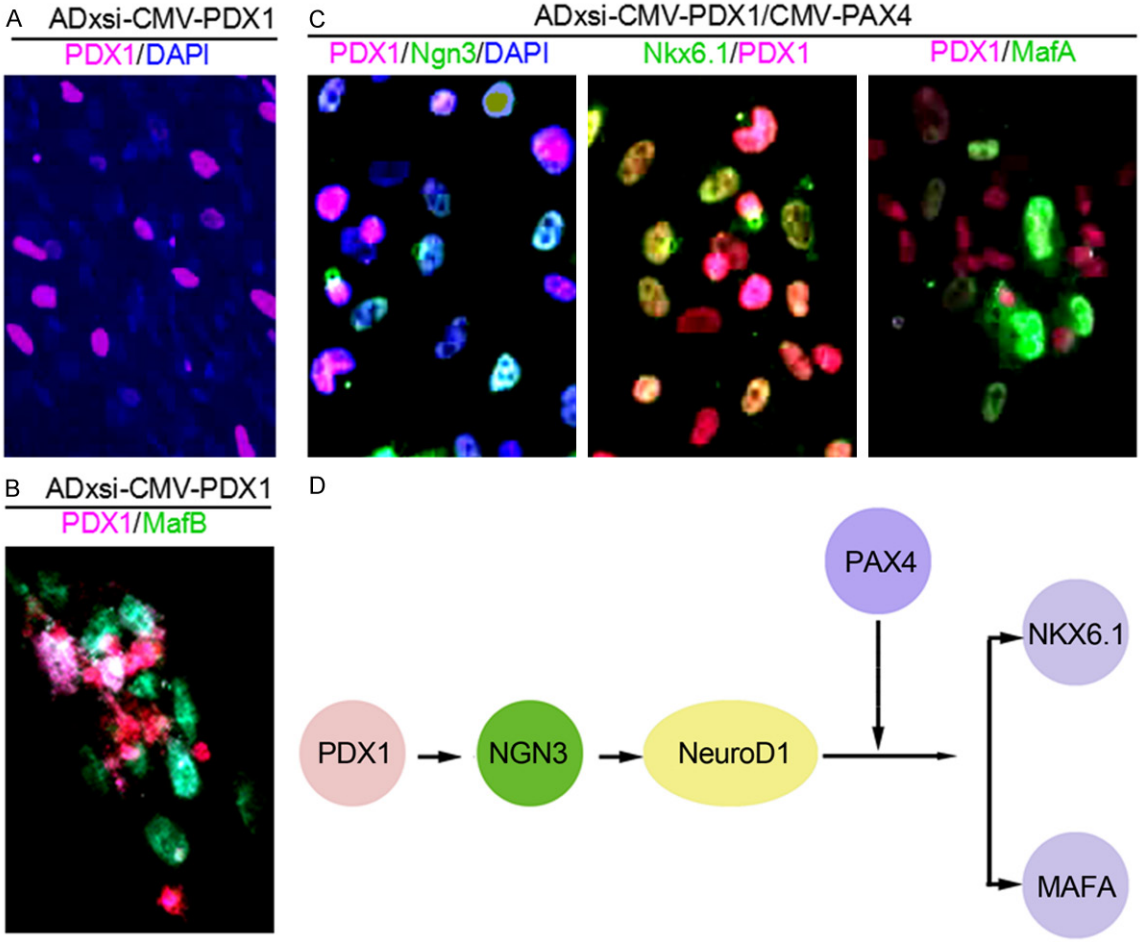

Figure 6. Expression and localization analysis of transcription factors in islet-like clusters. Immunofluorescence analysis detects localization of PDX1, Ngn3, Nkx6.1, MafA and MafB in the nucleus of differentiated cells after 30 days of recombinant virus infection. A. PDX1 is expressed stably and located only in the nucleus. B. PDX1 and MafB are not present in the same cellular nucleus in the islet-like clusters of the Adxsi-CMV-PDX1 infection group. C. Transcription factor PDX1 with transcription factors Ngn3, MafA, and Nkx6.1 are all located in the same cell nucleus of the Adxsi-CMV-PDX1/CMV-PAX4 infection group. D. PAX4 promotes transcription factor cascade expression during PDX1^{+} cell development. Counterstaining of nucleus (*blue*) is by DAPI.

