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Establishment of a porcine pancreatic stem cell line using T-REx™ system-inducible *Wnt3a* expression

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Abstract

Objectives: Porcine pancreatic stem cells (PSCs) are highly valuable in transplantation applications for type II diabetes. However, there are still many problems to be solved before they can be used in the clinic, such as insufficient cell number availability and low secretion level of insulin. It has been reported that *Wnt3a* plays pivotal roles during cell proliferation and differentiation. Here, we have aimed to establish an ideal research platform using the T-REx™ system, to study mechanisms of *Wnt3a* during PSC proliferation and differentiation.

Materials and methods: Construction of the recombinant plasmid and cell transfection were used for establishment of a porcine PSC line. Related gene expressions were examined using quantitative real-time PCR (QRT-PCR), western blotting, immunostaining and flow cytometry. BrdU incorporation assay and cell cycle analysis were used to investigate *Wnt3a* roles in PSCs.

Results: *Wnt3a*-expressing clones regulated by T-REx™ were successfully obtained. Wnt3a and GFP expression were strictly regulated by Dox in a time- and dose-dependent manner. Furthermore, we found that *Wnt3a*-expressing porcine PSCs induced by Dox exhibited raised proliferative potential. After Dox stimulation, expression of PCNA, C-MYC and active β-catenin were higher, but were down-regulated after Dkk1 addition.

Conclusion: We established a porcine PSC line that dynamically expressed *Wnt3a*, and we found that *Wnt3a* promoted PSC proliferative potential. This inducible expression system thus provides an important tool for further study on porcine PSC development and differentiation.

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Introduction

Statistics obtained from the International Diabetes Federation (IDF) indicate that there were 381.8 million diabetic patients worldwide in 2013, which is predicted to reach 591.9 million by 2035 (1). Generally, diabetes mellitus (DM) is classified into two major types, type 1 and type 2.

Type 2 diabetes mellitus (DM2) is a heterogeneous metabolic disorder characterized by insulin resistance and relative insulin deficiency (2). Side effects of immunosuppressive treatment and low availability of organ donors limit application of traditional DM2 methods of therapy (3). Porcine PSCs are currently viewed to be one of the most promising alternative sources for diabetes treatment due to their highly conserved insulin structure and similar physiological glucose levels to those of humans (4–6). However, low quantities and their mechanism of proliferation have not yet been fully elucidated and resolved; these need to be overcome before porcine PSCs can be used in clinical applications for human diabetes.

The canonical Wnt/β-catenin signalling pathway controls a number of biological phenomena such as cell population expansion, development and stem cell maintenance (7,8). It is a critical regulatory pathway during embryonic development and it maintains gut homoeostasis (9). The system can be activated by an upstream Wnt secretory protein, such as *Wnt3a*; then Wnt molecules bind their corresponding transmembrane receptors and cause β-catenin accumulation in the cytoplasm followed by its translocation to the nucleus. Finally, target gene transcription is regulated and biological characteristics are devolved (10). Aberrant Wnt signalling pathway regulation has been suggested to play a vital role in tumourigenesis. Dkk1 is a predominant antagonist of the Wnt/β-catenin signal (10).

Recently, several studies have demonstrated involvement of Wnt pathway activation in pancreatic β cell development and proliferation (4). *Wnt3a*, a prominent member of the Wnt family, can induce accumulation of β-catenin and activation of the canonical Wnt signalling pathway (11). Gui *et al.* reported that *Wnt3a* regulated proliferation and apoptosis of pancreatic NIT-1 beta cells (12). However, little is known of whether *Wnt3a* has any effect on proliferation and survival of porcine PSCs (6,12).

The T-REx™ system is a tetracycline-regulated mammalian expression structure where regulatory elements are *Escherichia coli* Tn10-encoded (13,14). Tetracycline regulation in the T-REx™ system is based on binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest (15). Gene expression can be tightly control by adding different doses of tetracycline or its analogues, such as doxycycline (Dox). Miyazaki *et al.* studied the function of retinoid X receptor during glucose-stimulated insulin secretion of pancreatic ß-cells using T-REx™ system (16), but up to now, little has been reported concerning application of the system during study of the function of the Wnt pathway and PSCs.

In this study, we aimed to establish an ideal technical platform for exploring function and mechanism of the Wnt signalling pathway on PSCs, by establishing our porcine PSC line with *Wnt3a* expression regulated by T-REx™ system.

Materials and methods

Cells, plasmids and reagents

Porcine PSCs were established and maintained by our laboratory (5). The following materials were used: T-REx™ system pCDNA6/TR and pCDNA4/TO plasmids, low-glucose DMEM, glutamine, zeocin and blasticidin (Invitrogen, Carlsbad, CA, USA); Transfection Reagent Mega Tran 1.0 (ORIGEN, Rockville, MD, USA); reverse transcription (RT) kit and SYBR Green kit (TaKaRa, Dalian, China); Dkk1 (Peprotech, Rocky Hill, NJ, USA); anti-actin and anti-tublin antibodies (Beyotime, Haimen, Jiangsu, China); anti-*Wnt3a* antibody R&D Systems; anti-glut2 antibody, anti-active-β-catenin and anti-PCNA (Millipore, Billerica, MA, USA); anti-MYC antibody (Chemicon, Temecula, CA, USA); anti-PDX1 antibody (Abcam, Cambridge, MA, USA); anti-BrdU antibody (Santa Cruz Biotechnology, CA, USA).

