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RESEARCH ARTICLE

SALL4 maintains self-renewal of porcine pluripotent stem cells through downregulation of OTX2

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Abstract Sall4 as one of the spalt family members contains several alternative splicing variants, which are differentially expressed and has a key role in maintaining pluripotent stem cells. However, the molecular features and function of SALL4 have not been well elucidated in porcine induced pluripotent stem cells (piPSCs). In this study, we identified *SALL4* splice variants and found two *SALL4* splicing variants through analysis of the porcine transcriptome data derived from piPSCs. SALL4A was only detected in piPSCs but SALL4B was globally expressed in porcine tissues and piPSCs. The level of SALL4B was significantly reduced when piPSCs differentiation occurred, however, the expression of SALL4A was not affected, indicating that SALL4B may be essential for the maintenance of piPSCs self-renewal. Overexpression of SALL4A and SALL4B in PEF cells could significantly stimulated expression of endogenous pluripotent genes, when SALL4B significantly promoted OCT4 expression. Conversely, SALL4A significantly promoted KLF4 expression. Additionally, both SALL4A and SALL4B could repress *OTX2* promoter activity in a dose-dependent manner. Conversely, OTX2 also negatively regulated SALL4 expression. These observations indicate that a negative feedback regulatory mechanism may exist between SALL4 and OTX2, which is useful for the maintenance of the self-renewal of piPSCs.

Keywords OTX2, pluripotency, pig, SALL4, transcription regulation

1 Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst and retain the unique features for self-renewal and pluripotent potential allowing ESCs to be used as 'seed' cells in the field of regenerative medicine. Porcine pluripotent stem cells are especially important for regenerative medicine because they can be applied as models for human diseases[1,2]. Porcine induced pluripotent stem cells (piPSCs) have been generated by using mouse and human transcription factors OSKM (Oct4/Sox2/Klf4/c-Myc)[3,4]. However, most piPSC lines do not fulfill all criteria of putative ESCs[2–5]. The regulatory networks of ESCs in human and mouse have been extensively investigated and a large number of pluripotent genes have been confirmed as being essential for fate decision in ESCs. Investigating the functions of these pluripotent genes will help to establish the naive state of piPSCs and to uncover the mechanisms that restrict piPSCs potency.

A set of transcription factors, such as Oct4, Nanog, Esrrb and Sall4, have been shown to regulate pluripotent states[6,7]. Sall4 works as a key regulator to achieve the precise control of other transcription factors required for ESCs[8–12]. Therefore, to clarify the relationship between SALL4 and some of these factors would help to understand mechanisms that control the self-renewal and differentiation of piPSCs. Sall4 is vital for the maintenance of an undifferentiated state in both human and mouse ESCs[13]. Chromatin immunoprecipitation (ChIP) assays indicate that Sall4 binds to multiple loci in the genome, suggesting that Sall4 is a guardian of pluripotency that regulates many genes[7,11,14]. Previous studies indicated that Oct4, Sall4, and Nanog shared a close functional relationship[7]. Co-immunoprecipitation experiments showed that these factors interacted with one another *in vivo*[15,16]. Additionally, the three transcription factors co-occupy the promoters of many downstream target genes[16,17], indicating that these transcription factors form an integrated network in ESCs.

Since Sall4, as one of the spalt family members, is expressed in the 2-cell stage of embryos during human and mouse early development, showing a similar expression pattern to Oct4[8,18], and regulates the transcription of Oct4[16], we proposed to characterize porcine *SALL4* gene. There are two alternative splicing variants in humans and

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mouse and we identified two alternative splicing variants of porcine *SALL4*, *SALL4A* and *SALL4B,* and showed that both variants could individually regulate their downstream target genes and maintain the pluripotency of piPSCs. So far, porcine ESCs and naive piPSCs have been difficult to generate[19]. The critical problems are improper culture conditions used to generate and maintain piPSCs, and unclear state-specific regulatory circuitries. Thus, naive state piPSCs generation will benefit from an understanding of the mechanisms that control the self-renewal and differentiation of piPSCs. In this study, we explored whether porcine SALL4 splicing variants were functionally relevant to the pluripotency of piPSCs. Also, we dissected the relationship between SALL4 and OTX2 in such regulation.

2 Materials and methods

2.1 Molecular cloning of porcine *SALL4* and vector construction

Total RNAs were extracted from piPSCs by TRIzol Reagent (#15596-026, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Pig *SALL4* coding DNA sequence (CDS) was amplified by reverse transcription polymerase chain reaction (RT-PCR). The PCR fragments were cloned into pGEM-T Easy vector (A1360, Promega, Madison, WI, USA) and confirmed by DNA sequencing (Sangon Biotech, Shanghai, China). To construct *SALL4A* and *SALL4B* overexpression vector, CDS fragments of *SALL4A* and *SALL4B* were subcloned into *Bam*HI/*Xho*I sites of pEGFP-C1 (6084-1, Clontech, Palo Alto, CA, USA) to generate the recombinant expression vectors pSALL4A and pSALL4B, which produce fusion proteins of EGFP-SALL4A and EGFP-SALL4B[20].