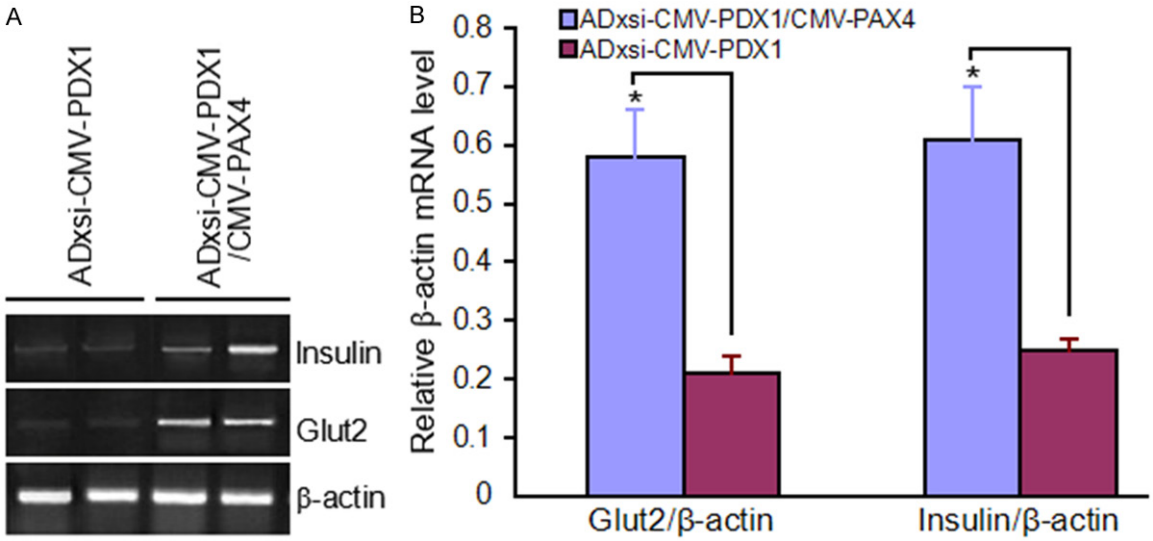

Figure 7. Co-expression of PAX4 and PDX1 in MSCs promotes the activation of *insulin* and *Glut2* expression. A. RT-PCR analysis of *insulin* and *Glut2* gene expression. *Insulin* and *Glut2* mRNA levels in the Adxsi-CMV-PDX1 group are markedly lower than those in the Adxsi-CMV-PDX1/CMV-PAX4 group. B. Results are expressed for the different groups as the ratio of *insulin* or *Glut2* mRNA to β-actin mRNA levels. Data represent the mean ± SEM; n = 3 in two different experiments. *$P < 0.001$.

in the Adxsi-CMV-PDX1 group. Neither PDX1 nor PAX4 was detected in the Adxsi-CMV-EGFP control group. Immunofluorescence analysis further confirmed that the expressed target proteins were localized in the cell nucleus (**Figure 4C**).

Pancreatic endocrine function-related molecules detected by Western blotting during the differentiation of MSCs into insulin-secreting cells

Protein expression levels for PDX1, PAX4, NeuroD1, neurogenin-3 (Ngn3), Nkx6.1, MafA, and MafB were measured 5, 10, 15, 20, 25, and 30 days after the recombinant virus infection. We found that the Adxsi-CMV-PDX1/CMV-PAX4-infected cells stably expressed PDX1 and PAX4, whereas Adxsi-CMV-PDX1-infected cells continuously expressed PDX1 (**Figure 5**). These results confirmed that this recombinant virus carrying target genes can be effectively expressed in infected MSCs. Compared with those in the Adxsi-CMV-PDX1 infected group, higher levels of intracellular NeuroD1, Ngn3, MafA, and Nkx6.1 were expressed earlier in the Adxsi-CMV-PDX1/CMV-PAX4 group. However, higher levels of MafB, a transcription factor related to pancreatic α-cell function, rather than β-cell function, were expressed earlier in Adxsi-CMV-PDX1 infected cells.