Construction of recombinant plasmids

Mouse *Wnt3a* primers designed according to the published *Wnt3a* mRNA sequence, were synthesized, length of amplification product being 1085 base pairs (Sangon Biotech, Shanghai, China). *Wnt3a* was amplified from mouse tail vein-derived samples using Vazyme Blood Direct PCR Kit (Vazyme, Nanjing, China), by PCR. PCR steps included denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 40 s and 72 °C for 1 min, and final extension at 72 °C for an additional 10 min. Specific fragments were then cloned into pIRES2-AcGFP1 to obtain pIRES2-*Wnt3a*-GFP1 recombinant plasmid. After bacterial PCR screening, positive clones were identified by PCR, restriction enzyme digestion and sequencing. Fragments were then cloned into pMD18-T cloning vector (TaKaRa). The full-length coding sequence for *Wnt3a-GFP* was obtained using restriction enzyme digestion and was cloned into pCDNA4T/O vector. The plasmid (pCDNA4T/O-*Wnt3a*) was verified by *Eco*R I and *Xho* I double restriction enzyme digestion, and sequenced. Enzyme digestion products were analysed in 1% agarose gel electrophoresis, stained with ethidium bromide and visualized using UV illumination.

Generation of porcine PSCs stably expressing Wnt3a regulated by T-REx™

Porcine PSCs cultured in low-glucose DMEM containing 10% FBS, 0.1 mM β-mercaptoethanol and 2 mM glutamine were refreshed every 2–3 days. Mega Tran 1.0 (ORIGEN) was used for transfection. Porcine PSC in the logarithmic growth phase, cultured in 6-well plates and grown to 50–70% fusion, were used for transfection. Medium needed to be changed to Opti-MEM or L-DMEM medium without serum 1 h before transfection. For preparation of the solution, in a 1.5 ml sterile Eppendorf tube, 240 μl serum-free medium (Opti-MEM) and 10 μl Mega Tran were mixed gently and allowed to stand at room temperature for 5 min; 4 μg control plasmid (pCDNA6T/R) and 10 μg induced expression plasmid (pCDNA4T/O-*Wnt3a*) in serum-free medium base (Opti-MEM), to a total volume of 250 μl, were gently mixed and allowed to stand for 20 min at room temperature. After washing the cells, serum-free L-DMEM medium was added directly at 37 °C, 5% CO_2 for 6 h. Transfection liquid was discarded, transfected again 24 h after replacement of medium, with 500 μg/ml zeocin and 1 μg/ml blasticidin to continue screening. Approximately 2 weeks after observation of cloning, concentration of zeocin was reduced to 50 μg/ml. Blasticidin was maintained at 1 and 5 μg/ml Dox included after 24–48 h to observe expression of green fluorescent protein (GFP). In the presence of Dox, the tetracycline controlled transactivator advanced (tTA) is complexed with Dox and is able to activate. In the presence of Dox, the tetracycline

controlled transactivator advanced (tTA) is complexed with Dox and is able to activate *Wnt3a* expression. The obtained condition-inducible expression plasmids pCDNA4T/O-*Wnt3a* and pCDNA6T/R, which also contains zeocin and blasticidin resistance genes, were co-transfected into porcine PSC. Forty-eight hours after transfection, zeocin and blasticidin were added to the culture medium for selection of transfected cells.

Immunostaining

Adherent cells were washed in PBS and subsequently fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 for 10 min, blocked with 10% goat serum in PBS at room temperature for 1 h, and incubated with the primary antibodies (1:100 dilution) overnight at 4 °C. After washing three times in PBS, samples were incubated in the secondary antibodies (1:500 dilution) at room temperature for 1 h. Samples were then washed in PBS three times, and cell nuclei were counterstained with Hoechst33342 (final concentration: 5 μg/ml) for 10 min at room temperature. Images were captured and analysed using a Leica fluorescence microscope (5,6).

Quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNAs for RT-PCR analysis were extracted from induced porcine pancreatic stem cells from different time periods and doses after treatment with Dox, and using Trizol (TaKaRa). cDNAs were synthesized (based on 500 ng RNA) with a commercially available kit (TaKaRa). Primers were designed based on sequences of the open reading frame from NCBI GenBank (Table 1). QRT-PCR reactions were set up in 25 μl reaction mixtures containing 12.5 μl 1 × SYBR@ PremixExTaqTM (TaKaRa), 0.5 μl sense primer, 0.5 μl antisense primer, 11 μl distilled water and 0.5 μl template. Reaction conditions were as follows: 95 °C for 30 s, followed by 35 cycles of 95 °C for 5 s, and 58 °C for 20 s. All expression levels were normalized to β-*actin* in each well. Expression was quantified as ratio of mRNA levels obtained from samples treated with Dox at different time periods and doses. To make quantitative comparisons between samples, we normalized time and dose *Wnt3a* to expression of β-*actin* in each well. Double ΔCt method was used to measure expression alteration; fluorescence signal was collected every 0.5 °C for 10 s (5,6).

