

Pdx1 expression up-regulated by Tet-on system induces the mouse embryonic stem cells to pancreatic-like cells through Notch pathway

Wa Zhong

Sun Yat-Sen Memorial hospital, Sun Yat-Sen University

Yu Lai

Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University

Ying Lin

Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University

Zhong-Sheng Xia

Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University

Chu-Yan Ni

Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University

Zhong Yu

Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University

Jie-Yao Li

Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University

Tao Yu

Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University

Qi-kui Chen (✉ chenqik@mail.sysu.edu.cn)

Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University

Research

Keywords: Pdx1, Notch signaling pathway, embryonic stem cell, pancreatic-like cell, differentiation

Posted Date: May 7th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-24808/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

The specific precursor cells derived from embryonic stem cells (ESCs) after induced differentiation offers great potential for repairing damaged pancreas. Therefore, it is of crucial importance to know whether pancreatic precursor cells with Pdx1⁺ could be induced to differentiate into pancreatic-like cells in vitro as well as the probable mechanism underlying.

Methods

In this study, mouse ESCs (ES-E14TG2a) were divided into the blank control group (ESC), the blank vector group (Pdx1⁻ ESC), and the Pdx1 lentiviral vector group (Pdx1⁺ ESC). We constructed lentiviral vectors with overexpressed Pdx1 in Tet-on system and screened the stably transfected cell lines after transfection. CXCR4⁺ (C-X-C chemokine receptor type 4) DE cells of the three groups were seeded (day zero [d0]) after being sorted with immune beads, and expression of Pdx1 was induced on the second day (d1). Then, markers for the differentiation of Pdx1⁺ pancreatic precursor cells and molecules in the Notch pathway were detected at d3, d7, d10, and, d14.

Results

We found that expressions of Ptf1a, CK19, and amylase increased at d3 and d7, Neuro D1 increased at d10 and d14, Pax6 and insulin increased at d14, as well as Notch1, Notch2, Hes1, and Hes5 increased at d3 and thereafter declined at d14 in the Pdx1⁺ ESC group. These expressions were significantly higher than those in the ESC group and Pdx1⁻ ESC group, but no marked differences were observed between the ESC group and Pdx1⁻ ESC group.

Conclusions

Our study indicates that Pdx1⁺ pancreatic precursor cells can differentiate into pancreatic-like cells in which the Notch pathway plays important roles.

Background

Embryonic stem cells (ESCs) are groups of pluripotent stem cells with potent multidirectional differentiation potential and self-renewal capability and can differentiate into almost all cell types in vivo and in vitro. It has been reported that mouse ESCs can differentiate into neural stem cells, pancreatic β cells, hematopoietic stem cells, cardiomyocytes, and osteoblasts after induction in vitro. ESCs can be used for tissue repair and functional replacement therapy, which may provide a new strategy for future

treatment of some irreversible diseases such as heart failure, hereditary diseases, diabetes, and neuronal degeneration [1–3].

Acute severe pancreatitis usually has a sudden onset, is often severe, and is associated with a mortality of about 20–40% [4]. The incidence of chronic pancreatitis has also been increasing in recent years. After pancreatic injury, lifelong endocrine and exocrine replacement therapy is often needed, which significantly affects patients' quality of life. Currently, there is still controversy about post-injury repair of the pancreas. Available studies reveal that pancreatic repair depends on duct cells and B cells [5–7]. In recent years, some investigators have postulated that pancreatic stem cells or very small embryonic-like stem cells in the pancreas are involved in insulin secretion after B cell injury and in pancreatic regeneration after pancreatectomy [8, 9]. However, further studies are warranted to investigate post-injury repair in the pancreas after cell transplantation.

The number of stem cells in pancreatic tissues is small, and their proliferation and differentiation are also limited. However, the ideal and timely repair of the pancreas at structural and functional levels requires sufficient amounts of pluripotent precursor cells or similar stem cells in order to achieve post-injury regeneration of the pancreas. Thus, the repair of injured pancreatic cells with ESCs is imperative. However, ESCs have a high risk of tumorigenicity in vivo and are not suitable for tissue repair in vivo. After in vitro induction, ESCs may differentiate into specific precursor cells that can be used for tissue repair, which provides a safer and more promising strategy. Pancreatic stem cells belong to the group of undifferentiated cells; have the potential to differentiate into endocrine cells, acinar cells, and ductal cells; and can express some markers of stem cells. However, no specific markers of pancreatic stem cells have been found [10].

Studies reveal that pancreatic duodenal homeobox1 (Pdx1) is one of the pancreatic stem cell markers. In the pancreatic development of mice, early expression of Pdx1 is a marker of pre-pancreatic endoderm before the formation of pancreatic buds [11]. Pdx1 is an important transcription factor in the early development of the pancreas, β cell differentiation, and mature islet cells [12, 13]. Point mutations in the Pdx1 transactivation domain will impair human β -cell development and function [14]. Pdx1 can induce the formation of pancreatic progenitor cells, the differentiation of endodermal tissues into pancreas, and its maturation; Pdx1 activation may promote the expression of some important genes such as those for insulin, somatostatin, glucokinase, glucose transport factor, and amylin [15, 16].

Our previous study found that, the addition of Wnt3a (25 ng/mL) and Activin A (50 ng/mL) can activate the Wnt and Notch signaling pathways to cooperatively promote the differentiation of ESCs into definitive endoderm (DE) [17]. In addition, inhibition of sonic hedgehog (Shh) signaling promotes pancreatic development. We also showed that $Pdx1^+/Shh^-$ cells from $CXCR4^+$ DE derived ESCs can improve the repair of pancreatic injury in mice [18]. In this study, we controlled the Pdx1 expression in DE by Tet-On and obtained the $Pdx1^+$ pancreatic precursor cells. We also evaluated the potential and possible mechanism of $Pdx1^+$ cells to differentiate into pancreatic-like cells. The findings will provide the experimental evidence about post-injury repair of the pancreas with ESCs.

Materials And Methods

Culture of ESCs

Mouse ESCs (ES-E14TG2a) from 129/Ola mice were purchased from the American Type Culture Collection (ATCC). ESCs were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, USA) with 10% fetal bovine serum (FBS; Gibco BRL, USA), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.12% sodium bicarbonate (NaHCO_3), 0.1 mM nonessential amino acids, 0.1 mM β -mercapto-ethanol, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 1000 U/mL leukemia inhibitory factor (LIF) at 37 °C in an environment with 5% CO_2 . The medium was refreshed once daily, and cells were passaged once every 2–3 days.

Culture of embryoid body (EB)

The EB culture medium was based on ESCs culture medium except for LIF and contained an additional 25 ng/mL Wnt3a and 50 ng/mL Activin A[17].

Tet-On system

The Tet-On system was used to construct the Pdx1 over-expressing lentivirus vector with green fluorescent protein (GFP) and puromycin resistance. The Pdx1 recombinant plasmid was constructed and then transfected into 293T cells, and fluorescence microscopy was done to observe the fluorescent marker (GFP). Western blotting was done to detect Pdx1 protein expression. The recombinant lentivirus plasmid and two original vector plasmids were co-transfected into 293T cells with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. At 8 h after transfection, the medium was refreshed with complete medium, and the supernatant was harvested 48 h later. The supernatant was then concentrated to obtain the lentivirus concentrate. The viral titer was determined in 293T cells.