Indirect fluorescence analysis of NeuroD1, Ngn3, Nkx6.1, MafA, and MafB expression

As shown in **Figure 6A** and **6B**, PDX1 and MafB were expressed in Adxsi-CMV-PDX1-infected cells 30 days after the infection. PDX1 and MafB were localized in nuclei, albeit in different nuclei, suggesting that MafB and PDX1 expression levels were not directly related. After 30 days of infection, Adxsi-CMV-PDX1/CMV-PAX4 induced islet-like cells (**Figure 6C**). The expression of Ngn3, Nkx6.1, and MafA induced in these cells was localized within the same nucleus, indicating the presence of these endo-

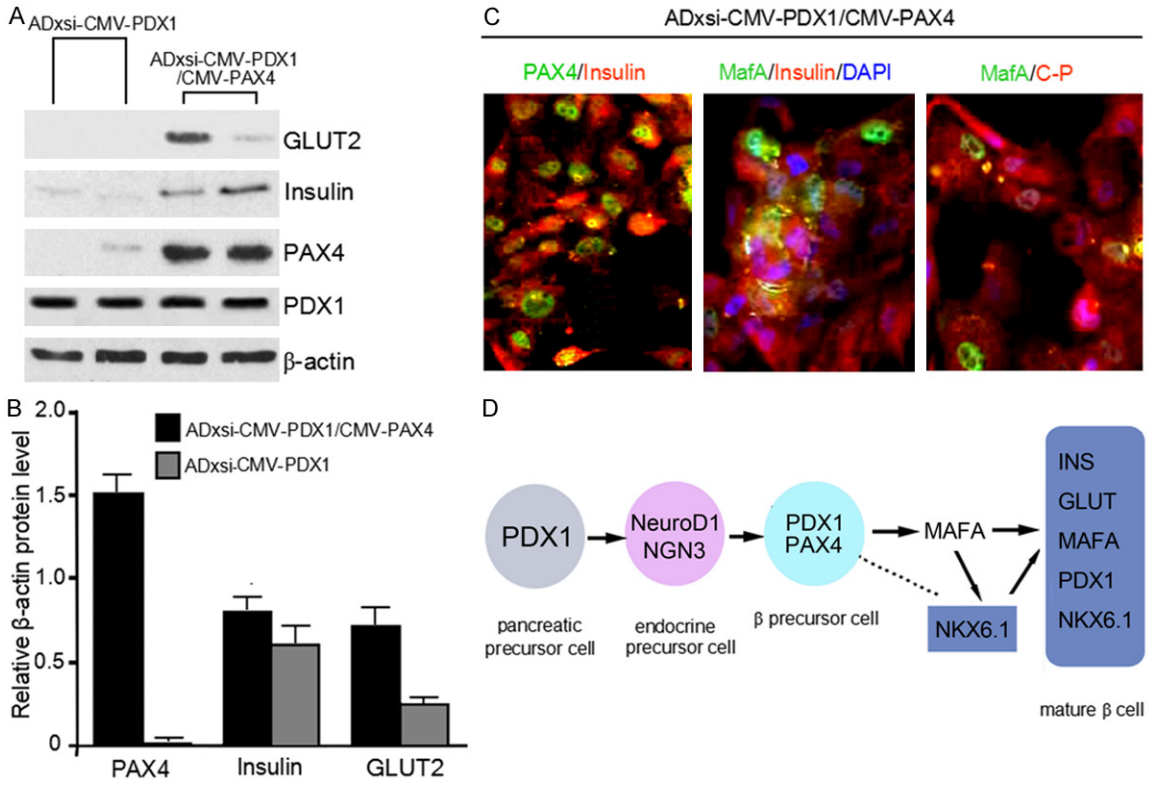

Figure 8. Co-expression of β-cell function-related molecules in cells of the Adxsi-CMV-PDX1 and Adxsi-CMV-PDX1/CMV-PAX4 groups. A, B. Characterization of GLUT2, Insulin, PAX4, and PDX1 protein expression in Adxsi-CMV-PDX1/CMV-PAX4 and Adxsi-CMV-PDX1 group cells. C. Immunofluorescence detection of both insulin or C-peptide (C-P, red) and PAX4 or MafA (green) shows co-expression in the cells of Adxsi-CMV-PDX1/CMV-PAX4 group. D. The schematic diagram is a simplified model indicating that transcription factors play roles in β-cell development. PAX4 is required for the terminal differentiation of β-cell function.

crine-related transcription factors may be associated with the expression of PDX1 and PAX4. These results indicate that PAX4 can induce the differentiation of PDX1$^+$ MSCs into Nkx6.1$^+$-MafA$^+$ cells (**Figure 6D**).

