

Synchronously Transgene of Therapeutic Intercalated Disc-related CX43 and ZO1 Genes and Reprogramming Genes does not Affect The Pluripotent of Pig IPS

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Key words: Transgene; Inducible pluripotent stem cells; CX43, ZO1, Intercalated disc

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ABSTRACT

AM: To explore the feasibility of reprogramming pig somatic cells into inducible pluripotent stem cells carrying therapeutic intercalated disc-related CX43 single gene and CX43/ZO1 dual genes.

METHODS: The retroviral vectors of CX43, ZO1, Oct-4, Sox2-HUMAN, Nanog, and Lin28 were synchronously transfected into the pig dermal fibroblasts to construct three iPS cell lines: iPS cell, iPS cell carrying CX43 single gene and iPS cell carrying CX43/ZO1 dual genes. The iPS cells' surface markers including SSEA-3, SSEA-4, nanog, REX-1, TRA-1-60, and TRA-1-81 were detected by immunohistochemical staining. In-vitro EB formation and in-vivo teratoma formation were done to observing the pluripotent differentiation abilities of iPS cells.

RESULTS: Four~five weeks after transduction, colonies resembling ES cells of three iPS lines emerged. Cells of three iPS cell lines all expressed REX-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. All of the three iPS cell lines showed alkaline phosphatase activity. Suspension cultures of the three iPS cell lines witnessed Ebs formation. After injecting cells of each iPS cell line into the back of NOD/shi-scid and IL-2R γ null (NOG) mice, teratomas formed, including ectodermal, mesodermal, and endodermal tissues.

CONCLUSIONS: Reprogramming pig iPS cells carrying CX43 single gene or CX43/ZO1 dual genes is feasibility, and synchronously

INTRODUCTION

The induced pluripotent stem cells (iPSCs) is one of the most exiting areas in cell biology. Grown from adult somatic cells, iPSCs are genetically transformed to have the same function as embryonic stem cells. Reprogramming somatic cells into inducible pluripotent stem cells by viral transduction of Oct4, SOX2-HUMAN, c-Myc and Klf4 has gained success not only in small experimental animals like mouse^[1] and rat^[2], but also in human race^[3]. iPSCs can not only differentiate into all kinds of terminally differentiated cells including motoneurons^[4], sensory neurons^[5], dopamine-producing neurons^[6], insulin-secreting cells^[7,8], endoderm cells^[9], and cardiomyocytes^[10,11], *in vitro*, but also differentiate into hematopoietic progenitors^[12], dopamine-producing neurons^[13], factor VIII-producing Liver cells^[14] and cardiac myocytes^[15-17], *in vivo* after transplantation. In domain of cardiac regeneration, although it's reported that iPS cells can differentiate into cardiac myocytes both *in vitro* and *in vivo*^[18], yet it's never reported whether the transplanted stem cells can form intercalated disc (ID).

Intercalated disc is a unique structure of the cardiac myocytes. It's

a construction comprising three kinds of special connections—gap junction, adhering junction and desmosomes connection. Intercalated disc plays an important physiological role in myocardial mechanical and electrical coupling. The desmosomes connection and adhering junction serves as the mechanical connection structure between the myocardial cells, while the function of electrical coupling is mainly completed by gap junctions, which is a transmembrane channel made of proteins named connexin 43 (CX43). Although Cx43 is essential for cell–cell contact^[19] and Cx43 transgenic strategy has been confirmed to confer cytoprotection of stem cells and improve infarcted heart function^[20], it's never reported that Cx43 transgenic strategy can effectively form complete intercalated disc structure. The quite possible reason is that the integrity of intercalated disc functions is also dependent on the adhering junction^[21], lackage of which will have a profound impact on the normal size and number of gap junctions, resulting impairing impulse conduction^[22-25]. Zonula occludens-1 (ZO-1), also known as tight junction protein -1 (TJP1), is the most important adhering junction protein^[26]. The PDZ domain binding motif in ZO-1, a member of guanylate kinase family, plays an important role in the localization of proteins, signal transduction and cell polarity^[27]. By the interaction of the PDZ domain binding motif of ZO-1 and the PDZ domain of Cx43, Cx43 protein is fixed to intercalated disk area and anchored to the cytoskeleton, thus maintaining the normal function of gap junctions^[28]. The molecular crosstalk mechanisms between Cx43 and ZO-1 have become a very fashionable concept, yet effect of the strategy of transferring CX43/ZO-1 dual-gene into stem cells, especially the most popular iPS cells, has never been reported. To compare the effects in reconstructing intercalated disk between CX43 single gene strategy and CX43/ZO-1 dual genes strategy, the first task is to observe this strategy's feasibility and safety, especially about the potential effect on the pluripotent of iPS cells.