Table 1. Primers for QRT-PCR

Gene	Prime sequence
Wnt3a	Forward: 5′-GGAGGGAGAAATGCCACTGTGTTT-3′
	Reverse: 5′-ACTTGCAGGTGTGCACGTCATAGA-3′
β-*actin*	Forward: 5′-GCGGCATCCACGAAACTAC-3′
	Reverse: 5′-TGATCTCCTTCTGCATCCTGTC-3′

Western blotting

Total cell extracts were prepared from porcine PSC and lysed in buffer [50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40 supplemented with protease inhibitor cocktail (Thermo, Waltham, MA, USA)]. The supernatant was collected from cell lysate after centrifugation with 12, 000 rpm for 15 min at 4 °C. For analysing protein expression of cell supernatant, supernatant of PSC was collected after treatment with 5 μg/ml Dox for 72 h, then concentrated in the region of 50 times. Total cell protein or supernatant were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20) supplemented with 5% (w/v) non-fat milk, then incubated with primary antibodies including anti-*Wnt3a* (1:1000, R&D) at 4 °C overnight. The membrane was then washed three times in TBST at room temperature and incubated with secondary antibody (1:1000) for 1 h. After the membrane was washed three times in TBST, detection was performed using BM-Chemiluminescence blotting substrate (Roche, Shanghai, China) (5,6).

BrdU incorporation assay

Effects of *Wnt3a* on proliferation of porcine PSC were assayed by BrdU incorporation. First, cells treated with 5 μg/ml Dox for 48 h indicated *Wnt3a* overexpression; cells with absence of Dox were controls. Dkk1 alone or in combination with 5 μg/ml Dox were treated with 30 μg/ml BrdU for 4–6 h then subjected to BrdU immunostaining. More specifically, cells were fixed in methanol (CH_3OH) for 15 min at room temperature and washed three times in PBS. Anti-BrdU antibody dissolved in PBS containing 5% normal goat serum was added and cells were incubated overnight at 4 °C. Cells were washed three times in PBS, then were incubated in secondary antibody for 1 h at room temperature. Three more washes were carried out and cells were visualized using a Leica fluorescence microscope then analysed for BrdU uptake. Level of BrdU positivity was assessed by manual counting under fluorescence microscopy (5,6).

Flow cytometric analysis of cell cycle

Cells were harvested by trypsin digestion and fixed in 70% ethanol at −20 °C. Ahead of analysis, cells were

incubated in RNaseA (20 μg/ml) and stained with propidium iodide (PI) (50 mg/ml) for 5 min. Samples were then immediately analysed using flow cytometry using a FACS Calibur flow cytometer. A total of 10 000 events were recorded per sample at FL2 peak emission values (FL2-H) (wave length, 575 ± 26 nm), and cell fractions in G1, S and G2/M cell cycle phases were quantified in histograms, using WinMDI software.

Statistical analysis

Data are presented as mean ± SEM and standard errors of the mean in this study were calculated for three replicates in each of three independent experiments. Statistical comparisons were assessed using analysis with Student's test. $P < 0.05$ was considered to be a statistically significant difference and $P < 0.01$ was considered to be a highly significant difference.

Results

Establishment of porcine PSCs stably expressing Wnt3a, using T-REx™ system

To establish an ideal and convenient platform for studying the function of *Wnt3a* in PSCs, we aimed to establish a porcine PSC line using T-REx™ System-inducible *Wnt3a* expression. As shown in Fig. 1a, in this system, the Tet repressor forms a homodimer that binds (with extremely high affinity) to each Tet operator sequence in the promoter of the inducible expression vector, and in the absence of tetracycline, causes inhibition of