Adxsi-CMV-PDX1/CMV-PAX4-induced expression of insulin and GLUT2

GLUT2, a membrane transport protein, transports glucose into cells and regulates ion channels on the cell membrane. It also promotes the release of intracellular insulin stores into the circulation. The presence of GLUT2 is a sign of mature pancreatic β cells. Although we found that Adxsi-CMV-PDX1/CMV-PAX4 induced small islet-like cells that expressed β-cell function-associated transcription factors, it was unknown whether these small islet-like cells were capable of insulin synthesis and GLUT2 expression. Thus, we used RT-PCR to determine mRNA levels of *Glut2* and *insulin* (**Figure 7A**). We found that although Adxsi-CMV-PDX1 induced small islet-like cells, the transcription levels of *insulin* and *Glut2* were significantly lower than those in Adxsi-CMV-PDX1/CMV-PAX4-transfected cells (**Figure 7A**, **7B**).

The protein levels of GLUT2 and insulin as determined by Western blotting were consistent with their respective mRNA levels (**Figure 8A**, **8B**). Indirect fluorescent analysis demonstrated that PAX4/MafA and insulin as well as MafA and C-peptide (C-P) were present in the same cell (**Figure 8C**). Thus, Adxsi-CMV-PDX1/CMV-PAX4-infected cells expressed proinsulin, which was further hydrolyzed into functional insulin and C-P. The GLUT2 expression levels in Adxsi-CMV-PDX1-infected MSCs were significantly lower than those in Adxsi-CMV-PDX1/CMV-PAX4-infected MSCs, suggesting that PAX4 enhanced the PDX1 induction of MSCs into islet-like cells. We hypothesized that the expression of MafA and PAX4 may play key

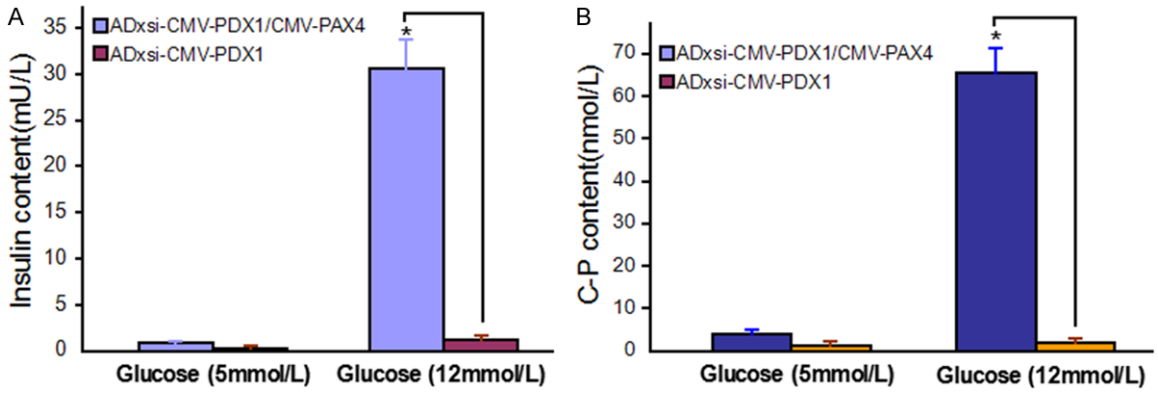

Figure 9. Co-expression of PDX1 and PAX4 improves glucose-regulated insulin and C-peptide secretion and increases the expression of β-cell-related genes in cells 30 days after Adxsi-CMV-PDX1/CMV-PAX4 infection. A. Insulin secretion levels at 5.0 and 12.0 mM glucose in response to Adxsi-CMV-PDX1 or Adxsi-CMV-PDX1/CMV-PAX4. B. C-peptide (C-P) secretion levels at 5.0 and 12.0 mM glucose in response to Adxsi-CMV-PDX1 or Adxsi-CMV-PDX1/CMV-PAX4. n = 3 in two different experiments. *$P < 0.001$.

roles in upregulating Nkx6.1 and other transcription factors in the induced cells. Thus, proinsulin, GLUT2, and related intracellular hydrolases are further increased in the induced MSCs, which ultimately differentiate into functional insulin-secreting cells (**Figure 8D**).

Glucose-stimulated insulin secretion and C-P levels

The culture fluid levels of insulin and C-P following stimulation of cells with 12.0 mmol/L glucose were measured by ELISA. The medium from the Adxsi-CMV-PDX1/CMV-PAX4 group contained significantly higher concentrations of C-P and insulin than that from the Adxsi-CMV-PDX1 group under high glucose stimulation (**Figure 9**), indicating that PAX4 and PDX1 play an important role in the differentiation of MSCs into mature β cells capable of insulin synthesis and release in response to high glucose stimulation.