Transfection of ESCs with lentivirus

Cells were divided into the blank control group (ESC), the blank vector group (Pdx1^- ESC), and the Pdx1 lentiviral vector group (Pdx1^+ ESC). ESCs were seeded into 6-well plate. Sixteen h later, confluence reached 20–30%, and then transfection was done. In brief, the medium was removed, and serum-free medium (894 μL) was added. Then, 100 μL of Polybrene (concentration: 5 $\mu\text{g}/\text{mL}$) and 6 μL of viral solution (titer: $1\text{E} + 9\text{TU}/\text{mL}$) were added at a final volume of 1 mL. After 12-h transfection, the medium was refreshed with 2 mL of medium containing 10% FBS. The medium was refreshed once daily. After 48-h transfection, cells were passaged at a ratio of 1:5 when confluence reached > 80%. At 24 h after passaging, puromycin was added at a final concentration of 800 ng/mL (total volume of the medium: 2 mL). After puromycin screening for 2 days, the medium was refreshed with puromycin-free medium.

Detection of transfection efficiency by flow cytometry and construction of ESCs with stable Pdx1 expression and negative control ESCs

Cells in the Pdx1⁻ ESC group and the Pdx1⁺ ESC group were screened with 800 ng/mL puromycin for 2 days and induced with 2 µg/mL doxycycline (DOX). Then, cells were harvested from the ESC group, the Pdx1⁻ ESC group, and the Pdx1⁺ ESC group, and washed in phosphate-buffered saline (PBS) once. 1.0×10^6 cells were resuspended with 200 µL of PBS and then loaded for the detection of transfection efficiency by flow cytometry. The other cells were resuspended with PBS at a density of 1×10^7 cells/mL. Flow cytometry was used to sort GFP⁺ cells in the Pdx1⁻ ESC group and the Pdx1⁺ ESC group, and cells in the ESC group served as controls. After sorting, positive cells were seeded into 6-well plates, followed by incubation at 37 °C in an environment with 5% CO₂ (d0). On the second day (d1), puromycin was added at a final concentration of 800 ng/mL. On day 3 (d3), puromycin-resistant colonies could be observed. Cells surrounding the colonies were digested with trypsin, and the cell colonies were collected into 96-well plates for further culture. Twenty-four later, puromycin was added at a final concentration of 400 ng/mL to maintain the colonies. Then, cells were sequentially transferred into 24-well plates, 12-well plates, and 6-well plates for culture and expansion, and cell lines with stable transfection were harvested.

Observation of Pdx1 gene expression under fluorescence microscope

Cells of the three groups were seeded into 6-well plates (2 wells/group). In one well for each group, DOX was added at a final concentration of 2 µg/mL 24 h later. Cells were then observed under the fluorescence microscope.

Cell cycle and apoptosis of ESCs before and after transfection

Cells of the three groups were seeded into 6-well plates. Cells were cultured with ESC medium followed by incubation at 37 °C in an environment with 5% CO₂. They were harvested and washed in PBS 48 h later. Cells were resuspended in 2 mL of PBS. In total, $2-5 \times 10^5$ cells were resuspended in 400 µL of binding buffer containing Ca²⁺ after centrifugation. The cell suspension was divided into two tubes (200 µL of suspension in each tube). In one tube, cells were not treated and served as blank controls; in the other tube, FITC-conjugated Annexin V (5 µL) was added, followed by incubation at room temperature for 15 min in the dark. Then, 10 µL of propidium iodide (PI) was added, and cells were subjected to flow cytometry.

Cells were collected and single-cell suspensions were prepared with 200 µL of PBS after centrifugation. Cells were then fixed in 2 mL of 75% ice-cold ethanol (absolute ethanol: water = 7:3; - 20 °C) at 4 °C overnight. After washing in PBS once, cells were treated with 50 µL of RNase and 450 µL of PI staining solution, followed by detection of cell cycles in 4 h.

Sorting of CXCR4⁺ definitive endodermal cells with immune beads

Cells of the three groups were maintained for 5 days until a high proportion of DE cells at the EB stage was present after differentiation. Cells were collected and resuspended in PBS after centrifugation. Cells were then treated with phycoerythrin (PE)-conjugated anti-mouse CXCR4 monoclonal antibody (10 $\mu\text{L}/1.0 \times 10^6$ cells) at 4 °C for 20 min. After washing in running buffer twice, cells were resuspended in running buffer (80 $\mu\text{L}/1 \times 10^7$ cells), and anti-PE immune beads (Miltenyi Biotec) were added (20 $\mu\text{L}/1 \times 10^7$ cells), followed by incubation at 4 °C for 15 min. After washing in running buffer twice and centrifuging, cells were resuspended in running buffer at a density of 1×10^7 cells/mL. Cells were then subjected to flow cytometry (FACSVerse) for sorting CXCR4⁺ cells. The data were collected with EXPO32 MultiCOMP *ver.* 1.1C software, and analyzed with EXPO32 analysis *ver.* 1.2B software.

Differentiation of CXCR4⁺ DE cells into pancreatic-like cells

After sorting, CXCR4⁺ DE cells in the ESC group, the Pdx1⁻ ESC group, and the Pdx1⁺ ESC group were seeded into 6-well plates ($0.5\text{--}1.0 \times 10^5$ cells/well) on day 0. On the second day (d1), DOX was added at the final concentration of 2 $\mu\text{g}/\text{mL}$. The Tet-On system was used to initiate Pdx1 expression in DE cells. Cells in the three groups were cultured for 4 (d3), 8 (d7), 11 (d10), and 15 (d14) days.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from samples was extracted using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA), and the concentration was calculated from the absorbance at 260 nm as determined using an ND-2000 instrument (NanoDrop Technologies, Wilmington, DE, USA), followed by reverse transcription with PrimeScript™ Master Mix (TaKaRa Bio, Kusatsu, Shiga, Japan). RT-qPCR was performed on a CFX Connect™ real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using SYBR® Premix Ex Taq™ (TaKaRa Bio, Kusatsu, Shiga, Japan). The primers are shown in Table 1–3. Data were analyzed using the $\Delta\Delta\text{Ct}$ method with β -actin as an internal control. The RT-qPCR experiments were performed in sextuplicate using independent samples.

Table 1
Primer for Mouse PDX1:

Gene	Primer
mouse Pdx1	Forwar: 5'-GGCAGGAGGTGCTTACACAG-3' Reverse: 5'-GGCCGGGAGATGTATTTGTT-3'
mouse 18sRNA	Forward: 5'-GCTAGGAATAATGGAATAGG-3' Reverse: 5'-ACTTTCGTTCTTGAGGAATG-3'

Table 2
Primer for Mouse CK19, Ptf1a, Pax6, Neuro D1, Insuline, Amylase

Gene	Primer
Mouse CK19	Forward: 5'-AGGTCAGTGTGGAGGTGGA - 3'
	Reverse: 5'-CTCAATCCGAGCAAGGTAGG - 3'
Mouse Ptf1a	Forward: 5'-AAACGGGCAGTAACCCTCTT - 3'
	Reverse: 5'-TCAGCAACAGCAGCAGAAGT - 3'
Mouse Pax6	Forward: 5'-ATCGGAGGGAGTAAGCCAAG - 3'
	Reverse: 5'-TAGCCAGGTTGCGAAGAACT - 3'
Mouse Neuro D1	Forward: 5'-TTGTCCCAGCCCCTACCAATTTG - 3'
	Reverse: 5'-TCGGCGGATGGTTCGTGTTTG - 3'
Mouse Insuline	Forward: 5'-CGGAGACCCACAAAGAGAGA - 3'
	Reverse: 5'-GGAGTCACAGCGAGAAGACC - 3'
Mouse Amylase	Forward: 5'-ACTGCCAACAGCATAGCAAA - 3'
	Reverse: 5'-TCAAACAGGTGGACAATAGCA - 3'