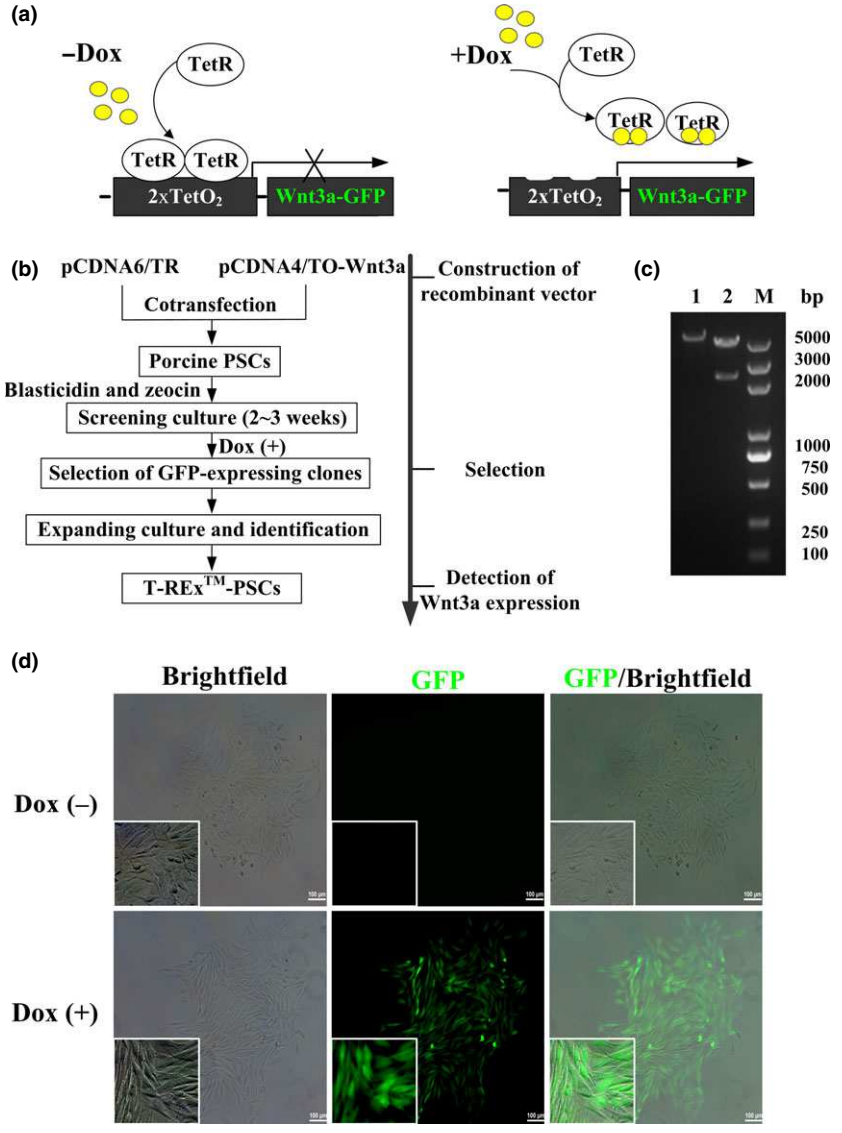

Figure 1. Schematic outline for obtaining PSCs line with inducible expression of *Wnt3a* using T-REXTM system. (a) The mechanism map of Wnt 3a expression regulated by T-RexTM system. (b) A brief schematic outline for obtaining PSCs line stably expressing *Wnt3a* regulated by T-RexTM system. The regulatory expression vector pcDNA6/TR and inducible expression vector pcDNA4/TO-wnt3a constructed successfully were cotransfected into PSCs then screened by blasticidin and zeocin until single cell clone appeared. After about 2–3 weeks, clones expressing GFP protein with Dox treatment were picked for further expanding culture and identified. (c) The inducible expression vector pcDNA4/TO-wnt3a was identified by EcoRI and XhoI double restrictive enzyme digestion. (d) Screening culture for 10 days, 5 μg/ml Dox was added to the L-DMEM containing zeocin and blasticidin, the clones expressing GFP were observed under fluorescence microscope (bar = 100 μm).

Wnt3a expression. In contrast, in the presence of tetracycline, Tet repressor homodimers bind and become released from the Tet operator sequence, then induced *Wnt3a* transcription. Brief workflow of this study is shown in Fig. 1b, and details are described in the Materials and methods section, above. Figure 1c shows that the inducible expression vector pcDNA4/TO-Wnt3a was identified by double restrictive enzyme digestion using *Eco*R I and *Xho* I. Fragment length was in accordance with expectation. GFP protein expression was observed after Dox induction when we screened the culture for 2 weeks after transfecting pcDNA6/TR and pcDNA4/TO-Wnt3a into PSCs (Fig. 1d). This result indicated that we had, indeed, obtained a porcine PSC line regulated by the T-REx™ system.

Wnt3a expression in PSCs was regulated by Dox in a time- and dose-dependent manner

To further determine effectiveness of the T-REx™ system-inducible *Wnt3a* expression, we detected GFP expression in PSCs using fluorescence microscopy, after treatment with different concentrations (0, 0.1, 1, 5, 10 μg/ml) of Dox for different time periods (0, 24, 48, 72 h). As shown in Fig. 2a, we observed that GFP expression was clearly enhanced and percentage of