Discussion

PDX1 contains a number of transcriptional regulatory regions, such as the N-terminus transcriptional regulatory elements related to the functional structure of the GG and insulin A3 domains, and is a key gene necessary for the development of pancreatic endocrine cells [19-21]. However, this development requires additional molecules. PDX1 also combines with the GLUT2 TATA box, contributing to GLUT2 transcription [21, 22]. Recent studies have shown that PDX1 induces pancreatic differentiation and development. *PDX*1 activation induced by the combined effects of the relevant factors and the activation of the related signaling pathways leads to the differentiation of MSCs in the pancreatic β-cell-like direction [23, 24], thereby indicating that PDX1 plays a role as a transcriptional master switch in pancreatic cell differentiation [25]. However, studies have also shown that PDX1 alone cannot efficiently induce pancreatic precursor cells to differentiate into mature pancreatic β cells; thus, the combination of PDX1 and other related transcription factors is required [26].

PAX4, an important transcription factor for the development of the pancreas and regulation of endocrine differentiation, encourages the expression of MafA, Nkx6.1, and other transcription factors and induces pancreatic progenitor cells to differentiate into endocrine cells, thereby enhancing the transcription and biosynthesis of insulin [27, 28]. During pancreas development, PDXI and PAX4 are essential for activating a series of transcription factors [27, 28] and for directing the differentiation of pancreatic endocrine cells toward a β-cell fate [28]. Our present results confirmed that PDX1 and PAX4 effectively promoted MSCs to differentiate into functionally active β cells enriched with zinc.

Zinc enrichment is a basic physiological function of islet β-cell precursor cells. When we observed morphological changes in the MSCs and they were arranged in small islet-like structures, DTZ stained the cytoplasm red, suggest-

ing that the cytoplasm was enriched with zinc. Zinc plays a critical role in the action of insulin because interactions between metal ions and proteins change the structure of insulin and its biological functions [29, 30]. However, proinsulin-producing cells must synthesize proteins, and zinc ions alone cannot synthesize insulin with physiological functions. Proinsulin is hydrolyzed to C-P in the Golgi apparatus, where insulin complexes with zinc ions are formed with biological activity [31].

Transcription and expression of proinsulin requires the regulation of a series of transcription factors, including PDX1, NeuroD1, Ngn3, PAX4, and Nkx6.1, which play important roles in pancreatic β-cell differentiation and development [32]. NeuroD1 is involved in the differentiation of pancreatic endocrine precursor cells. In mice, NeuroD1 expression is first observed on embryonic day 9.5 in pancreatic progenitor cells and persists throughout β-cell development; however, NeuroD1 expression is limited in β cells in the mature pancreas [32, 33]. NeuroD1/Beta2-deficient mice exhibit severe diabetes, because of the severely reduced β cells, resulting in high perinatal mortality [33, 34]. NeuroD1 also increases the concentration of intracellular Ca^{2+} by activating the Sur1 promoter. The *insulin* activation by glucose stimulation is controlled by NeuroD1 at transcriptional and post-transcriptional levels [35].

Ngn3 is an important islet progenitor cell marker [36]. In the formation of the pancreas, all endocrine cells are derived from $Ngn3^{+}$ cells, and Ngn3 initiates the differentiation of endocrine progenitor cells [36]. The binding of the bHLH domain of Ngn3 with the upstream promoter of the E-box domain activates the transcription of *NeuroDI* and *PAX4* [36, 37]. By contrast, the binding between Ngn3's own promoters can inhibit the transcription of Ngn3 itself to regulate the differentiation of islet cells [36, 37].

Nkx6.1 inhibits *glucagon* transcription, but promotes *insulin* activation, whereas the downregulation of Nkx6.1 induces the opposite effect. Nkx6.1 gene-deficient homozygous mice cannot develop β cells [38]. The Nkx6.1-null mouse presents a selective reduction in β cells, while other endocrine cells remain normal [39], which is similar to PAX4-negative mice, suggesting that Nkx6.1 is necessary for functional β-cell development. Furthermore, MafA directly activates *insulin* in β-cell lines and plays an important role in the cellular transcription factor cascade in adult β cells [40]. Therefore, the development of insulin-producing cells into functional β cells requires cooperation among multiple transcription factors, which sheds light on functional β-cell induction *in vitro*.