Gene	Primer
Mouse 18srRNA	Forward: 5'-GCTAGGAATAATGGAATAGG-3' Reverse: 5'-ACTTTCGTTCTTGAGGAATG-3'

Table 3
Primer for Mouse Notch1,Notch2,Hes1,Hes5

Gene	Primer
Mouse Notch1	Forward: 5'-CGTGGATGAGTGTGCTCTG - 3' Reverse: 5'-TCCCGTGTAGCCCTGTAGAC-3'
Mouse Notch2	Forward: 5'-GACTGGCGACTTCACTTTCG - 3' Reverse: 5'-CATCCACACAAACTCCTCCA-3'
Mouse Hes1	Forward: 5'-GTGGGTCCTAACGCAGTGTC - 3' Reverse: 5'-TCAGAAGAGAGAGGTGGGCTA - 3'
Mouse Hes5	Forward: 5'-TCCTCTGGATGTGGGAAGAC - 3' Reverse: 5'-CTAAGAATGACCCTCCTGCTG - 3'
Mouse 18srRNA	Forward: 5'-GCTAGGAATAATGGAATAGG-3' Reverse: 5'-ACTTTCGTTCTTGAGGAATG-3'

Western blotting

The samples were incubated in RIPA buffer (Thermo Scientific, Carlsbad, CA, USA). And the protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (CW Biotech, China). Protein separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred onto poly-vinylidene difluoride (PVDF) membranes. The membranes incubated with a 1:1000 dilution of Pdx1, CK19, Ptf1a, Pax6, Neuro D1, insulin, amylase, Notch1, Notch2, Hes1, or Hes5 (Abcam, Cambridge, UK) primary antibody at 4 °C overnight. After washing in TBST (tris-buffered saline with Tween 20), the membranes incubated with a 1:1000 dilution of secondary antibody, at room temperature, for one hour. The data were analyzed using relative intensity with β -actin as the internal control. The Western blot experiments were performed in three repetitions using independent samples.

Statistical analysis

Statistical analysis was performed with SPSS version 17.0. Quantitative data are expressed as mean \pm standard deviation (mean \pm SD). Comparisons were done using Student's *t* test between groups and analysis of variance among groups, followed by Student's *t* test with Bonferroni correction for comparisons of means between two groups. A value of $p < 0.05$ was considered statistically significant.

Results

The transfection efficiency of lentiviral was determined and the positive cells were selected.

The transfection efficiency of the cells in mock-lentiviral vector group (Pdx1⁻ ESC group) was 90.7% (Fig. 1A) and that in Pdx1⁺ lentivirus transfection group (Pdx1⁺ ESC group) was 94.0% (Fig. 1B). The transfection efficiency of the cells in Pdx1⁻ ESC group was 97.8% (Fig. 1C) and Pdx1⁺ ESC group was 98.13% (Fig. 1D) after selected by flow cytometry.

Expression of Green fluorescent protein was induced by activating the Tet-on system.

Induction was done with 2 μ g/mL DOX. In the ESC group, green fluorescence was not observed, but cells with green fluorescence were observed in the Pdx1⁻ ESC group and the Pdx1⁺ ESC group. In the absence of treatment with DOX, cells with green fluorescence were not observed in any of the three groups (Fig. 2A).

Expression of Pdx1 transfection gene was induced by activating the Tet-on system.

After DOX treatment, the mRNA and protein expressions of Pdx1 were not comparable between the ESC group and the Pdx1⁻ ESC group ($p > 0.05$, Fig. 2B-D). However, that increased significantly in the Pdx1⁺ ESC group compared with the ESC group and the Pdx1⁻ ESC group ($p < 0.05$, Fig. 2B-D), and not only the Pdx1 protein at 46 kDa but also the Pdx1 fused protein at 75 kDa were observed (Fig. 2B). In the absence of DOX, the mRNA and protein expressions of Pdx1 were not comparable among the three groups ($p > 0.05$, Fig. 2B-D).

There was no significant change in cycles and apoptosis of the ESC after virus transfection

The G0/G1 rate of cell cycles compared with 37.714 ± 3.955 , 37.332 ± 4.262 and 39.103 ± 5.294 , the S rate of cell cycles compared with 54.331 ± 7.065 , 54.359 ± 5.954 and 52.314 ± 7.315 and the G2/M rate of cell cycles compared with 7.947 ± 1.080 , 8.309 ± 1.304 , and 8.583 ± 1.117 for the ESC group, the Pdx1⁻ ESC group and the Pdx1 + ESC group respectively (Fig. 3A-G). The cell apoptosis of the ESC group, the Pdx1⁻ ESC group, and the Pdx1 + ESC group were 3.167 ± 0.314 , 3.050 ± 0.388 and 3.244 ± 0.420 respectively (Fig. 3H-J). There were no marked differences in the cell cycles and apoptosis among the three groups ($p > 0.05$).

The molecular marker were expressed in the precursor cells with Pdx1⁺ to differentiate into pancreatic-like cells.

In the Pdx1⁺ ESC group, the mRNA and protein expressions of Ptf1a increased at d7 (Fig. 4A,G,H); the mRNA and protein expressions of CK19 and amylase increased at d3 and d7 (Fig. 4B,E,G,I,L); the mRNA and protein expressions of Pax6 and insulin increased at d14 (Fig. 4C,F,G,J,M); the mRNA and protein expressions of Neuro D1 increased at d10 and d14 ($p < 0.05$ vs the ESC group and the Pdx1⁻ ESC group, Fig. 4D,G,K). There were no marked differences in the expressions of these mRNA and proteins between the ESC group and the Pdx1⁻ ESC group ($p > 0.05$ Fig. 4A-M).

The molecular marker of Notch signaling pathway were expressed in the precursor cells with Pdx1⁺ to differentiate into pancreatic-like cells.

In the Pdx1⁺ ESC group, the mRNA and protein expression of Notch1, Notch2, Hes1, and Hes5 increased at d3, thereafter declined gradually, and reached the lowest level at d14 ($p < 0.05$ vs the ESC group and Pdx1⁻ ESC group, Fig. 5A-I), but there was no marked difference in the expression of these mRNA and proteins between the ESC group and the Pdx1⁻ ESC group ($p > 0.05$, Fig. 5A-I).

Discussion

ESCs in the pluripotency state can differentiate into the ectoderm, mesoderm, and endoderm, which are the sources of all cell types. The endoderm may differentiate into DE and visceral endoderm. During embryonic development, DE may form a primitive gastrointestinal tract and relevant organs such as digestive organs, including liver and pancreas [19, 20]. On the eighth day of embryonic development in mice, tissues with high expression of pancreas-specific transcription factor 1 α (Ptf1 α) and Pdx1 and low Shh expression will develop into pancreatic tissues [21]. Gu et al. [22] found that, during the formation of embryonic pancreas (days 9.5–11.5), Pdx1 was extensively expressed in immature pancreatic tissues of mice, including endocrine cells, acinar cells, and ductal cells, which suggests that pancreatic parenchymal cells, including ductal cells, are derived from Pdx1⁺ cells. In early embryonic development,

Pdx1 knockout may cause pancreatic dysplasia in mice, and the pancreas may have an abnormal structure and diffused B cells and A cells although the pancreatic germ still forms [23]. In late embryonic development, Pdx1 knockout may affect the maturation of β cells, causing impaired glucose tolerance and an increase in blood glucose [22]. Thus, Pdx1⁺ cells can be regarded as pancreatic precursor cells. Pdx1 expression in DE may determine the differentiation of DE into pancreatic cells. Thus, up-regulating Pdx1 expression in DE may be helpful for the collection of greater numbers of pancreatic-like cells for transplantation.