To construct the porcine *SALL4* reporter vector, a 2.1 kb *SALL4* promoter fragment was amplified by PCR, cloned into pGEM-T Easy vector, and confirmed by DNA sequencing[21]. The sequence of the *SALL4* promoter was subcloned into *Xho*I/*Hind*III sites of pGL3-basic (U47295, Promega) to form the recombinant vector pL2.1. To construct a series of truncated reporter constructs, fragments were amplified from pL2.1, inserted into pGEM-T Easy vector, and confirmed by DNA sequencing. These *SALL4* promoter fragments were then subcloned into *Xho*I/*Hind*III sites of pGL3-basic to construct pL0.1, pL0.5, pL0.7, pL1.0, and pL1.9 vectors. The 2 kb *OTX2* promoter vector (pG-OTX2) and the truncated constructs of *OTX2* promoter (pOX-80, pOX-915, pOX-1327, and pOX-1742) have been reported[22].

2.2 Cell culture and transfection

A porcine iPSC line generated in this laboratory was cultured in piPS medium, including Knockout DMEM (KO-DMEM, #10829, ThermoFisher) supplemented with 20% FBS (#16000-044, Gibco, Grand Island, NE, USA), 0.1 mmol·L^{-1} nonessential amino acids (NEAA, #11140-050, Invitrogen), 1 mmol·L^{-1} L-glutamine (#32571-093, Gibco), 10 ng·mL^{-1} LIF (ESG1106, Millipore, Temecula, CA, USA), 10 ng·mL^{-1} bFGF (GF003, Millipore), 0.1 mmol·L^{-1} β-mercaptoethanol, 50 U·mL^{-1} penicillin/streptomycin, at 37°C, 5% CO_2 in a humidified atmosphere. The piPSCs were maintained on feeders made by mitotically inactive mouse embryonic fibroblasts (MEF) derived from ICR mice, and passaged using 1 mg·mL^{-1} Collagenase type IV (#17104-019, Gibco) and 0.05% Trypsin (#27250-018, Gibco) every 2 to 3 days. For the differentiation of piPSCs, cells were treated by retinoic acid (RA) for various time points. The differentiated piPSCs was named piPS^{+RA}. The 293T cells were cultured in DMEM with 10% FBS at 37°C, 5% CO_2. Porcine embryonic fibroblasts (PEF), which were made by following the procedure described previously[22], were cultured in DMEM with 15% FBS. To perform the transfection, cells were seeded in 6-well culture dishes 24 h prior to transfection. When they reached 80% confluence, 3.5 μg plasmids of pSALL4A and pSALL4B were transfected into 293T cells by using Lipofectamine 2000 Reagent (#11668-019, Invitrogen) following the manufacturer's instructions. At 36 h post-transfection, GFP-positive cells were examined and collected for western blotting. For overexpression of SALL4 in piPSCs, cells plated on 6-well plates were transfected with 3.5 μg pSALL4A, pSALL4B, and pEGFP-C1 using Lipofectamine 2000 Regent for 36 h, respectively. To knockdown SALL4 expression, 200 nmol·L^{-1} RNA interfering fragments, si-254, si-446, and si-1724, and negative control were transfected into piPSCs, with X-treme GENE siRNA Transfection Reagent (04 476 093 001, Roche, Basel, Switzerland) for 36 h (Table S1).

To investigate the interaction of SALL4A and SALL4B with the *OTX2* promoter, 0.125 μg pSALL4A or pSALL4B with 0.125 μg pG-OTX2, pOX-1742, pOX-1327, pOX-915, pOX-80 and the internal control pRT-TK (0.025 μg) were cotransfected into 293T cells on a 48-well plate, using Lipofectamine 2000 Regent. To determine the regulatory function of OTX2, the truncated constructs, including pL0.1, pL0.5, pL0.7, pL1.0, pL1.9, and pL2.1 were cotransfected with pE-OTX2 and pRT-TK into 293T cells on a 48-well plate. For time-dependent luciferase assays, 0.125 μg pG-OTX2 and 0.125 μg of *SALL4* constructs with pRT-TK (0.025 μg) were cotransfected into 293T cells. The luciferase activity was detected at 12 h intervals (from 0 to 48 h). To knockdown OTX2 expression, 200 nmol·L^{-1} RNA interfering fragments, si-543, si-863 and si-1115, or negative control were transfected into piPSCs (Table S1). For dose-dependent luciferase assays, 0.125 μg of pG-OTX2 and different amounts of *SALL4* constructs (from 0 to 0.5 μg) were

cotransfected into 293T cells for 36 h. Control experiments were performed by transfection of pG-OTX2 only, without adding *SALL4* constructs.