We compared the expression of NeuroD1, Ngn3, Nkx6.1, and MafA in various groups of recombinant virus-infected cells. We found significant expression of NeuroD1, Ngn3, Nkx6.1, and MafA earlier in the Adxsi-CMV-PDX1/CMV-PAX4 group than in the Adxsi-CMV-PDX1 group, and the expression levels of NeuroD1, Ngn3, Nkx6.1, and MafA induced by PAX4/PDX1 were higher than those induced by PDX1. The upregulation of Nkx6.1 and MafA in particular favors directing MSCs to differentiate into endocrine β cells, given that Nkx6.1 inhibits the differentiation of islet endocrine cells into α-cell, while MafA directly activates *insulin* transcription and the transcription factor cascade reaction required for β-cell development. Analysis of molecules related to β-cell function using Western blotting and indirect fluorescence assays in the present study indicated that the expression of GLUT2 was higher in the Adxsi-CMV-PDX1/CMV-PAX4 group than that in the Adxsi-CMV-PDX1 group. The differentiated β-cells from MSCs induced by PAX4/PDX1 responded to high glucose stimulation and released insulin and C-P molecules, suggesting that PAX4 promotes the PDX1-induced differentiation of MSCs into functional β cells. The mechanism for this promotion may be a synergist interaction between PAX4 and PDX1 and increased endogenous expression of these and other endocrine-related transcription factors, including NeuroD1, Ngn3, Nkx6.1, and MafA. These transcription factors increased insulin and GLUT2 expression levels and eventually induced MSCs to differentiate and develop into functional β-like cells that were capable of producing insulin and responding to high-glucose stimulation. However, verification of this suggested mechanism will require further study.

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Disclosure of conflict of interest

None.

Address correspondence to: Yongqiang Zhu, Department of Medical Genetics, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, P.R. China. E-mail: zyq666999@aliyun.com; Xiaolong Tang, Stem Cell Engineering Research Center, School of Medical, Anhui University of Science & Technology, Huainan 232001, P.R. China. E-mail: txljd2006@126.com

References

[1] Roma LP, Pascal SM, Duprez J, Jonas JC. Mitochondrial oxidative stress contributes differently to rat pancreatic islet cell apoptosis and insulin secretory defects after prolonged culture in a low non-stimulating glucose concentration. Diabetologia 2012; 55: 2226-2237.

[2] Radichev IA, Maneva-Radicheva LV, Amatya C, Salehi M, Parker C, Ellefson J, Burn P, Savinov AY. Loss of peripheral protection in pancreatic islets by proteolysis-driven impairment of VTCN1 (B7-H4) presentation is associated with the development of autoimmune diabetes. J Immunol 2016; 196: 1495-1506.

[3] Xu X, Browning VL, Odorico JS. Activin, BMP and FGF pathways cooperate to promote endoderm and pancreatic lineage cell differentiation from human embryonic stem cells. Mech Dev 2011; 128: 412-427.

[4] Shiroi A, Yoshikawa M, Yokota H, Fukui H, Ishizaka S, Tatsumi K, Takahashi Y. Identification of insulin-producing cells derived from embryonic stem cells by zinc-chelating dithizone. Stem Cells 2002; 20: 284-292.

[5] Kim JH, Shin KH, Li TZ, Suh H. Potential of nucleofected human MSCs for insulin secretion. J Tissue Eng Regen Med 2011; 5: 761-769.

[6] Rackham CL, Vargas AE, Hawkes RG, Amisten S, Persaud SJ, Austin AL, King AJ, Jones PM. Annexin A1 is a key modulator of mesenchymal stromal cell-mediated improvements in islet function. Diabetes 2016; 65: 129-139.

[7] Wang H, Luo X, Yao L, Lehman DM, Wang P. Improvement of cell survival during human pluripotent stem cell definitive endoderm differentiation. Stem Cells Dev 2015; 24: 2536-2546.

[8] Pezzolla D, López-Beas J, Lachaud CC, Domínguez-Rodríguez A, Smani T, Hmadcha A, Soria B. Resveratrol ameliorates the maturation process of β-cell-like cells obtained from an optimized differentiation protocol of human embryonic stem cells. PLoS One 2015; 10: e0119904.