Gossen et al constructed the Tet-Off gene expression system in 1992 and formally established the Tet-On gene expression system in 1995 [24, 25]. In the Tet-On gene expression system, the expression of an exogenous gene increases with the increase in tetracycline or DOX and the system is responsive only to tetracycline or DOX. So the exogenous gene can be expressed quantitatively and regularly. In this study, we constructed a single-plasmid Tet-On regulatory system with Pdx1 and GFP expression and puromycin resistance. From this results, we can see that the Tet-On system we built was regulated by doxycycline and worked well(Fig. 2). Memon B et al enhanced the proportion of pancreatic progenitors co-expressing PDX1 and NKX6.1 by dissociating densely formed endodermal cells and re-plating them at different densities [26]. However in our study, under normal conditions, DOX can regulate the quantity of Pdx1 at a predesigned time point, which provides a basis to investigate the differentiation of Pdx1⁺ cells into pancreatic cells. We are also concerned about whether Pdx1 transfection affects the growth and proliferation of ESCs. Thus, after construction of cells with stable transfection, we used flow cytometry to detect the cell cycle and apoptosis of ESCs. The results confirmed that Pdx1 transfection had little influence on the cell cycle and apoptosis of ESCs(Fig. 3).

Our previous study found that ESCs can be induced to differentiate into DE with expression of Gsc, Tm4sf2, Gpc1, Sdc4, and CXCR4 (markers of DE), and the proportion of DE peaked at 5 days after induction of differentiation [27]. So in this study, the CXCR4⁺ cells were isolated from stable transfected ESC by using immunomagnetic beads (IMBs) at the 5th day of differentiation. Previous research found that tissues with high Ptf1 α expression may differentiate into pancreatic acinar cells in pancreatic development[28]. CK19 is a marker of pancreatic exocrine cells and is expressed mainly in the duct [29]. Neuro D and Pax6 are involved in the regulation of differentiation and maturation of pancreatic endocrine cells [30], and gene mutations of both are related to the pathogenesis of diabetes mellitus [31, 32]. Our results showed that ESCs with Pdx1 transfection differentiated into DE and, after induction of Pdx1 expression, further differentiated into cells with increased expression of markers of exocrine and ductal cells (CK19, Ptf1 α , and amylase) and endocrine cells (Neuro D1, Pax6, and insulin). Moreover, the expressions of markers of exocrine cells and ductal cells peaked at d3 and d7, and those of endocrine cells peaked at d10 and d14(Fig. 4). Pdx1 is not only an important marker of pancreatic precursor cells, but it is also a crucial transcription factor in the differentiation of pancreatic cells. Our study indicates that Pdx1 can activate downstream target genes to induce differentiation into pancreatic cells. In recent years, ESCs have been induced to differentiate into insulin-secreting cells and islet-like cells [33, 34] in vitro. Our results revealed that pancreatic precursor cells (Pdx1⁺ cells) can differentiate into several types

of pancreatic-like cells: exocrine cells and ductal cells during the early stage and endocrine cells at a later stage.

In the differentiation of Pdx1⁺ cells into different pancreatic-like cells, Ptf1 α , functioning as a DNA-binding molecule, may promote the expression of acinar-cell digestive enzymes such as amylase and protease. Activation of the Notch pathway and up-regulation of Ptf1 α expression may induce the differentiation of Pdx1⁺ cells into acinar cells [35]. Inhibition of the Notch pathway may induce the differentiation of Pdx1⁺ cells into pancreatic endocrine cells [36]. Swales et al [35] found that neurogenin 3 (Ngn3) can inhibit the Notch signaling pathway to regulate the differentiation of pancreatic ductal cells into pancreatic endocrine cells. These findings suggest that the Notch pathway plays important roles in the differentiation of Pdx1⁺ cells into different pancreatic cells. Available studies focus mainly on the Notch pathway in the differentiation of Pdx1⁺ cells into exocrine cells or endocrine cells alone. But, how the Notch pathway changes in Pdx1⁺ cells differentiating into exocrine and endocrine cells is unknown. Our results showed that the mRNA and protein expression of Notch1, Notch2, Hes1, and Hes5 increased at early differentiation, thereafter declined gradually later in the Pdx1⁺ ESC group. So the results indicated that, in the differentiation of DE into pancreatic cells, the Notch pathway was first activated and thereafter inhibited (Fig. 5). Findings also revealed that the Notch pathway can regulate the differentiation of Pdx1⁺ pancreatic precursor cells into pancreatic-like cells, which is consistent with previous results. However, the specific target gene that is regulated by Pdx1 to affect the Notch pathway is still unclear, and more studies are needed to elucidate it.

Conclusion

Altogether, our study indicates that Pdx1⁺ pancreatic precursor cells can differentiate into pancreatic-like cells in which the Notch pathway plays important roles. However, the mechanism by which the Pdx1 gene affects the Notch pathway is still unknown, and further investigations are needed.

Abbreviations

Embryonic stem cells (ESCs), pancreatic duodenal homeobox1 (Pdx1), definitive endoderm (DE), sonic hedgehog (Shh), leukemia inhibitory factor (LIF), embryoid body (EB), green fluorescent protein (GFP), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), poly-vinylidene difluoride (PVDF) membranes, pancreas-specific transcription factor 1 α (Ptf1 α), immunomagnetic beads (IMBs), neurogenin 3 (Ngn3).

Declarations

Ethical Approval and Consent to participate Not applicable. Consent for publication All authors are consent to publish the manuscript. Availability of supporting data All data showed in this manuscript was available. The data supporting our findings can be found in the tables and figures in this manuscript. If

readers want the details, can send an email for us to get. Competing interests The authors declare that they have no competing interests. All authors read and approved the final manuscript. All informed consent written by participants have been obtained. Funding The fund for study design and data collection, data analysis were supported by the National Natural Science Foundation of China (No.81270442 and No. 81370475), the Science and Technology and Social Development Project of Guangdong Province (No. 2012B031800030) and the Natural Science Foundation of Guangdong Province (2017A030313600) were funding in interpretation of data and in writing the manuscript. Authors' contributions Wa Zhong and Yu Lai were in charge of study design, statistical analysis and manuscript preparation; Ying Lin and Zhong-Sheng Xia were responsible for data collection and statistical analysis; Chu-Yan Ni, Zhong Yu, Jie-Yao Li were responsible for data interpretation and literature search; Tao Yu and Qi-Kui Chen took charge for study design and funds collection. All authors read and approved the final manuscript. Acknowledgements The study was technically supported by grants from the Key Laboratory of Malignant Tumor Molecular Mechanism and Translational Medicine of Guangzhou Bureau of Science and Information Technology (Grant [2013]163) and the Key Laboratory of Malignant Tumor Gene Regulation and Target Therapy of Guangdong Higher Education Institutes (Grant KLB09001).