2.3 Luciferase assay

Cells were harvested after transfected for 36 h and lysed for 10 min at room temperature using passive lysis buffer (E1910, Promega). Luciferase activity was detected by luciferase assay reagents (E1910, Promega) and BHP9504 microplate illuminometer (D04407H, Hamamatsu, Beijing, China). Triplicates were measured for each treatment, and the average values of the ratio of firefly luciferase units to renilla luciferase units were used for data analysis.

2.4 Reverse transcription and polymerase chain reaction

Total RNAs from piPSCs were extracted using TRIzol Reagent (#15596-026, Invitrogen) following the manufacturer's instructions. RNA samples were examined by the measurement of OD260/280 ratio. RNAs with a ratio of 2.0 were used for reverse transcription. One microgram RNA was reverse-transcribed using Revert Aid Reverse Transcriptase (EP0732, ThermoFisher Scientific, Rockford, IL, USA). PCR was performed for 35 cycles at 94°C 30 s, 56°C 30 s, and 72°C 45 s. PCR products were analyzed by 1.5% agarose gel. GAPDH was used as internal control. Quantitative RT-PCR (qRT-PCR) was performed in triplicates using SYBR Green PCR Master Mix (DRR420, Takara Bio Inc., Kusatsu, Shiga, Japan), and detected with CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The reaction condition was as follows: 95°C 30 s as the first cycle, and 40 cycles of 95°C 5 s, and 60°C 30 s. Relative expression levels of genes were normalized to GAPDH and calculated using $2^{-\Delta\Delta C_T}$. To perform genomic DNA PCR, one microgram genomic DNA extracted from piPSCs using TIANamp Genomic DNA Kit (DP304-02, Tiangen Biotech, Beijing, China) was used as the template. PCR reactions were performed for 35 cycles at 94°C 30 s, 60°C 30 s, and 72°C 1 min. The PCR products were separated by 1.5% agarose gel electrophoresis. Primers used in this study are listed in Table S2.

2.5 Immunostaining analysis

For immunocytochemical analysis, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min and 0.1% TritonX-100 for 10 min at room temperature. After washing twice with ice-cold PBS, cells were blocked in BSA-blotting buffer (1% BSA and 0.1% Tween 20 in PBS) for 1 h, and then incubated in BSA-blotting buffer with primary antibodies, including OCT4 (1:300, SC-5279, Santa Cruz Biotechnology, Dallas, TX, USA), SALL4 (1:500, GeneTex, Irvine, CA, USA), and SOX2 (1:400, #3579, Cell Signaling Technology, Danvers, MA, USA), at 4°C overnight. After washing three times, cells were stained with a secondary antibody conjugated with Alexa Fluor 488 (1:800, A-21202, ThermoFisher Scientific) for 1 h. For nuclear staining, the fixed cells were incubated with 10 $mg \cdot mL^{-1}$ DAPI solution for 2 min. The images were documented using a fluorescence microscope (ECLIPSE Ti-U, Nikon, Tokyo, Japan).

2.6 Alkaline phosphatase staining

To perform alkaline phosphatase (AP) staining, piPSCs were washed twice using ice-cold PBS, fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature, followed by washing three times using ice-cold PBS. Cells were then incubated at room temperature in 0.1 $mol \cdot L^{-1}$ Tris buffer with 1.0 $mg \cdot mL^{-1}$ Fast Red TR, 0.4 $mg \cdot mL^{-1}$ Naphthol AS-MX Phosphate (#1596-56-1, Sigma, St Louis, Mo, USA). After 10 min incubation, the AP positive piPSC colonies appeared red in color.

2.7 Western blotting

Thirty-six-h post-transfected cells were collected by centrifugation at 10000 *g* for 5 min. The 1×10^6 cell pellet was resuspended in SDS-PAGE loading buffer (50 $mmol \cdot L^{-1}$ Tris-HCl pH 6.8, 2% SDS, 10% glycerin, 2% β-mercaptoethanol and 0.05% bromophenol blue) and heated at 100°C for 5 min. Twenty μg of the protein sample was loaded onto 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to a PVDF membrane (LC2002, Invitrogen) by semidry electrophoretic transfer (Bio-Rad Laboratories) for 45 min at 15–20 V. The membrane was blocked with the blocking buffer (20 $mmol \cdot L^{-1}$ Tris/HCl pH7.6, 137 $mmol \cdot L^{-1}$ NaCl, 0.1% Tween 20 and 8% dried skim milk) at 4°C overnight, and then incubated with the primary anti-GFP antibody (1:5000, KM8009, Sungene Biotech, Tianjin, China) or primary anti-SALL4 antibody that targets both SALL4A and SALL4B (1:2000, GTX109983, GeneTex) at 37°C for 2 h. After washing three times with TBS-T buffer (20 $mmol \cdot L^{-1}$ Tris/HCl pH 7.6, 137 $mmol \cdot L^{-1}$ NaCl, 0.1% Tween 20), the membrane was incubated with a HRP-conjugated secondary antibody (1:3000, A0258, Beyotime, Shanghai, China) at 37°C for 1 h. After washing in TBS-T three times at room temperature, the membrane was incubated in the enhanced chemiluminescent substrate (32106, ECL, Pierce Biotechnology Inc., Rockford, IL, USA) for 1 min and detected with a Chemiluminescent Imaging System (ZY058176, Tanon-4200, Shanghai, China).