[9] Wang A, Yue F, Li Y, Xie R, Harper T, Patel NA, Muth K, Palmer J, Qiu Y, Wang J, Lam DK, Raum JC, Stoffers DA, Ren B, Sander M. Epigenetic priming of enhancers predicts developmental competence of hESC-derived endodermal lineage intermediates. Cell Stem Cell 2015; 16: 386-399.

[10] Marty-Santos L, Cleaver O. Pdx1 regulates pancreas tubulogenesis and E-cadherin expression. Development 2016; 143: 1056.

[11] Kimmel RA, Dobler S, Schmitner N, Walsen T, Freudenblum J, Meyer D. Diabetic Pdx1-mutant zebrafish show conserved responses to nutrient overload and anti-glycemic treatment. Sci Rep 2015; 5: 14241.

[12] Qin Y, Xiao L, Zhan XB, Zhou HX. Pdxl and its role in activating Ngn3 and Pax6 to induce differentiation of iPSCs into islet β cells. Genet Mol Res 2015; 14: 8892-8900.

[13] Donelan W, Li S, Wang H, Lu S, Xie C, Tang D, Chang LJ, Yang LJ. Pancreatic and duodenal homeobox gene 1 (Pdx1) down-regulates hepatic transcription factor 1 alpha (HNF1α) expression during reprogramming of human hepatic cells intoinsulin-producing cells. Am J Transl Res 2015; 7: 995-1008.

[14] Wang Q, Elghazi L, Martin S, Martins I, Srinivasan RS, Geng X, Sleeman M, Collombat P, Houghton J, Sosa-Pineda B. Ghrelin is a novel target of Pax4 in endocrine progenitors of the pancreas and duodenum. Dev Dyn 2008; 237: 51-61.

[15] Smith SB, Ee HC, Conners JR, German MS. Paired-homeodomain transcription factor PAX4 acts as a transcriptional repressor in early pancreatic development. Mol Cell Biol 1999; 19: 8272-8280.

[16] Sun J, Xu M, Ortsäter H, Lundeberg E, Juntti-Berggren L, Chen YQ, Haeggström JZ, Gudmundsson GH, Diana J, Agerberth B. Cathelicidins positively regulate pancreatic β-cell functions. FASEB J 2016; 30: 884-894.

[17] Gage BK, Baker RK, Kieffer TJ. Overexpression of PAX4 reduces glucagon expression in differentiating hESCs. Islets 2014; 6: e29236.

[18] Chen M, Hu C, Zhang R, Jiang F, Wang J, Peng D, Tang S, Sun X, Yan J, Luo Y, Bao Y, Jia W. Association of PAX4 genetic variants with oral antidiabetic drugs efficacy in Chinese type 2 diabetes patients. Pharmacogenomics J 2014; 14: 488-492.