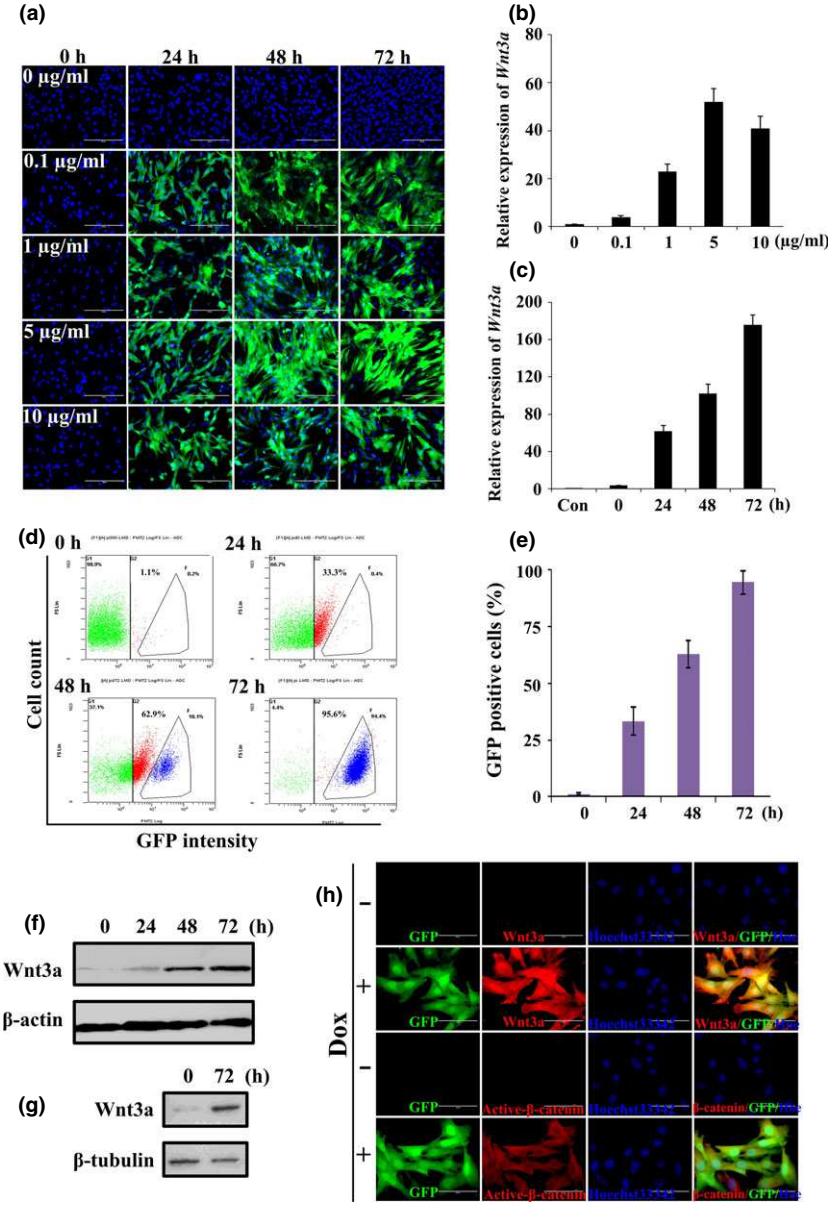

Figure 2. The expression of GFP and *Wnt3a* was regulated by Dox in a time- and dose-dependent manner. (a) The expression of GFP in PSCs was observed under fluorescence microscope after treatment with different concentrations of Dox (0, 0.1, 1, 5, 10 μg/ml) for different times (0, 24, 48 and 72 h) (bar = 200 μm). (b) The relative *Wnt3a* mRNA expression level in PSCs after Dox treatment for different times analyzed by QRT-PCR. (c) The relative *Wnt3a* mRNA expression level in PSCs after treatment with different concentrations of Dox analysed by QRT-PCR. (d) GFP-positive PSCs were quantified by flow cytometric after induced by Dox. (e) The histogram of statistical result for Fig. d. (f) The *Wnt3a* protein expression in PSCs after Dox addition for different times detected by Western blotting. (g) The *Wnt3a* protein expression in the supernate of cultured PSC after Dox treatment for 72 h. (h) Immunostaining of *Wnt3a* and active β-catenin in PSCs after treatment with or without Dox (bar = 100 μm).

GFP-positive cells increased with extension of treatment time with Dox, but no significant difference was found when different concentrations of Dox were added. To find the most suitable induction concentration and time, we induced *Wnt3a* expression in PSCs with different concentrations of Dox (0, 0.1, 1, 5, 10 μg/ml) for 48 h. Results of QRT-PCR showed that 5 μg/ml was the optimal induction concentration for *Wnt3a* expression even if 0.1 μg/ml Dox had the induction effect shown in Fig. 2b. Moreover, we induced *Wnt3a* expression with 5 μg/ml Dox for different time periods (0, 24, 48, 72 h), as shown in Fig. 2c. QRT-PCR results indicated that *Wnt3a* mRNA expression was enhanced gradually with extension of Dox treatment time. In addition, we analysed GFP expression using flow cytometry, after Dox induction for different time periods. We found that percentage of GFP-positive cells increased with extension of Dox treatment time (Fig. 2d, e). Further, we noticed that *Wnt3a* protein expression was also elevated with extension of Dox treatment time, detected by western blotting (Fig. 2f). Then, we tested *Wnt3a* protein expression level in the PSC supernatants. Results indicated that *Wnt3a* expression clearly increased after Dox treatment for 72 h compared to controls (Fig. 2g).When we induced PSCs with 5 μg/ml Dox for 48 h, we found that expression *Wnt3a* and active β-catenin were clearly observed, compared to the control group, according to results of fluorescence staining (Fig. 2h). All results illustrated that *Wnt3a* expression in PSCs was regulated by the T-REx™ system.