Here we explored the the feasibility of reprogramming pig somatic cells into inducible pluripotent stem cells carrying therapeutic intercalated disc-related CX43 single gene or CX43/ZO1 dual genes and observe whether synchronous transgene of both reprogramming genes and therapeutic genes affect the pluripotent of iPS cells.

METHODS

Animals

A tree-month-old male pig was maintained in cages in our laboratory at the Central Institute for Experimental Animals of Xiangtan City Central Hospital affiliated to Central South University. This study was approved by the animal ethics committees and gene recombination experiment safety management committees of Central South University and was performed in accordance with Central South University guidelines.

Cell culture

Pig dermal fibroblasts were isolated from the pig's ear skin by digestion with dispase and trypsin. After minced with iris scissors on a 60-mM culture dish, the pig ear skin tissues were dissociated in 0.25% trypsin-EDTA solution (Invitrogen, USA) at 37°C for 15 min. Then the digestion was terminated by DMEM containing 10% fetal calf serum. The single cells were separated by a 200-mesh filter, washed once by centrifugation at 190 g for 5 min and subsequently seeded into 100-mM plastic culture dishes. The seeded cells were cultured for 6-8 days in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (Sigma, U.S.A) and 1% antibiotic-antimycotic (Invitrogen, USA) at 37°C in a humidified atmosphere

of 5% CO₂ and 95% air. After 2 or 3 generations, fibroblasts were reserved for retroviral transfection.

Retroviral infection and iPS cell generation

Retroviral vectors for CX43, ZO-1, Oct-4, Sox2-HUMAN, Nanog, and Lin28 were kindly provided by Dr Lei XIAO (Zhejiang University). These retroviral vectors were prepared for three iPS cell lines as followed: iPS cell line (reprogramming by genes including Oct-4, Sox2-HUMAN, Nanog, and Lin28 genes, similarly hereinafter), iPS cell line carrying CX43 single gene (by CX43 and four reprogramming genes), and iPS cell line carrying CX43/ZO1 dual genes (by CX43, ZO1 and four reprogramming genes). Pig fibroblasts were seeded at 1×10^6 cells per 10-cm dish 1 day before transduction. The medium was replaced with different virus-containing supernatant (according to the corresponding iPS cell line reprogramming plan) supplemented with 4 µg/ml polybrene (Sigma, U.S.A) and incubated for 12 hours. Seven days after introduction, the cells were harvested by trypsinization and plated onto MEFs at 1×10^5 cells per 10-cm dish. And the DMEM containing 10% FBS was replaced with medium for ES cell culture that consisted of knockout DMEM supplemented with 10% knockout serum replacement (Life Technologies, U.S.A), 1 mM l-glutamine, 0.1 mM MEM nonessential amino acids, 0.1 mM β-mercaptoethanol (BioChemica, U.S.A), 1% antibiotic-antimycotic and 10 ng/ml leukemia inhibitory factor (Millipore, USA). Three to five weeks after transgene, colonies were picked up and dissociated mechanically into small clumps by pipetting up and down. The cell suspension was transferred onto MEFs in 12-well plates and cultured in 0.2 mL medium for ES cell culture. During cell splitting, undifferentiated cell colonies were detached from the feeder cells using 0.25% trypsin supplemented with 1 mM CaCl₂ and 20% KSR. The removed colonies were dissociated mechanically into 10~50 cells and replated on a new irradiated MEF feeder layer.