[19] Soleimanpour SA, Ferrari AM, Raum JC, Groff DN, Yang J, Kaufman BA, Stoffers DA. Diabetes susceptibility genes Pdx1 and Clec16a function in a pathway regulating mitophagy in β-cells. Diabetes 2015; 64: 3475-3484.
[20] Bonfanti P, Nobecourt E, Oshima M, Albagli-Curiel O, Laurysens V, Stangé G, Sojoodi M, Heremans Y, Heimberg H, Scharfmann R. Ex vivo expansion and differentiation of human and mouse fetal pancreatic progenitors are modulated by epidermal growth factor. Stem Cells Dev 2015; 24: 1766-1778.
[21] McKenna B, Guo M, Reynolds A, Hara M, Stein R. Dynamic recruitment of functionally distinct Swi/Snf chromatin remodeling complexes modulates Pdx1 activity in islet β cells. Cell Rep 2015; 10: 2032-2042.
[22] Wang X, Lei XG, Wang J. Malondialdehyde regulates glucose-stimulated insulin secretion in murine islets via TCF7L2-dependent Wnt signaling pathway. Mol Cell Endocrinol 2014; 382: 8-16.
[23] Boroujeni ZN, Aleyasin A. Insulin producing cells established using non-integrated lentiviral vector harboring PDX1 gene. World J Stem Cells 2013; 5: 217-228.
[24] Tonne JM, Sakuma T, Deeds MC, Munoz-Gomez M, Barry MA, Kudva YC, Ikeda Y. Global gene expression profiling of pancreatic islets in mice during streptozotocin-induced β-cell damage and pancreatic Glp-1 gene therapy. Dis Model Mech 2013; 6: 1236-1245.
[25] Talebi S, Aleyasin A, Soleimani M, Massumi M. Derivation of islet-like cells from mesenchymal stem cells using PDX1-transducing lentiviruses. Biotechnol Appl Biochem 2012; 59: 205-212.
[26] Wang J, Gao Y, Lu Y, Tang X, He D, Zhang Y. Induced differentiation of human umbilical cord mesenchymal stem modified by cells Pdx1gene into islet beta-like cells in vitro. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi 2011; 28: 1175-1180.
[27] Zhang Y, Fava GE, Wang H, Mauvais-Jarvis F, Fonseca VA, Wu H. PAX4Gene transfer induces α-to-β cell phenotypic conversion and confers therapeutic benefits for diabetes treatment. Mol Ther 2016; 24: 251-260.
[28] Napolitano T, Avolio F, Courtney M, Vieira A, Druelle N, Ben-Othman N, Hadzic B, Navarro S, Collombat P. Pax4 acts as a key player in pancreas development and plasticity. Semin Cell Dev Biol 2015; 44: 107-114.
[29] Abouzaripour M, Pasbakhsh P, Atlasi N, Shahverdi AH, Mahmoudi R, Kashani IR. In vitro differentiation of insulin secreting cells from mouse bone marrow derived stage-specific embryonic antigen 1 positive stem cells. Cell J 2016; 17: 701-710.
[30] Song WQ, Fu DZ, Cheng Y, Liu YF. Influence of adenosine on preservation of porcine pancreas in islet transplantation. Genet Mol Res 2015; 14: 18293-18301.
[31] Sun Y, Zhang M, Ji S, Liu L. Induction differentiation of rabbit adipose-derived stromal cells into insulin-producing cells in vitro. Mol Med Rep 2015; 12: 6835-6840.
[32] Gosmain Y, Marthinet E, Cheyssac C, Guérardel A, Mamin A, Katz LS, Bouzakri K, Philippe J. Pax6 controls the expression of critical genes involved in pancreatic alpha-cell differentiation and function. J Biol Chem 2010; 285: 33381-33393.
[33] Han X, Xiao J, Ren Q, Tang Y, Yang W, Ji L. Evaluation of variant A45T in NEUROD1/BETA2 for its association with type 2 diabetes mellitus. Endocrine 2013; 44: 99-106.
[34] Gong ZC, Huang Q, Dai XP, Lei GH, Lu HB, Yin JY, Xu XJ, Qu J, Pei Q, Dong M, Zhou BT, Shen J, Zhou G, Zhou HH, Liu ZQ. NeuroD1 A45T and PAX4 R121W polymorphisms are associated with plasma glucose level of repaglinide monotherapy in Chinese patients with type 2 diabetes. Br J Clin Pharmacol 2012; 74: 501-509.
[35] Bernardo AS, Hay CW, Docherty K. Pancreatic transcription factors and their role in the birth, life and survival of the pancreatic beta cell. Mol Cell Endocrinol 2008; 294: 1-9.
[36] Qin Y, Xiao L, Zhan XB, Zhou HX. Pdxl and its role in activating Ngn3 and Pax6 to induce differentiation of iPSCs into islet β cells. Genet Mol Res 2015; 14: 8892-8900.
[37] Ang XL, Xiao R, Wang YX, He M, Xie T, Zhang C, Liu SJ. Neurogenin 3 and paired box gene 4 promote PDX1-induced differentiation of mesenchymal stem cells into pancreatic secretory cells. Beijing Da Xue Xue Bao 2011; 43: 421-426.
[38] Hobson A, Draney C, Stratford A, Becker TC, Lu D, Arlotto M, Tessem JS. Aurora Kinase A is critical for the Nkx6.1 mediated β-cell proliferation pathway. Islets 2015; 7: e1027854.
[39] Nostro MC, Sarangi F, Yang C, Holland A, Elefanty AG, Stanley EG, Greiner DL, Keller G. Efficient generation of NKX6-1+ pancreatic progenitors from multiple human pluripotent stem cell lines. Stem Cell Reports 2015; 4: 591-604.
[40] Aguayo-Mazzucato C, Koh A, El Khattabi I, Li WC, Toschi E, Jermendy A, Juhl K, Mao K, Weir GC, Sharma A, Bonner-Weir S. MafA expression enhances glucose-responsive insulin secretion in neonatal rat beta cells. Diabetologia 2011; 54: 583-593.