2.8 Data sources and bioinformatics analysis

MeDIP-seq data of LDM (GSE26382)[23], piPS-L (EBI number: E-MTAB-2634)[5] and piPS-F(GSE36114)[19,24] were downloaded from National Center for Biotechnology

Information. The JASPAR database was used to search for putative binding sites of transcription factors in the porcine *SALL4* promoter sequence.

2.9 DNA methylation analysis and bisulfite genomic sequencing analysis

MeDIP reads were separately aligned to the pig genome (SGSC Sscrofa10.2/susScr3) using the Bowtie software[25]. We used MACS (version 1.4.0 beta) for peak detection and analysis of immunoprecipitated single-end sequencing data to find genomic regions that are enriched in a pool of specifically precipitated DNA fragments.

Genomic DNA from piPSCs, porcine longissimusdorsi muscle (LDM), liver, kidney and ovary were isolated by using TIANamp Genomic DNA Kit following the manufacturer's instructions. Bisulfite modification using EpiTect Bisulfite Kit (#59104, Qiagen, Hilden, Germany) was carried out following the manufacturer's instructions, and bisulfite PCR amplifications were performed as a regular PCR reaction and followed by DNA sequencing. The results were analyzed by BiQ Analyzer software.

2.10 Statistical analysis

Data are presented as mean±SD (n = 3). Means were compared by Student's t-test with statistical significance at $P < 0.05$.

3 Results

3.1 Identification of porcine *SALL4* alternative splicing variants

We found the porcine *SALL4* gene was highly and specifically expressed in pluripotent stem cells and porcine tissues (Fig. 1a). Previous reports indicated that the human and mouse SALL4 genes have two splicing variants (Fig. S1)[26,27]. To investigate porcine SALL4 isoforms, we analyzed the transcriptome data from three porcine iPSC lines, which include LIF-dependent piPSCs (piPS-L)[5], FGF2-dependent piPSCs (piPS-F)[19] and LIF/FGF2-dependent piPSCs (piPS-LF)[28], LDM and porcine liver tissue. Porcine SALL4 was found to encode the two alternative splicing variants *SALL4A* and *SALL4B* in piPSCs, showing a similar genomic structure to the human SALL4 gene (Fig. 1a; Fig. S1). The SALL4 alternative splicing variants were confirmed by RT-PCR analysis and DNA sequencing (Fig. 1b; Fig. S2). To investigate the function of SALL4A and SALL4B in pluripotent cells, we constructed the expression vectors pSALL4A and pSALL4B, which were confirmed by enzyme digestion (Fig. 1c). The pSALL4A, pSALL4B and a control pEGFP-C1 were transfected into HEK-293T cells for 48 h, and the expression of EGFP-SALL4 fusion protein was confirmed by western blotting (Fig. 1d). In a cell-based assay, EGFP positive and EGFP-SALL4 positive cells were observed with fluorescence microscopy. EGFP-SALL4 fusion protein was translocated into nuclei, whereas EGFP protein was present throughout whole cells (Fig. 1e). Thus, the cloned *SALL4A* and *SALL4B* can be translated into functional proteins and be used for further research.

3.2 Porcine SALL4 expression pattern

To investigate SALL4A and SALL4B expression in different porcine somatic and pluripotent cells, we analyzed the DNA methylation profile of SALL4. MeDIP-Seq data of SALL4 demonstrated that the SALL4 methylation level in LDM was higher than that in pluripotent stem cells (Fig. 2a). The bisulfite genomic sequencing further confirmed that SALL4 was highly methylated in porcine tissues, including liver, kidney, ovary and LDM, but was weakly methylated in piPSCs (Fig. 2b). The RT-PCR analysis showed that *SALL4B* was globally expressed in porcine tissues and pluripotent stem cells, however, *SALL4A* was mainly expressed in pluripotent stem cells and was absent in porcine tissues derived from ectoderm (skin and brain), mesoderm (LDM, heart, and ovary), and endoderm (lung, liver, and pancreas). Moreover, the expression level of *SALL4B* was higher than that of *SALL4A* in piPSCs (Fig. 2c). Since Sall4 was reported to be relevant to embryo development and pluripotent stem cells self-renewal[16,26,29,30], we then investigated SALL4 expression in undifferentiated (AP positive) and differentiated (AP negative) piPSCs (Fig. 2d upper). Immunofluorescence staining showed that high level expression of pluripotent factors OCT4, SOX2, and SALL4 was detected in piPSCs. However, the expression of SALL4 was obviously decreased, and the expression of OCT4 and SOX2 was absent in differentiated cells (Fig. 2d lower). These results indicated that SALL4 was an impotent factor for porcine iPSCs.