Characterization of PSC line of Wnt3a-induced expression by T-REx™ system

To confirm that biological characteristics of the PSCs were maintained, we examined expression of PDX1, Glut2, C-MYC and PCNA, specific markers of PSCs and proliferation, after Dox induction, using immunostaining. We found that almost all PSCs expressed these typical pancreatic stem cell and proliferation markers (Fig. 3). This result suggests that Dox induction did not affect biological characteristics of the PSCs.

Activation of the canonical Wnt signalling pathway promoted PSC proliferation potential induced by Dox

Furthermore, we sought to explore whether *Wnt3a* promoted cell proliferation after Dox induction. The PSCs were treated with Dox for different time periods (0, 24, 48 h with or without Dkk1), then DNA content was analysed by flow cytometry. As shown in Fig. 4a, percentage of cells in S phase was 10.29% (0 h), 28.40% (24 h) and 34.42% (48 h) respectively, that is, increasing. However, when we induced PSCs for 48 h using Dox in combination with Dkk1, the percentage of cells in S phase was reduced to 8.71%. Moreover, we observed that Dox clearly increase percentage of BrdU-positive cells, compared to the 'no induction' group, detected by BrdU incorporation assay. This phenomenon was consistent with that of exogenous *Wnt3a* on PSC proliferation (Fig. S1). The effect of *Wnt3a* after Dox induction on PSC proliferation was rescued by addition of Dkk1 (Fig. 4c, d). To study the implied mechanism of PSC proliferation, we examined expression of C-MYC, PCNA and active β-catenin, downstream target genes and the key regulation protein of the canonical Wnt signalling pathway, after Dox induction, by western blotting. Results indicated that all three protein expressions (C-MYC, PCNA, active β-catenin) were highly enhanced with Dox induction for 48 h in comparison to control and 0 h groups (Fig. 4b). This enhanced expression was rescued by Dkk1 addition (Fig. 4b). These results revealed that promotion of PSC proliferation capability could be caused by activation of the Wnt signalling pathway, and successive downstream target gene transcription related to proliferation, after Dox treatment.

Discussion

Human pancreatic islet cells are currently thought to be the most promising source for cell therapy for diabetes (17,18). However, shortage of donor quantities available for transplantation requires researchers to seek alternatives, such as porcine cells. In our previous studies, we have described establishment of an immortalized porcine pancreatic mesenchymal stem cell line (5,6). To further explore the mechanism of the Wnt signalling pathway during porcine PSC proliferation and differentiation, we established a PSC line with *Wnt3a* expression, regulated by T-REx™ system.

The T-REx™ system is a method of inducible gene expression where gene transcription is reversely turned-on/off in the presence of tetracycline or its derivatives (doxycycline) (5,6). The advantage of using this system, is that expression of the gene of interest can be tightly turned-on or turned-off. In our experiments, expression of GFP protein could be induced by addition of Dox after we transfected regulatory expression vector pcDNA6/TR and inducible expression vector pcDNA4/TO-Wnt3a into porcine PSCs. Moreover, expression of GFP and *Wnt3a* was regulated by Dox in a time- and dose-dependent manner, thus, suggesting successful establishment of *Wnt3a*-induced expression system in porcine PSCs. This would provide a real-time and dynamic cell model system for studying development and differentiation of insulin-secreting cells in the future.