Immunohistochemical staining

To observe the expression of cell-surface markers on cells of three iPS lines, we detected alkaline phosphatase using the alkaline phosphatase detection kit (Millipore, USA). IPS cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature and then incubated with 0.3% H₂O₂ for 10 min at room temperature. The primary antibodies against stage-specific embryonic antigen SSEA-3, SSEA-4 (invitrogen, USA), nanog, REX-1, TRA-1-60, and TRA-1-81 (Millipore, USA) were diluted with antibody diluent and incubated for 1 h at room temperature. The following primary antibodies were used: anti-SSEA-3 (1:100), anti-SSEA-4 (1:200), anti-nanog (1:200), anti-REX-1 (1:200), anti-TRA-1-60 (10µg/mL) and anti-TRA-1-81 (10 µg/mL). After three washes with PBS, the biotinylated secondary antibody (invitrogen, USA) was incubated with the cells for 30 min at room temperature. The samples were washed three times with PBS, and the bound monoclonal antibodies were located using the DAB horseradish peroxidase complex.

RT-PCR analysis of endogenous genes

RNA was isolated from cells of three iPS lines using the RNeasy mini kit (QIAGEN, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized from RNA from undifferentiated iPSCs using the QuantiTect Reverse Transcription kit (QIAGEN, Germany). As a negative control, RNA was allowed to react with the cDNA synthesis reaction mixture in the absence of reverse transcriptase. After cDNA synthesis, the cDNA synthesis reaction mixture was used as the template for the PCR. The primers

of endogenous genes, including CX43, ZO1, Oct-4, Sox2-HUMAN, Nanog and Lin28, are shown in Table1. The PCR reaction mixture (20 μ l) contained $\times 1$ PCR buffer, 0.2 mM dNTP, 0.5 μ M each primer, and 2.5 U Taq polymerase. The amplification was performed for 35 cycles of 98°C for 20 s, 60°C for 25 s, and 72°C for 25 s.

EB formation

To observing EBs formation, we removed undifferentiated iPS cells of each iPS line from the MEF feeder layer, dissociated them using 0.25% trypsin in PBS with 20% KSR and 1 mM CaCl₂, and cultured them in bacterial petri dishes for 7-28 days using DMEM supplemented with 10% FBS. The medium was changed every 2 days.

In vivo differentiation: teratoma formation

To examine teratoma formation in mice, 5×10^6 cells of each iPS line were subcutaneously injected into 5-week-old immunodeficient NOG mice. Four to eight weeks after the injection, the tumors were removed from the mice. The resected tumors were fixed in buffered formaldehyde, embedded in paraffin blocks, and subjected to immunohistochemical and histologic examination.

Table 1 The PCR primers of exogenous CX43, ZO1, Oct-4, Sox2-HUMAN, Nanog and Nanog genes.

Gene	Primers	Sequences
CX43	Forward	AGTGAGGAAAGTACCAAACAGCAG
	Reverse	CCCGGACACTACTCTTCCCT
ZO1	Forward	AAGGAACGTCAAGTGGGTAAAGTC
	Reverse	GTAICTTCTCGGTTTGGTGGTCTG
Oct-4	Forward	CCC CAG GGC CCC ATT TTG GTA CC
	Reverse	CAGCCAAGTTGTGAGGT
Sox2-HUMAN	Forward	GGC ACC CCT GGC ATG GCT CTT GGC TC
	Reverse	AATGTTTTAATATTGCAAGCAACTTTGT
Nanog	Forward	GTCGGAAATCCATCCGIGTC
	Reverse	AGTCTCAAGACT
Lin28	Forward	AACAATCAGGCCTGGAACAG
	Reverse	CAGTAAAGGCCGAGCTTACACATGTTCT