3.3 SALL4 regulates the expression of pluripotent genes

To explore the regulatory function of SALL4, we examined the expression of pluripotent genes in piPSCs and retinoic acid-induced piPS (piPS^{+RA}) cells. The endogenous *SALL4* expression level was significantly reduced in piPS^{+RA} cells versus normal piPSCs. During piPSCs differentiation, the expression of *OCT4* and *ESRRB* were also significantly reduced (Fig. 3a). However, the expression of *OTX2* was increased at the early stage of differentiation, but reduced significantly in the later stage of differentiation (Fig. 3a). We noticed that *SALL4B* was more significantly reduced than that of *SALL4A* during piPSCs differentiation (Fig. 3b). To further investigate *SALL4A* and *SALL4B* function, PEF cells were transfected by pSALL4A and pSALL4B, and qRT-PCRs were

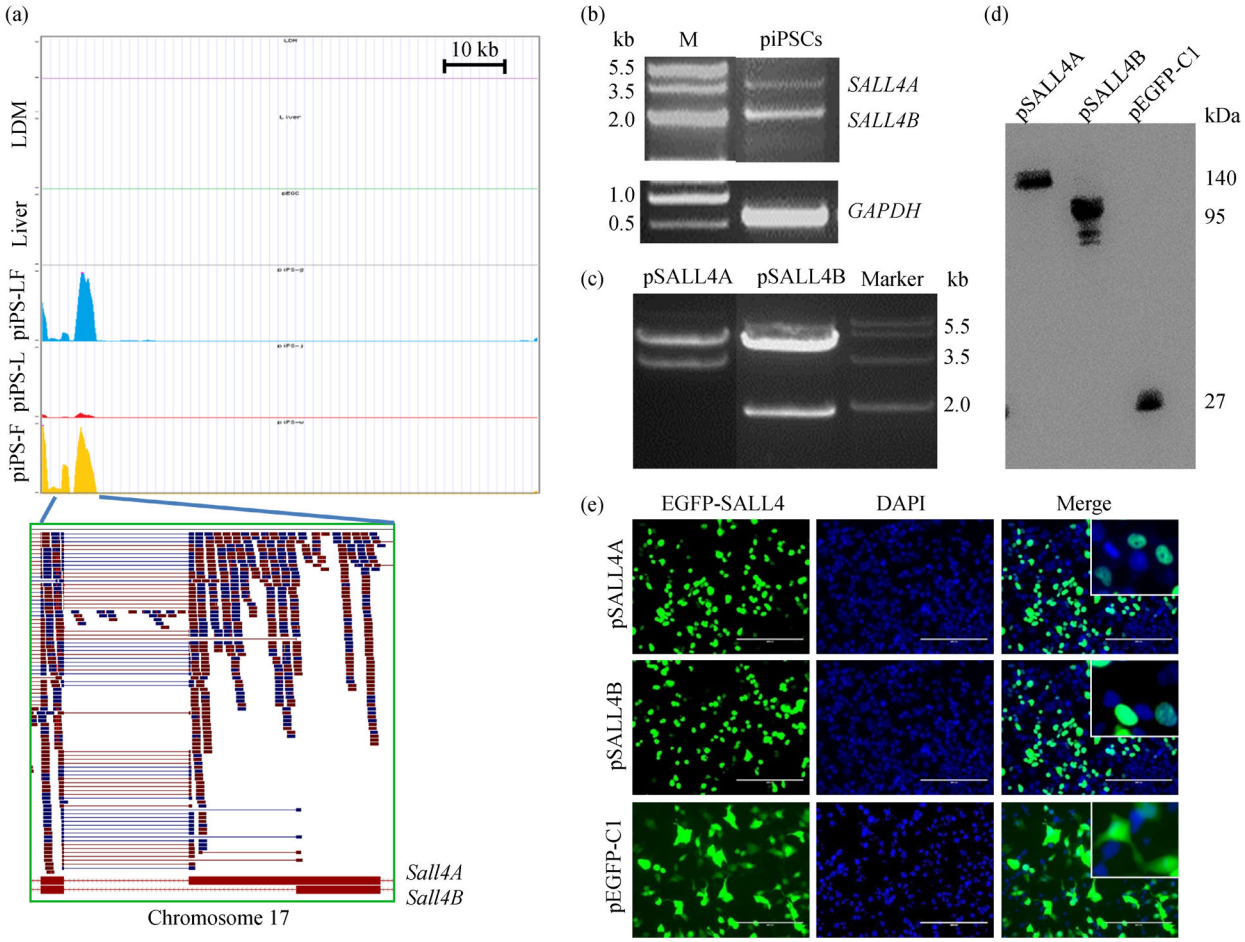

Fig. 1 Identification and cloning of porcine *SALL4* alternative splicing variants. (a) Transcriptome analysis of *SALL4* in porcine tissues and pluripotent cells. Sequencing reads for *SALL4A* and *SALL4B* in piPS-F cells are given in the green box; (b) RT-PCR analysis of *SALL4* splicing variants in piPSCs; (c) enzyme digestions (*Bam*HI/*Xho*I) to confirm the constructs of pSALL4A and pSALL4B; (d) western blot analysis of fusion proteins, EGFP-SALL4A (140 kDa) and EGFP-SALL4B (95 kDa), and EGFP (27 kDa) in 293T cells; (e) vectors of pSALL4A, pSALL4B, and pEGFP-C1 were transfected into 293T cells for 48 h. EGFP-SALL4 fusion proteins were translocated into nuclei.