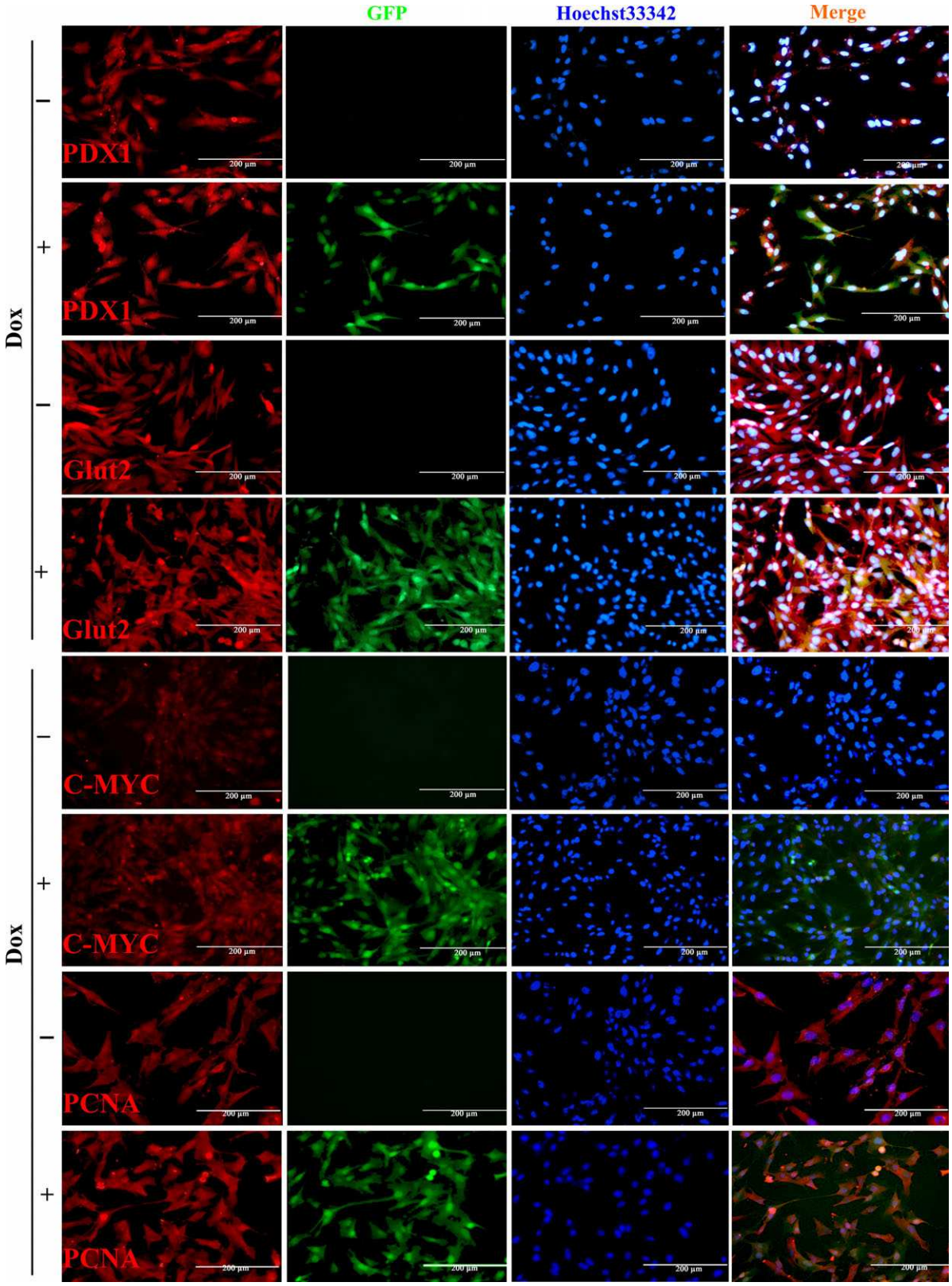

Figure 3. The expression of PSCs-specific markers and proliferation-related molecules. The expression of PSCs-specific markers (PDX1 and Glut2) and proliferation-related molecules(C-MYC and PCNA) detected by immunofluorescent staining (bar = 200 μm).