References

1. Dai W, Kay GL, Kloner RA. The Therapeutic Effect of Cell Transplantation Versus Noncellular Biomaterial Implantation on Cardiac Structure and Function Following Myocardial Infarction. *J Cardiovasc Pharmacol Therap.* 2014;19(4):350–7.
2. Fan X, Sun D, Tang X, Cai Y, Yin ZQ, Xu H. Stem-cell challenges in the treatment of Alzheimer's disease: a long way from bench to bedside. *Medicinal research reviews.* 2014;34(5):957–78.
3. Muir KR, Lima MJ, Docherty HM, Docherty K. Cell therapy for type 1 diabetes. *QJM: monthly journal of the Association of Physicians.* 2014;107(4):253–9.
4. Wu BU, Hwang JQ, Gardner TH, Repas K, Delee R, Yu S, et al. Lactated Ringer's solution reduces systemic inflammation compared with saline in patients with acute pancreatitis. *Clinical gastroenterology hepatology: the official clinical practice journal of the American Gastroenterological Association.* 2011;9(8):710-7.e1.
5. Rafaeloff R, Qin XF, Barlow SW, Rosenberg L, Vinik AI. Identification of differentially expressed genes induced in pancreatic islet neogenesis. *FEBS Lett.* 1996;378(3):219–23.
6. Zhao Y, Yu Q, Li Z, Qin G. Transdifferentiation of mouse pancreatic ductal epithelial cells into islet-like cells. *Journal of Clinical Rehabilitative Tissue Engineering Research.* 2010;32(14):6064–7.
7. Dor Y, Melton DA. Facultative endocrine progenitor cells in the adult pancreas. *Cell.* 2008;132(2):183–4.
8. Banakh I, Gonez LJ, Sutherland RM, Naselli G, Harrison LC. Adult pancreas side population cells expand after beta cell injury and are a source of insulin-secreting cells. *PloS one.* 2012;7(11):e48977.

9. Bhartiya D, Mundekar A, Mahale V, Patel H. Very small embryonic-like stem cells are involved in regeneration of mouse pancreas post-pancreatectomy. *Stem Cell Res Ther.* 2014;5(5):106.
10. Li J, Zhu L, Qu X, Li J, Lin R, Liao L, et al. Stepwise differentiation of human adipose-derived mesenchymal stem cells toward definitive endoderm and pancreatic progenitor cells by mimicking pancreatic development in vivo. *Stem Cells Dev.* 2013;22(10):1576–87.
11. Offield MF, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA, et al. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development.* 1996;122(3):983–95.
12. Babu DA, Deering TG, Mirmira RG. A feat of metabolic proportions: Pdx1 orchestrates islet development and function in the maintenance of glucose homeostasis. *Mol Genet Metab.* 2007;92(1–2):43–55.
13. Bastidas-Ponce A, Roscioni SS, Burtscher I, Bader E, Sterr M, Bakhti M, et al. Foxa2 and Pdx1 cooperatively regulate postnatal maturation of pancreatic beta-cells. *Molecular metabolism.* 2017;6(6):524–34.
14. Wang X, Sterr M, Ansarullah, Burtscher I, Böttcher A, Beckenbauer J, et al. Point mutations in the PDX1 transactivation domain impair human β -cell development and function. *Mol Metab.* 2019 Jun;24:80–97.
15. Hori Y. Insulin-producing cells derived from stem/progenitor cells: therapeutic implications for diabetes mellitus. *Med Mol Morphol.* 2009;42(4):195–200.
16. Balaji S, Zhou Y, Opara EC, Soker S. Combinations of Activin A or Nicotinamide with the Pancreatic Transcription Factor PDX1 Support Differentiation of Human Amnion Epithelial Cells Toward a Pancreatic Lineage. *Cellular reprogramming.* 2017;19(4):255–62.
17. Wa Zhong Yu, Lai T, Xia YZhong-Sheng, Yuan Y-H, Ouyang H, Shan T-D. Qi-Kui Chen. Wnt and Nodal signaling simultaneously induces definitive endoderm differentiation of mouse embryonic stem cells. *Rom J Morphol Embryol.* 2017;58(2):527–35.
18. Yu T, Qing Q, Deng N, Min XH, Zhao LN, Li JY, et al. CXCR4 positive cell-derived Pdx1-high/Shh-low cells originated from embryonic stem cells improve the repair of pancreatic injury in mice. *Cell Biol Int.* 2015;39(9):995–1006.
19. Lu CC, Brennan J, Robertson EJ. From fertilization to gastrulation: axis formation in the mouse embryo. *Curr Opin Genet Dev.* 2001;11(4):384–92.
20. Robb L, Tam PP. Gastrula organiser and embryonic patterning in the mouse. *Semin Cell Dev Biol.* 2004;15(5):543–54.
21. Lammert E, Cleaver O, Melton D. Induction of pancreatic differentiation by signals from blood vessels. *Science.* 2001;294(5542):564–7.
22. Gu G, Brown JR, Melton DA. Direct lineage tracing reveals the ontogeny of pancreatic cell fates during mouse embryogenesis. *Mechanisms of development.* 2003;120(1):35–43.

23. Yoshida T, Murata K, Shiraki N, Kume K, Kume S. Analysis of gene expressions of embryonic stem-derived Pdx1-expressing cells: implications of genes involved in pancreas differentiation. *Development growth differentiation*. 2009;51(4):463–72.
24. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA*. 1992;89(12):5547–51.
25. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science*. 1995;268(5218):1766–9.
26. Memon B, Karam M, Al-Khawaga S, Abdelalim EM. Enhanced differentiation of human pluripotent stem cells into pancreatic progenitors co-expressing PDX1 and NKX6.1. *Stem Cell Res Ther*. 2018 Jan 23;9(1):15.
27. Yu T, Lan SY, Wu B, Pan QH, Shi L, Huang KH, et al. Musashi1 and hairy and enhancer of split 1 high expression cells derived from embryonic stem cells enhance the repair of small-intestinal injury in the mouse. *Digestive diseases sciences*. 2011;56(5):1354–68.
28. Zhou Q, Law AC, Rajagopal J, Anderson WJ, Gray PA, Melton DA. A multipotent progenitor domain guides pancreatic organogenesis. *Developmental cell*. 2007;13(1):103–14.
29. Li R, Yu L, Zhang X, Zhou X, Wang M, Zhao H. Distribution of islet hormones in human adult pancreatic ducts. *Digestion*. 2015;91(2):174–9.
30. Pan FC, Wright C. Pancreas organogenesis: from bud to plexus to gland. *Developmental dynamics: an official publication of the American Association of Anatomists*. 2011;240(3):530–65.
31. Malecki MT, Jhala US, Antonellis A, Fields L, Doria A, Orban T, et al. Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. *Nat Genet*. 1999;23(3):323–8.
32. Yasuda T, Kajimoto Y, Fujitani Y, Watada H, Yamamoto S, Watarai T, et al. PAX6 mutation as a genetic factor common to aniridia and glucose intolerance. *Diabetes*. 2002;51(1):224–30.
33. Bose B, Sudheer PS. In Vitro Differentiation of Pluripotent Stem Cells into Functional beta Islets Under 2D and 3D Culture Conditions and In Vivo Preclinical Validation of 3D Islets. *Methods in molecular biology (Clifton NJ)*. 2016;1341:257–84.
34. Shim JH, Kim J, Han J, An SY, Jang YJ, Son J, et al. Pancreatic Islet-Like Three-Dimensional Aggregates Derived From Human Embryonic Stem Cells Ameliorate Hyperglycemia in Streptozotocin-Induced Diabetic Mice. *Cell Transplant*. 2015;24(10):2155–68.
35. Swales N, Martens GA, Bonne S, Heremans Y, Borup R, Van de Casteele M, et al. Plasticity of adult human pancreatic duct cells by neurogenin3-mediated reprogramming. *PloS one*. 2012;7(5):e37055.
36. Schneider KJ, Scheer M, Suhr M, Clemens DL. Ethanol administration impairs pancreatic repair after injury. *Pancreas*. 2012;41(8):1272–9.