conducted to determine the expression of pluripotent genes. Results indicated that both SALL4A and SALL4B could stimulate *OCT4*, *KLF4*, and *ESRRB* expressions, in which SALL4B significantly promoted *OCT4* expression, while SALL4A significantly promoted *KLF4* expression. *ESRRB* expression could be activated by either SALL4A or SALL4B (Fig. 3c). Importantly, *OTX2* expression was downregulated by overexpression of SALL4A and SALL4B.

Three SALL4 siRNAs, which include si-254, si-446, and si-1724, were synthesized and used for RNA interfering assay. *SALL4* expression level was reduced by 60% to 80% by the siRNAs treatment, especially by si-254 (Fig. 3d). Additionally, the morphology of piPSCs that were treated with siRNA si-254 produced cellular colonies that were much less compact compared with control cells (Fig. 3e). We found that downregulating *SALL4* could significantly reduce the expression of endogenous *OCT4* and *ESRRB* in piPSCs, but led to the remarkable increase of *OTX2* expression (Fig. 3d). These observations indicated that *SALL4* knockdown could disturb the self-renewal and pluripotent state of piPSCs. Also, SALL4 and OTX2 shared a negative regulatory correlation, which is worthy of further investigation.

3.4 SALL4 represses OTX2 expression

To understand the SALL4 regulatory relationship with OTX2, we cloned a 2 kb porcine *OTX2* promoter fragment and constructed several OTX2 reporter vectors as previously reported[22]. Luciferase assays showed that OTX2 reporter had strong promoter activity in 293T cells that were transfected by pG-OTX2, and the *OTX2* promoter activity was significantly repressed by over-expression of either SALL4A or SALL4B (Fig. 4a). To further monitor the SALL4 binding region on the *OTX2*