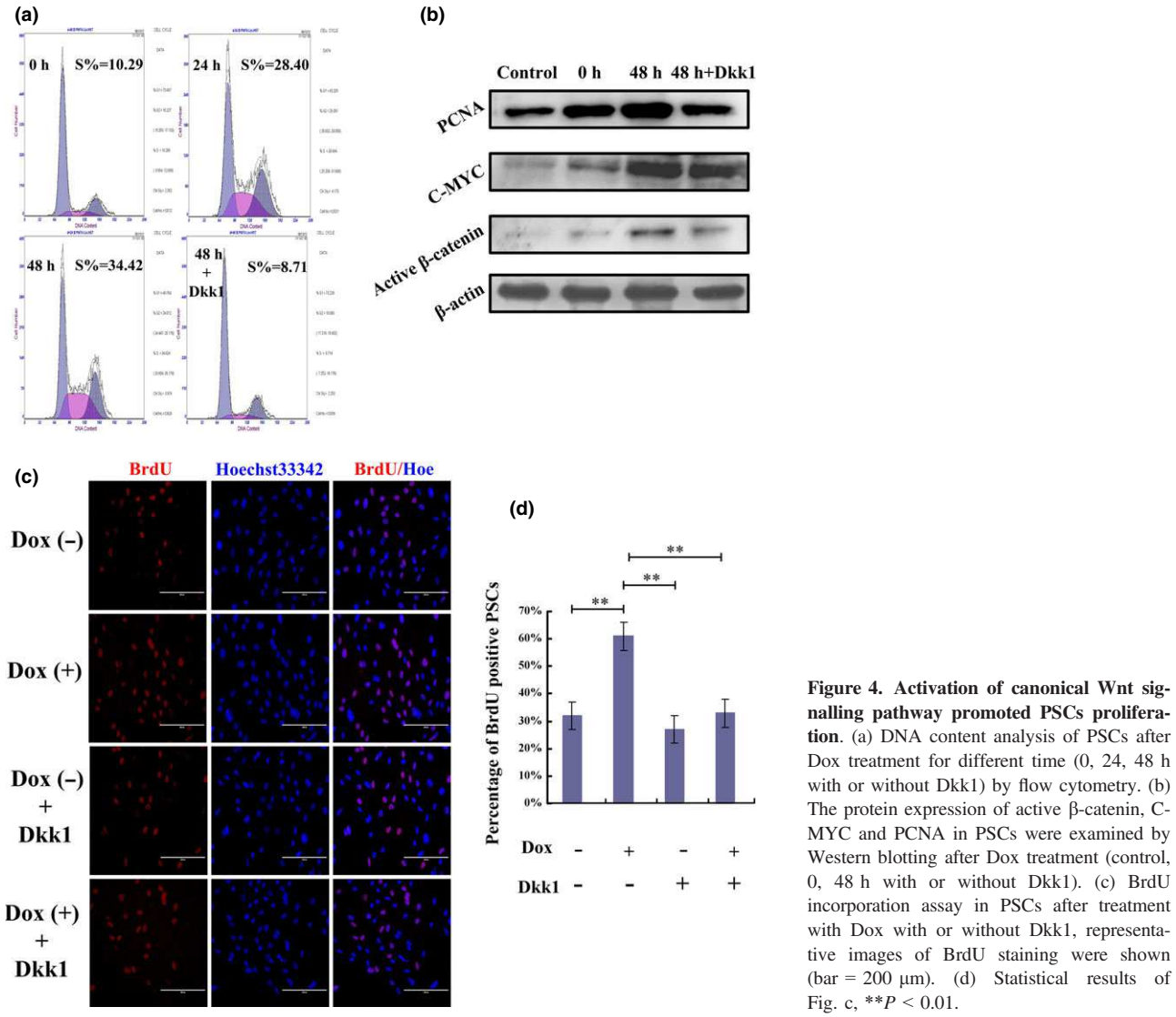

Figure 4. Activation of canonical Wnt signalling pathway promoted PSCs proliferation. (a) DNA content analysis of PSCs after Dox treatment for different time (0, 24, 48 h with or without Dkk1) by flow cytometry. (b) The protein expression of active β-catenin, C-MYC and PCNA in PSCs were examined by Western blotting after Dox treatment (control, 0, 48 h with or without Dkk1). (c) BrdU incorporation assay in PSCs after treatment with Dox with or without Dkk1, representative images of BrdU staining were shown (bar = 200 μm). (d) Statistical results of Fig. c, $^{**}P < 0.01$.

The Wnt signalling pathway involves a large number of proteins that are required for basic developmental processes, such as cell fate specification, progenitor cell proliferation and control of asymmetric cell division, in many different organs and species (19,20). Previous studies have produced evidence that pancreatic growth and pancreatic cell differentiation are regulated by Wnt signalling (21,22). However, these reports have not fully elaborated Wnt signalling function and mechanisms during PSC proliferation and differentiation, towards the insulin-secreting cells. Here, we found that porcine PSC proliferative ability was promoted after Dox stimulation, detected by cell cycle and BrdU assay. This increasing tendency could be reversed by Dkk1, an inhibitor of the canonical Wnt signalling pathway (11,23–25). This indicates that Dox induced activation of canonical Wnt signalling pathway in porcine PSCs. To further testify to the results, we examined expression of active β-catenin, key mediator of the canonical Wnt signalling pathway. Western blotting results suggested that expression of active β-catenin was enhanced for 48 h after Dox induction, and Dkk1 rescued this increasing tendency. We conclude that promotion of PSC proliferation was caused by activation of the canonical Wnt signalling pathway induced by addition of Dox. The effect of the canonical Wnt signalling pathway on PSC proliferation was in accordance with its function in other types of adult stem cells reported in previous studies, such as neural stem cells and bone marrow mesenchymal stem cells (11,23–26). Recently, many investigations have reported functions of canonical Wnt signalling in cell differentiation (27), but the increasing differentiation efficiency of PSCs into insulin-secreting cells is still an issue of research. We aim to further explore the effects

of canonical Wnt signalling on PSC differentiation towards insulin-secreting cells, utilizing the Dox-induced *Wnt3a* expression platform.

Many studies have shown that activation of canonical Wnt signalling up-regulates expression of *PCNA* and *C-MYC* genes, thus promoting proliferation ability in many kinds of cells, such as human Sertoli cells and cancer stem-like cells (28,29). Similarly, we found that in porcine PSCs, expression of PCNA and C-MYC proteins was up-regulated after Dox stimulation, while this increasing tendency was reversed by Dkk1. At the same time, we also observed that expression of active β-catenin was increased; hence, we concluded that *Wnt3a* expression induced by Dox activated the canonical Wnt signalling pathway effectively. There was intracellular accumulation of β-catenin, causing its entry into nuclei, inducing its regulation and expression of specific downstream genes, such as *PCNA* and *C-MYC*, which promoted the porcine PSC proliferation. Thus it had interested us to select *Wnt3a* to promote proliferation in our PSCs.

Taken together, we successfully established the porcine PSC line with *Wnt3a* expression induced by T-REx™. This will provide an ideal platform for further investigating mechanisms of PSC proliferation and differentiation. We also found that cell proliferation ability enhanced after *Wnt3a* expression, was induced by Dox. This partially solves the problem of poor vitality and low survival along with increase in PSC generation, during *in vitro* culture. Furthermore, we will be able to stimulate PSC proliferation potential *in vivo* after transplantation, by providing drinking water and food already containing Dox. Thus we could attempt to have a long-term treatment effect for diabetes.

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Competing interests

The authors have declared that no competing interests exist.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Exogenous *Wnt3a* protein promoted PSC proliferation. (a) BrdU incorporation assay in PSCs after treatment with 20 μg/ml *Wnt3a* for 24 h, representative images of BrdU staining were shown. bar = 200 μm. (b) Statistical results of Fig. a, $*P < 0.05$.