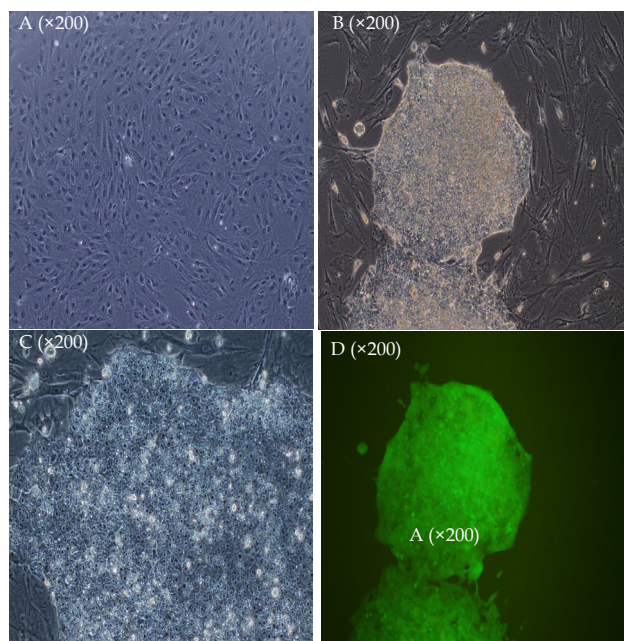


Figure 1 Induced Pluripotent Stem (iPS) Colonies. Four to five weeks after reprogramming and therapeutic retroviral vectors were introduced into pig dermal cells(A), colonies of three iPS lines resembling embryonic stem cells in morphologically were observed (B and C). All iPS cell lines had a flat, packed, tight colony morphology and a high nucleus-to-cytoplasm ratio (C), and expressed green-fluorescent protein fluorescence (D).

RESULTS

Generation of pig iPS cells from dermal cells

Isolation of pig dermal cells is successful (Figure 1A). After four weeks, we observed colonies resembling ES cells morphologically (Figure 1B), which had a flat, packed, and tight morphology. All of the cells of three iPS lines, including iPS cell (iPS 2), PS cell line carrying CX43 single gene (iPS 2-CX43) and iPS cell line carrying CX43/ZO1 dual genes (iPS 4-CX43/ZO1), had a high nucleus-to-cytoplasm ratio (Figure 1C). All of the cells colonies of each iPS line were green-fluorescent protein (GFP)- positive (Figure 1D). Picked-up colonies were plated on an irradiated mouse embryonic fibroblasts (MEFs) feeder layer. All the three iPS cell lines were cultured for more than 10 passages.

Immunohistochemical staining showed that cells of three iPS lines all expressed REX-1 (Figure 2A), SSEA-3 (Figure 2B), SSEA-4 (Figure 2D), TRA-1-60 (Figure 2D), and TRA-1-81 (Figure 2E). Besides, they all showed alkaline phosphatase activity (Figure 2F). Reverse transcription (RT)-PCR showed that cells of all three iPS lines expressed endogenous Oct4, Sox2, Nanog, and Lin28 genes, which were silenced or low-expressed in PF cells (Figure 3). RT-PCR showed that iPS 2-CX43 line expressed endogenous CX43 and did not expressed endogenous ZO1, whereas iPS 4-CX43/ZO1 line expressed both endogenous CX43 and endogenous ZO1(Figure 4).

In vitro differentiation of iPS cells

Suspension cultures of each iPS cell line formed EBs. Seven days after the start of suspension culture, simple EBs formed (Figure 5A), and cystic EBs formed within 2 weeks (Figure 5B).