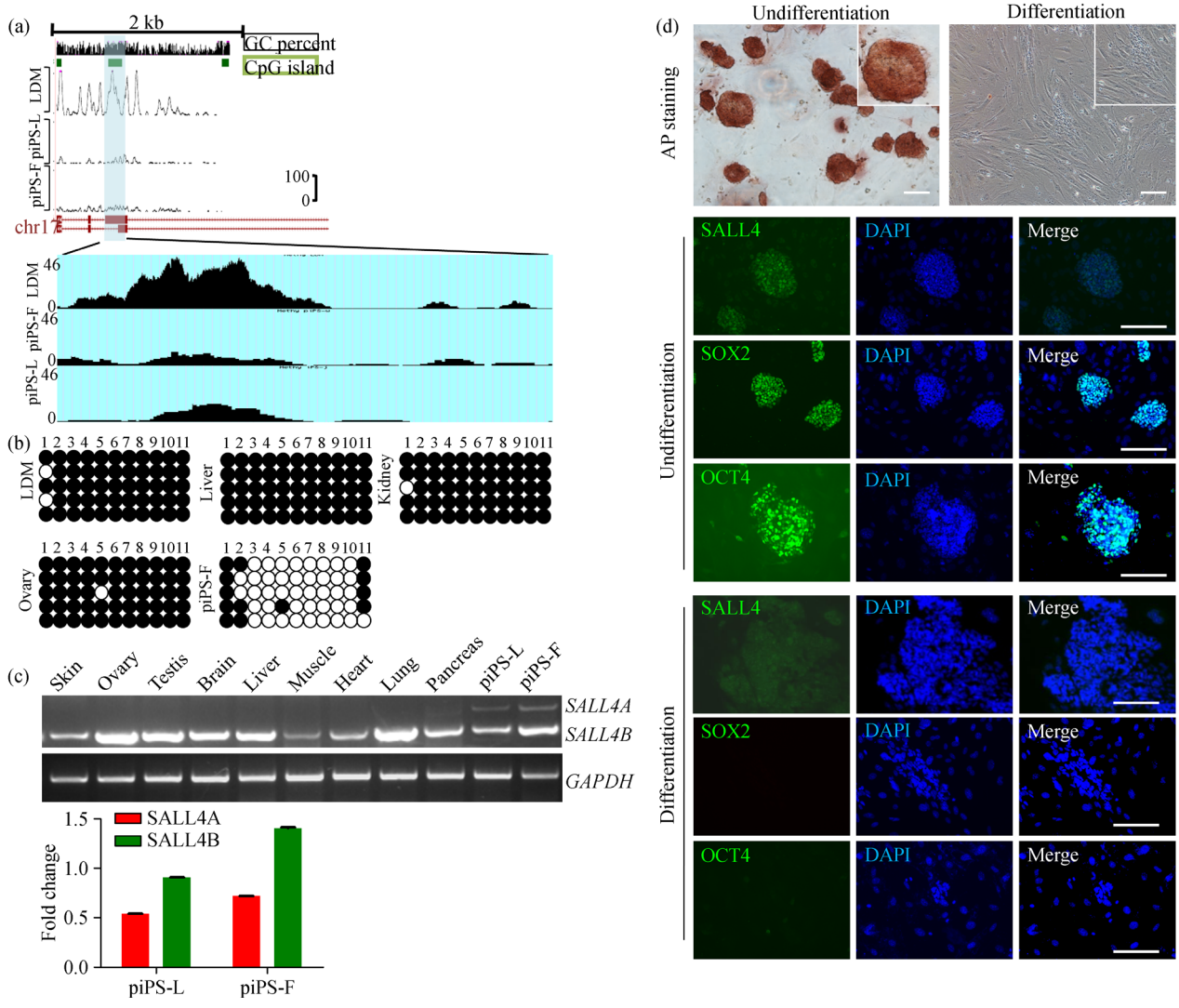

Fig. 2 SALL4 expression in porcine tissues and pluripotent cells. (a) Dynamic DNA methylation profile of porcine *SALL4*. MeDIP-Seq data of *SALL4* methylation in porcine *longissimusdorsi* muscle (LDM) and piPSCs were visualized in UCSC genome browser; (b) bisulfite genomic sequencing analysis of *SALL4* in LDM and piPS-F cells. Open and filled circles represent unmethylated and methylated CpGs; (c) RT-PCR (upper) and densitometry (lower) analyses of *SALL4* expression in porcine tissues and piPSCs; (d) alkaline phosphatase staining (upper) and immunofluorescence staining (lower) of SALL4, SOX2, and OCT4 in undifferentiated and differentiated piPSCs. Scale bar, 50 μm.

promoter, several truncated *OTX2* promoter constructs were made and the potential transcription factor binding sites for SALL4, NANOG, OCT4, and SOX2 were notated (Fig. 4b upper). Results of promoter activity assay showed that between –1327 to –915 regions the promoter activity displayed a significant change, indicating that this is a vital regulatory site (Fig. 4b lower). We then used the truncated constructs transfected with either pSALL4A or pSALL4B to determine whether SALL4 negatively regulates *OTX2* promoter activation. When the distal sequence (–1327 to –915 regions) of the *OTX2* promoter was removed, *OTX2* activity was significantly repressed by SALL4A or SALL4B (Fig. 4c). The time and dose dependent assays further proved that SALL4A and SALL4B negatively regulated *OTX2* promoter activity in a dose and time dependent manner (Fig. 4d). To investigate the regulatory function of SALL4A/B and OTX2 in piPSCs, the piPSCs were transfected with pE-OTX2, pE-OTX2 plus pSALL4A and pE-OTX2 plus pSALL4B. Alkaline phosphatase staining showed that overexpression of OTX2 in piPSCs could partially reduce the AP staining and the proportion of AP^+ colonies in $OTX2^+$piPSCs was significantly lower than that in the control group. In addition, the AP staining showed that piPSCs transfected with SALL4A/B exhibited much more compact colonies compared with $OTX2^+$piPSCs group (Fig. 4e). These observations indicate that SALL4 can negatively regulate the activity of porcine *OTX2* promoter.