Figures

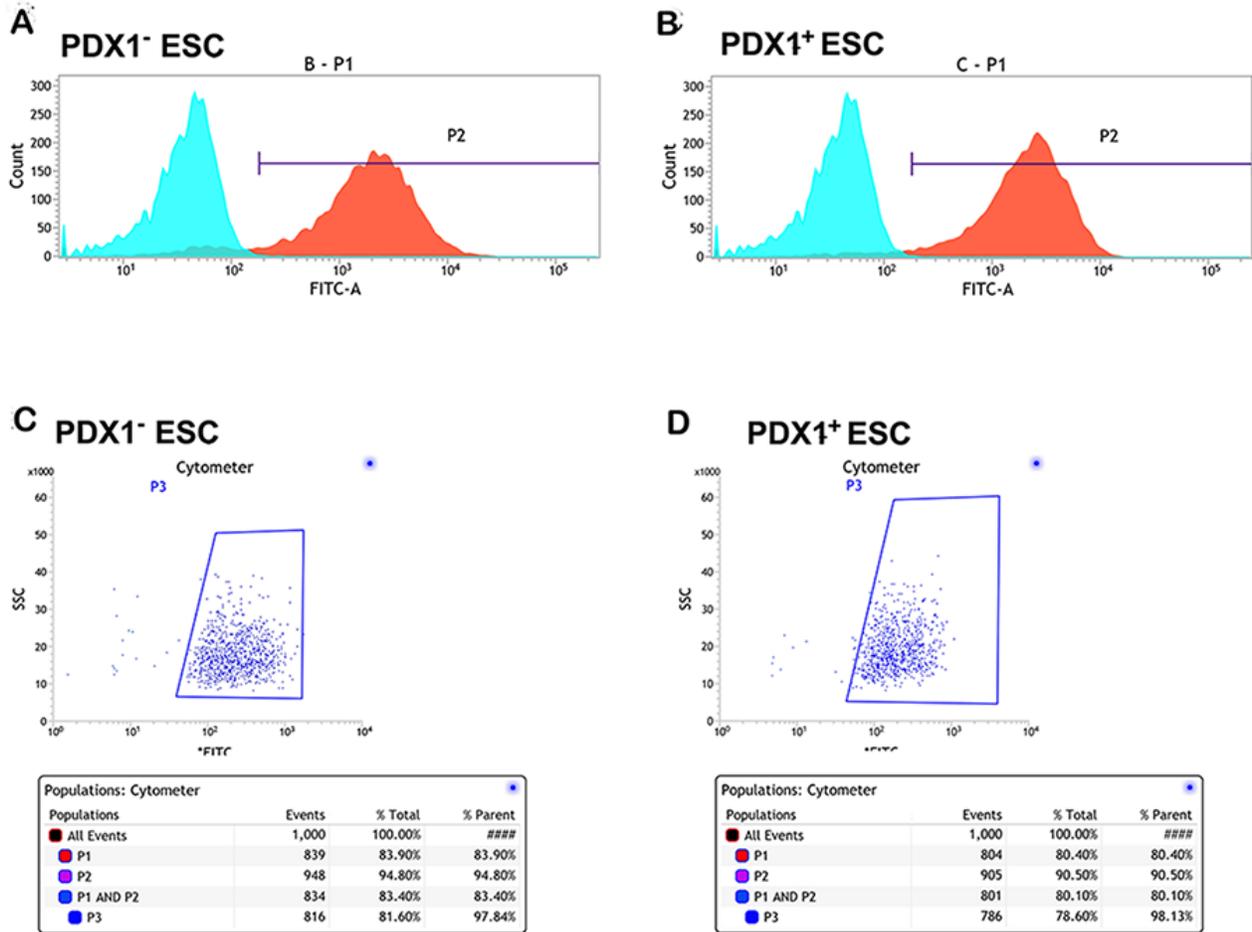


Figure 1

The transfection efficiency of lentiviral was determined by flow cytometry. The blue wave crest represents negative control cells, and red wave crest represents positive transfection cells (A, B) PDX1- ESC group (A), PDX1+ESC group (B). The positive cells were selected and exhibited in blue frame by flow cytometry (C, D), PDX1- ESC group (C), PDX1+ESC group (D).

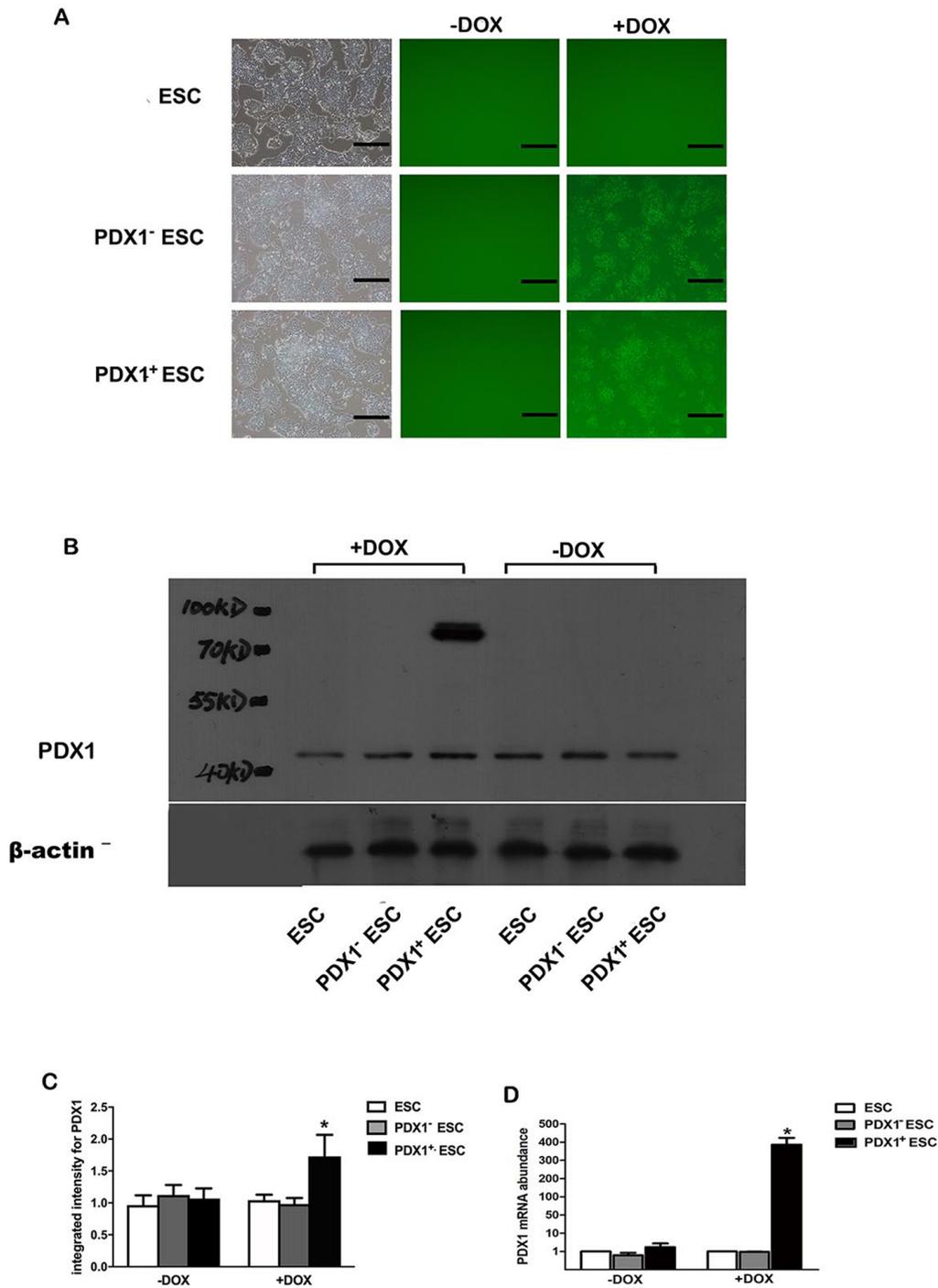


Figure 2

Expression of Green fluorescent protein and Pdx1 transfection gene was induced by activating the Tet-on system. Green fluorescence hasn't been observed in the three groups without DOX, including ESC group, PDX1- ESC group and PDX+ ESC group. Green fluorescence hasn't been observed in ESC group, but can be observed in PDX1- ESC group and PDX+ ESC group with DOX. Scale Bar=50 μ m(A). There were no differences in mRNA and protein expression of PDX1 without DOX among the cells in the three groups .