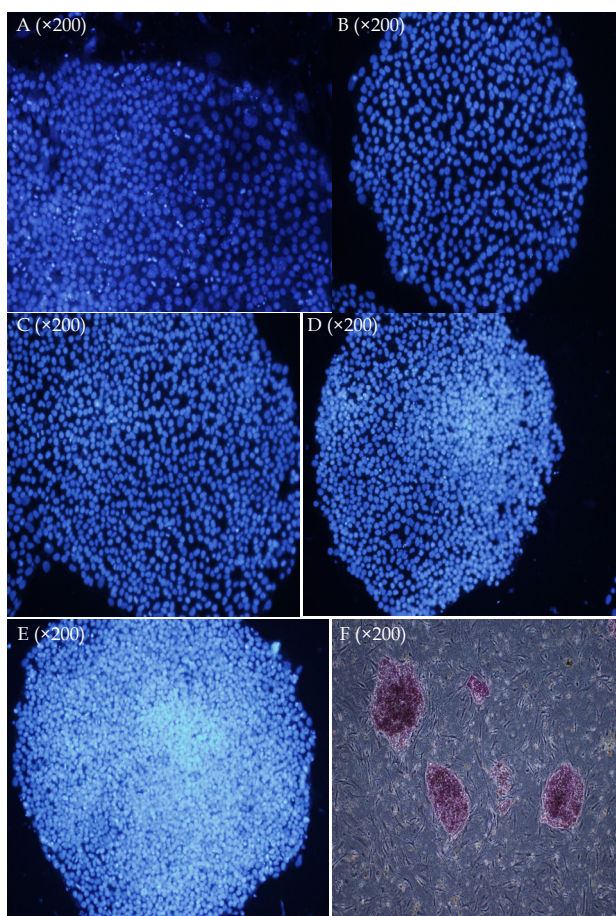


Figure 2 Immunohistochemical Staining. The cells of three iPS cell lines expressed REX-1 (A), SSEA-3 (B), SSEA-4 (D), TRA-1-60 (D), and TRA-1-81 (E) and showed alkaline phosphatase activity (F).

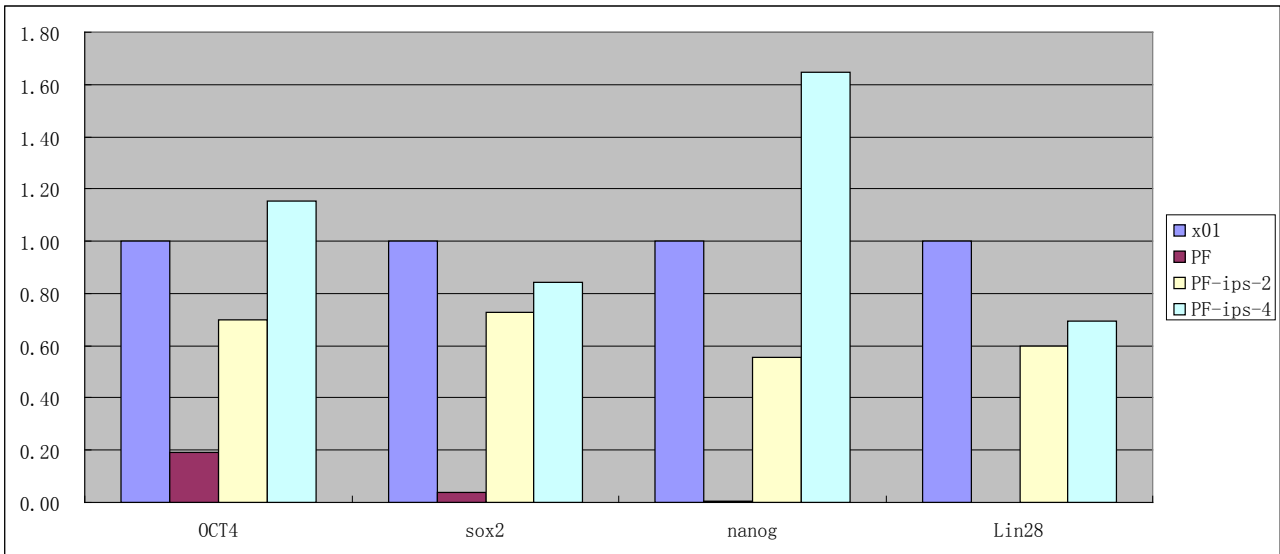


Figure 3 RT-PCR Analysis of Endogenous Reprogramming Genes. RT-PCR showed that all of the induced pluripotent stem (iPS) cell lines also expressed endogenous Oct-4, Sox2, Nanog and Lin28 genes, whereas all of four genes were silenced or low-expressed in PF cells.

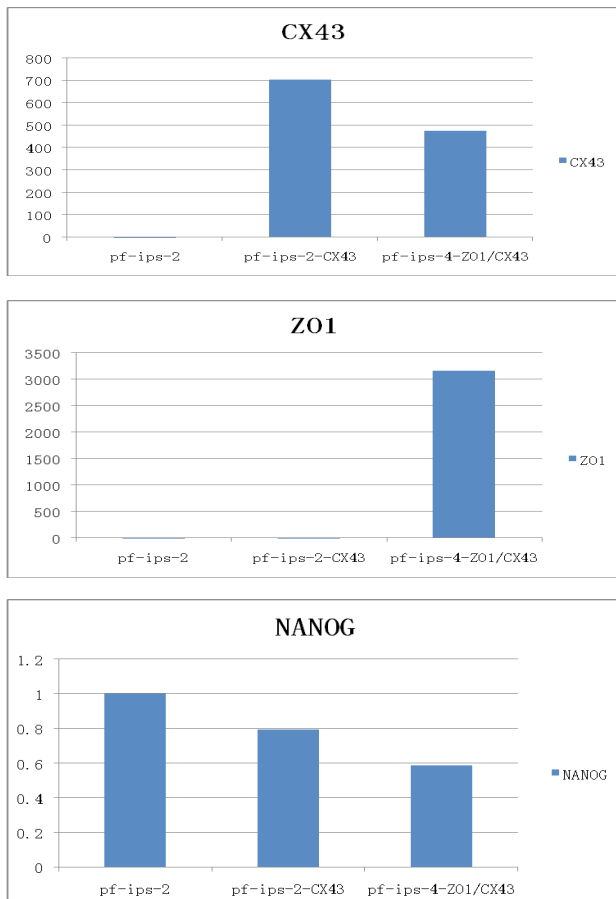


Figure 4 RT-PCR Analysis of Endogenous CX43 and ZO1 Genes. RT-PCR showed that iPS 2-CX43 line expressed endogenous CX43 genes, whereas iPS 4-CX43/ZO1 line expressed both endogenous CX43 and endogenous ZO1. As a control, NANOG gene was expressed in iPS 2 line, iPS 2-CX43 line and iPS 4-CX43/ZO1 line.

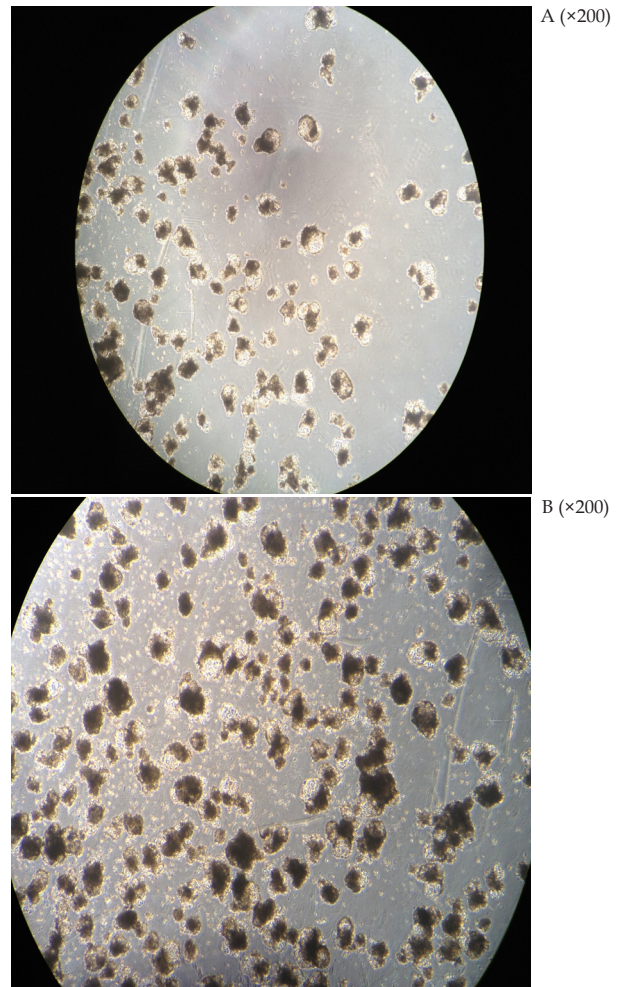


Figure 5 EBs formation of three iPS cell lines. Simple EBs formed several days after the start of the suspension cultures(A), and cystic EBs formed within 2 weeks (B).