However, there are significantly increased in the cells of PDX1+ESC groups with DOX . The protein expression of PDX1 was observed in 46KD and the fusion protein expression of PDX1 also can be observed in 75KD in PDX1+ESC group by Western blot (B) and quantification of immunoblot bands from Western blot (C) and qRT-PCR (D); mRNA and protein levels are expressed relative to β -actin $\ast P < 0.05$.

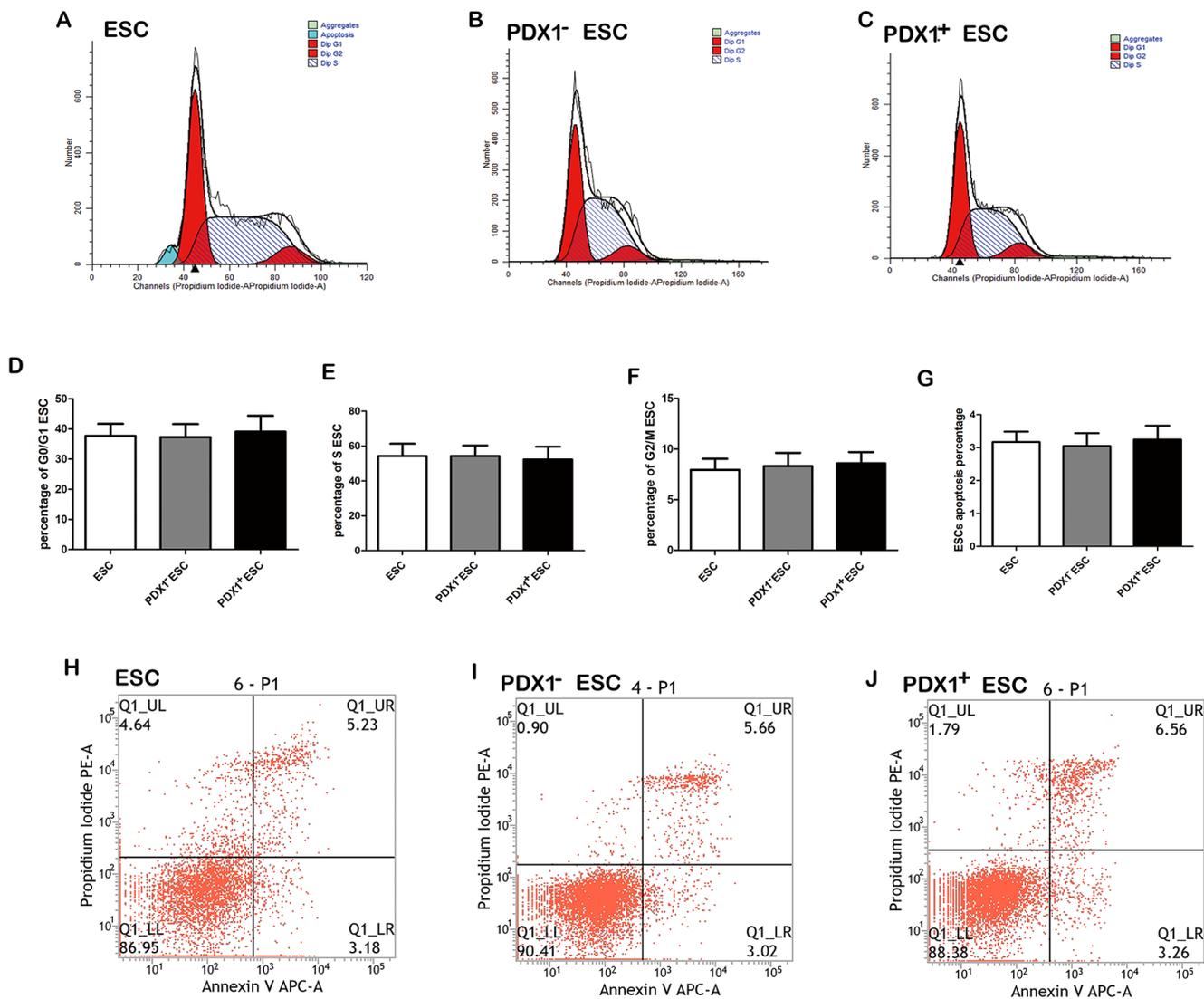


Figure 3

The cell cycle and apoptosis of cells in three groups were determined by flow cytometry after cultured 2 days. The flow cytometry graph of cells cycle(A-C). The first red wave stands for G1 phase of cell cycle, the second red wave for G2 phase and the blue wave for S phase. ESC group(A), PDX1- ESC group(B) and PDX+ ESC group(C). The histogram of proportions of different cell cycle phase(D-F). The histogram of apoptosis proportions (G). The flow cytometry graph of apoptosis(H-J). ESC group (H), PDX1-ESC group (I) and PDX1+ESC group (J). There were no any changes of the cell cycle and apoptosis in the cells in three groups before the expression of Pdx1 gene after the transfection ($P > 0.05$).