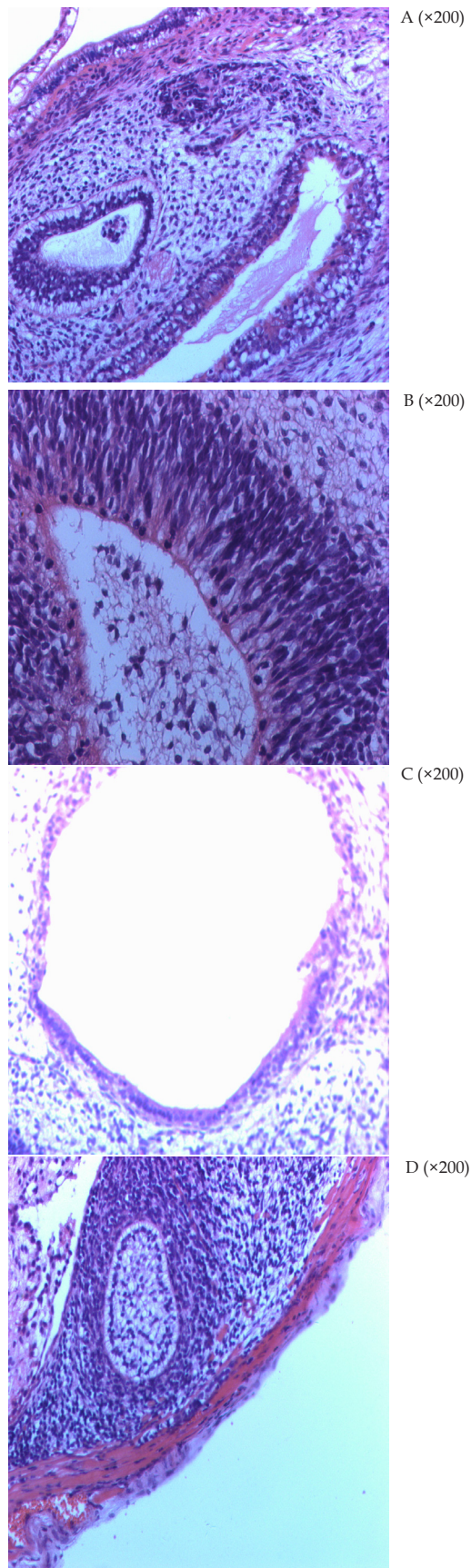


Figure 6 Teratoma Formation of three iPS cell lines. The NCAM-positive cells were observed as neuronal cells (A), suggesting ectodermal differentiation. The columnar epithelium (B) formed luminal-like structure, suggesting endodermal differentiation. The presence of blood vessels(C) and cartilage(D) suggests endodermal differentiation.

Teratoma formation of iPS cells

Eight weeks after the cells of each iPS line were injected into the back of NOD/shi-scid and IL-2R γ null (NOG) mice, teratomas formed, consisting of ectodermal, mesodermal, and endodermal tissues (Figure 6). Neural cell adhesion molecule (NCAM)-positive cells were observed (Figure 6A), suggesting ectodermal differentiation. And columnar epithelium (Figure 6B) formed luminal-like structure, suggesting endodermal differentiation. And the presence of blood vessels (Figure 6C) and cartilage (Figure 6D) suggests endodermal differentiation.

DISCUSSION

The technical difficulties in myocardial regeneration domain is how to rebuild intercalated disc so as to achieve an electrical coupling relationship between transplanted cells and residual myocardial cells. It's reported that iPS cells can differentiate into cardiac myocytes both *in vitro* and *in vivo*^[18], yet it's unclear whether the transplanted stem cells can form intercalated disc (ID). CX43 transgenic strategy has been confirmed to confer cytoprotection of stem cells and improve infarcted heart function^[29], yet it's never reported whether this strategy can effectively form complete intercalated disc structure. Recent studies showed that the CX43 can not construct a complete intercalated disc alone, because the molecular crosstalk mechanisms between connexin 43 and zonula occludens-1 are essential for the formation of intercalated discs^[30]. However, researches on the strategy of transferring CX43/ZO-1 dual-gene into stem cells has never been reported so far.

The presently study first verified the feasibility of reprogramming pig iPS cells carrying CX43 single gene and pig iPS cells carrying dual-gene CX43/ZO-1 dual-gene. It's confirmed that synchronously transferring both therapeutic genes and reprogramming genes does not affect the pluripotent of all three iPS cells lines. RT-PCR analysis witnessed that endogenous genes, including therapeutic CX43 and ZO1 genes and reprogramming Oct-4, Sox2-HUMAN, Nanog and Lin28 genes, are expressed in iPS cells carrying dual-gene CX43/ZO-1 dual-gene, indicating that gene transduction of both therapeutic genes and reprogramming genes is feasible. Cells of iPS line, iPS carrying CX43 single gene and iPS carrying CX43/ZO1 dual genes all had many similarities to ES cells in terms of morphology, surface antigens, and cellular characteristics. And they showed a normal ability to differentiate into three germ cell layers both *in vitro* and *in vivo* that was equivalent to marmoset ES cells. All mentioned above proved that the strategy of transferring both therapeutic CX43 single gene or CX43/ZO-1 dual genes and reprogramming Oct4, SOX2-HUMAN, Nanog and Lin28 genes does not affect the pluripotent of iPS.

Previous studies only reported the feasibility of transferring Cx43 genes into Human Bone Marrow Derived Stem Cells^[19,20], yet our study first use iPS cells as the target cells for therapeutic gene transferring, and first constructed iPS line carrying CX43 single gene and iPS line carrying CX43/ZO1 dual genes. For sake of the importance of the molecular crosstalk mechanisms between connexin 43 and zonula occludens in formation of intercalated disc, CX43/ZO1 dual transgene might be a promising strategy for reconstruction of intercalated disc. Previous researches only focus on the effectiveness of transferring CX43 gene, and never involved the possible effect on the pluripotent of stem cells. Our study first witnessed that the strategy of transferring both therapeutic CX43 single gene or CX43/ZO-1 dual genes and reprogramming Oct4, SOX2-HUMAN, Nanog and Lin28 genes does not affect the pluripotent of iPS.

Besides, our study has another superiority—adopting valuable animal species. Previous reported iPS cell are derived either from small animals or from human beings. Each has its limitations. As for small animals, their anatomical and physiological features are far away from human beings', which discounts the value of related iPS studies. As for human beings, their iPS cells inevitably face ethical problem and therefore are far away from use for transplantation so far. Big mammal animals have closer anatomical and physiological characteristics to humans' than mouse and rat, and related iPS cells researches avoid ethical concern. So big animals like pig and dog are quite valuable candidates for iPSCs research.

Suggested meaning of our study is that, now that the feasibility of reprogramming pig iPS cells carrying CX43 single gene and iPS cells carrying CX43/ZO-1 dual-gene is confirmed and this strategy does not affect the iPS cells' pluripotent, whether iPS cells carrying CX43/ZO-1 dual-gene is better than iPS cells carrying CX43 single gene in regenerating myocardium and reconstructing intercalated disc can be verified in the next experiments in pig myocardial infarction model.

LIMITATIONS

Although the present study has revealed that there are high levels of CX43 and ZO-1 mRNAs in iPS cells carrying disc-related CX43/ZO-1 dual-gene, yet the immunohistochemical staining image for gap (Cx43) junction on the iPS cells and the functional study for gap junction of iPS cells remain to be furtherly researched in the next stage. Also, Whether iPS cells carrying CX43/ZO-1 dual-gene can regenerate myocardium and reconstruct intercalated disc better than iPS cells carrying CX43 single gene is still to be observed in the next animal transplantation experiments.

CONCLUSIONS

Reprogramming pig iPS cells carrying both therapeutic CX43 single or gene CX43/ZO-1 dual genes and reprogramming Oct4, SOX2-HUMAN, Nanog and Lin28 genes is feasibility, and this strategy does not affect the pluripotent of iPS cells.

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CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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