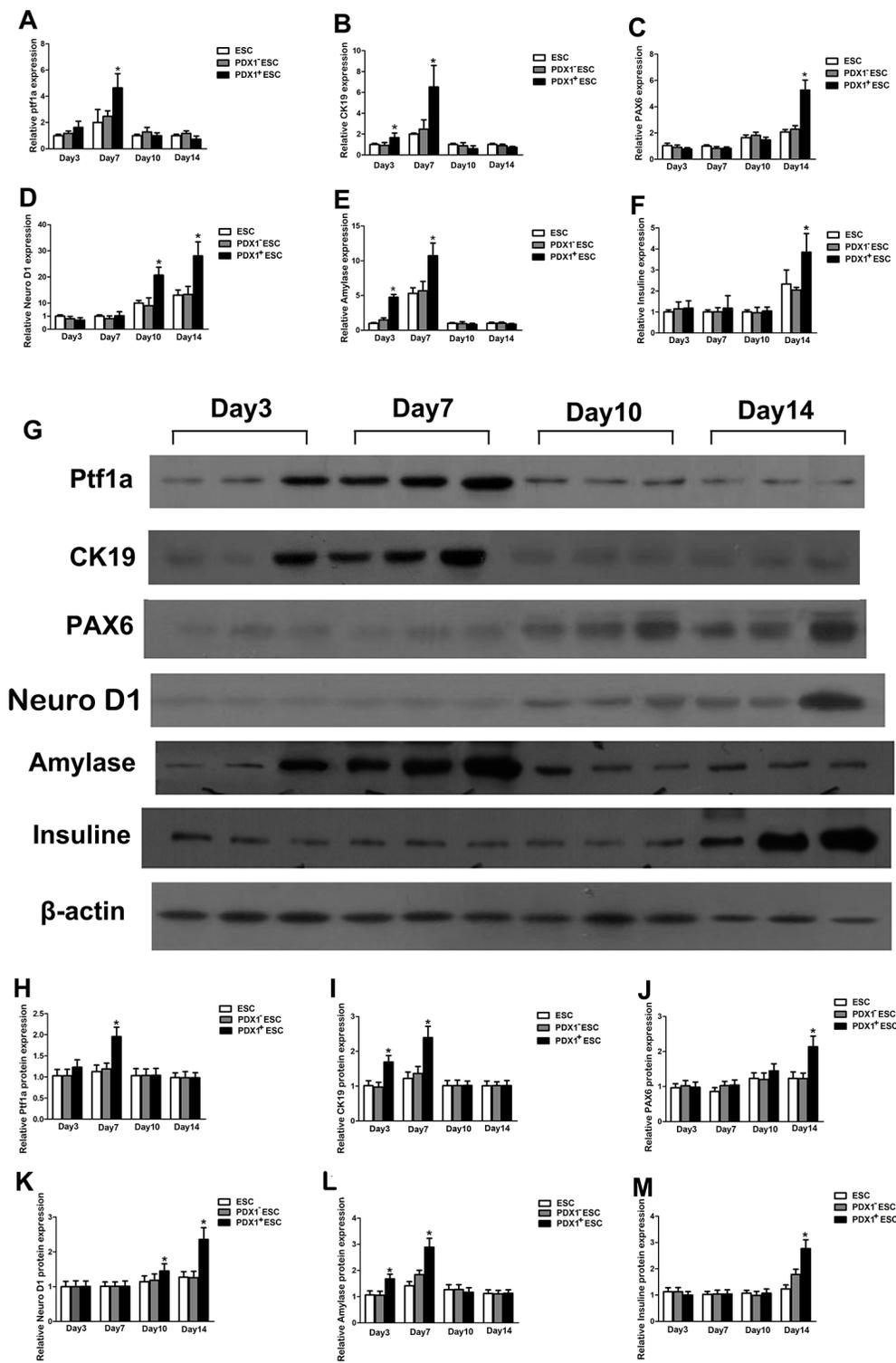


Figure 4

The mRNA and protein expression of Ptf1a, CK19, Pax6, Neuro D1, Amylase and Insulin were observed in ESC group, PDX1-ESC group and PDX1+ESC group by Western blot (G) and quantification of immunoblot bands from Western blot (H-M) and qRT-PCR (A-F). The mRNA and protein expressions of Ptf1a were higher in the cells in PDX1+ESC group than those in ESC group and PDX1-ESC group at d3 (A, G, H), and the expression of CK19 and Amylase were higher in the cells in PDX1+ESC group than those in ESC group

and PDX1-ESC group from d3 to d7 (B,E G, I, L). The mRNA and protein expressions of Pax6 and Insulin were higher in the cells in PDX1+ESC group than those in ESC group and PDX1-ESC group at d14 and the expressions of Neuro D1 was higher in the cells in PDX1+ESC group than those in ESC group and PDX1-ESC group from d10 to d14 . (C, D,F, G, J,K,M). The mRNA and protein levels are expressed relative to β -actin, *P<0.05.

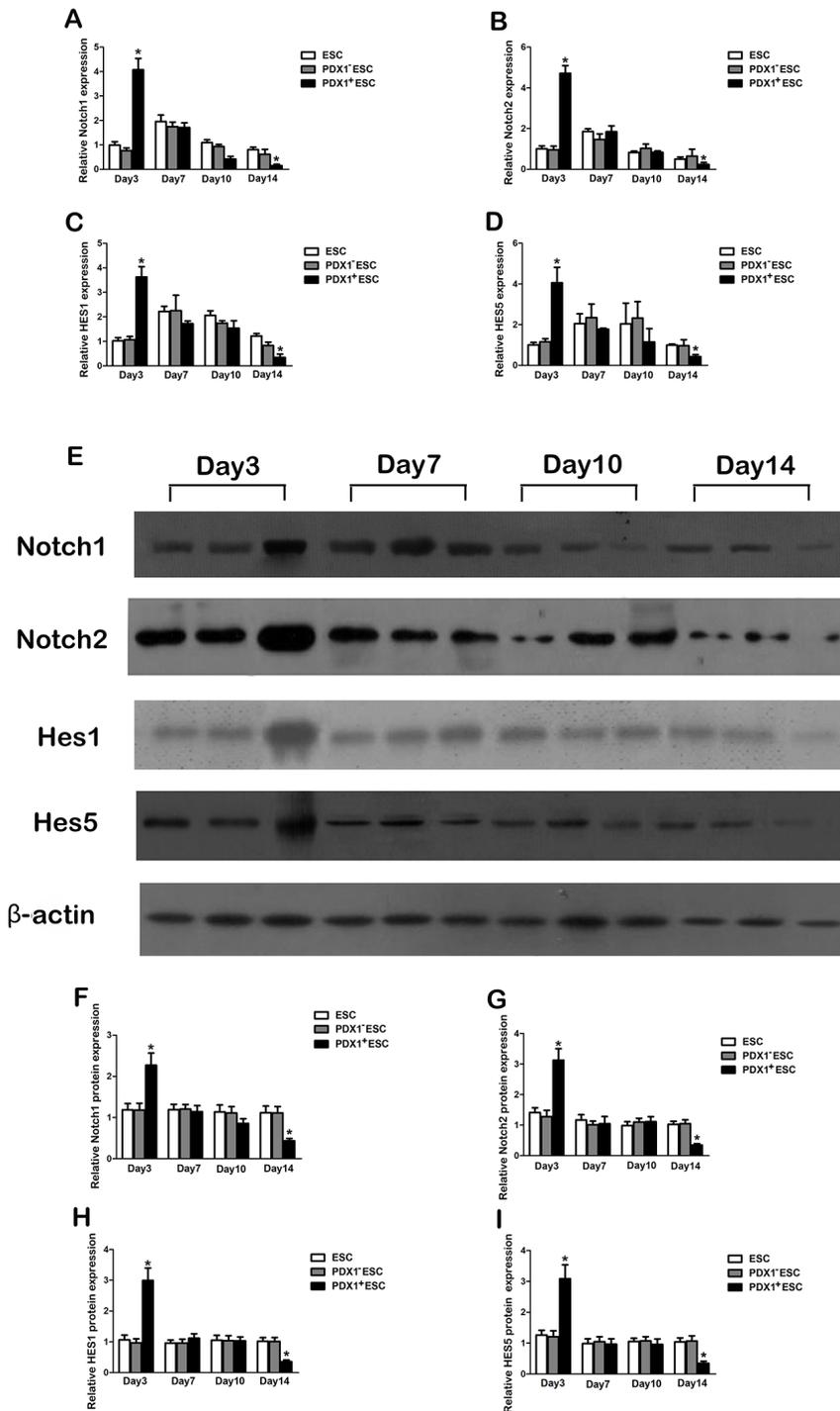


Figure 5

The mRNA and protein expressions of Notch1, Notch2, Hes1 and Hes5 were. Observed in ESC group, PDX1-ESC group and PDX1+ESC group by Western blot I and quantification of immunoblot bands from Western blot (F-I) and qRT-PCR (A-D). The mRNA and protein expression levels of these genes were higher at d3 and then lower at d14 in the cells in PDX1+ESC group than those in ESC group and PDX1-ESC group. The mRNA and protein levels are expressed relative to β -actin, *P<0.05.