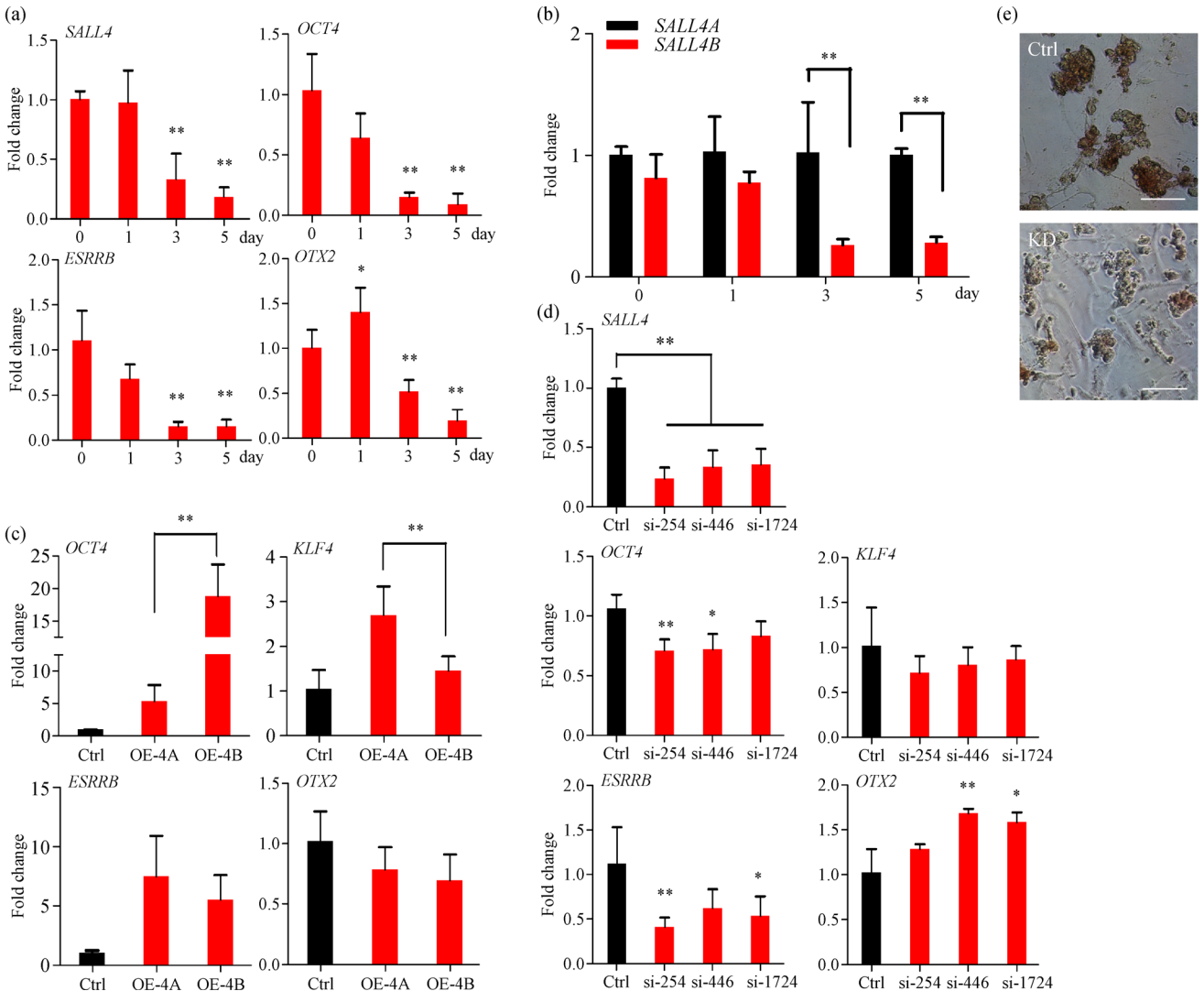

Fig. 3 SALL4 regulates the expression of pluripotent genes. Quantitative RT-PCR analyses were applied to determine the expression of pluripotent genes in piPSCs and PEF cells. (a) Expression of *SALL4* and pluripotent genes in the differentiated piPSCs (piPS^{+RA}) that were treated by retinoic acid (RA) for various time points; (b) expression of *SALL4A* and *SALL4B* in piPS^{+RA}; (c) overexpression of SALL4A (OE-4A) and SALL4B (OE-4B) in PEF cells for 48 h. Ctrl, cells were transfected by pEGFP-C1; (d) knockdown (KD) *SALL4* expression by siRNAs affected the expression of pluripotent genes. Ctrl, cells were transfected with an unspecific siRNA. Data are presented as mean±SD, * $P < 0.05$, ** $P < 0.01$, $n = 3$; (e) morphology of piPSCs with (KD) and without (Ctrl) siRNA treatment. Scale bar, 100 μm.

3.5 OTX2 regulates SALL4 expression

In mice, loss of Otx2 can severely affect *Sall4* expression[31]. In OTX2 overexpressing piPSCs, we found that AP positive colonies of OTX2^{+}piPSCs were reduced compared with the control group, which displayed a more compact morphology with uniform AP staining (Fig. 5a). RT-PCR and qRT-PCR analysis showed that overexpression of *OTX2* in piPSCs significantly decreased the expression level of both SALL4A and SALL4B (Fig. 5b, Fig. 5c). Conversely, when OTX2 was knocked down by siRNAs in piPSCs, the level of SALL4 protein was significantly increased, which was confirmed by western blotting assay (Fig. 5d). This result suggests that OTX2 might have a role in negatively regulating SALL4 expression. To determine the effect of OTX2 on *SALL4* expression, a 2.1 kb *SALL4* promoter fragment was cloned from piPSCs, and confirmed by DNA sequencing (Fig. S3). Within the promoter sequence, multiple putative OTX2 binding sites, and several OCT4 and SOX2 binding sites were found in the JASPAR database (Fig. S3). Based on 2.1 kb DNA fragment, several truncated SALL4 promoter fragments, including 0.1, 0.5, 0.7, 1.0, 1.9, and 2.1 kb, were amplified (Fig. S4) and subcloned into pGL3-basic plasmid to construct several Luciferase reporter vectors. The constructs were then transfected into 293T

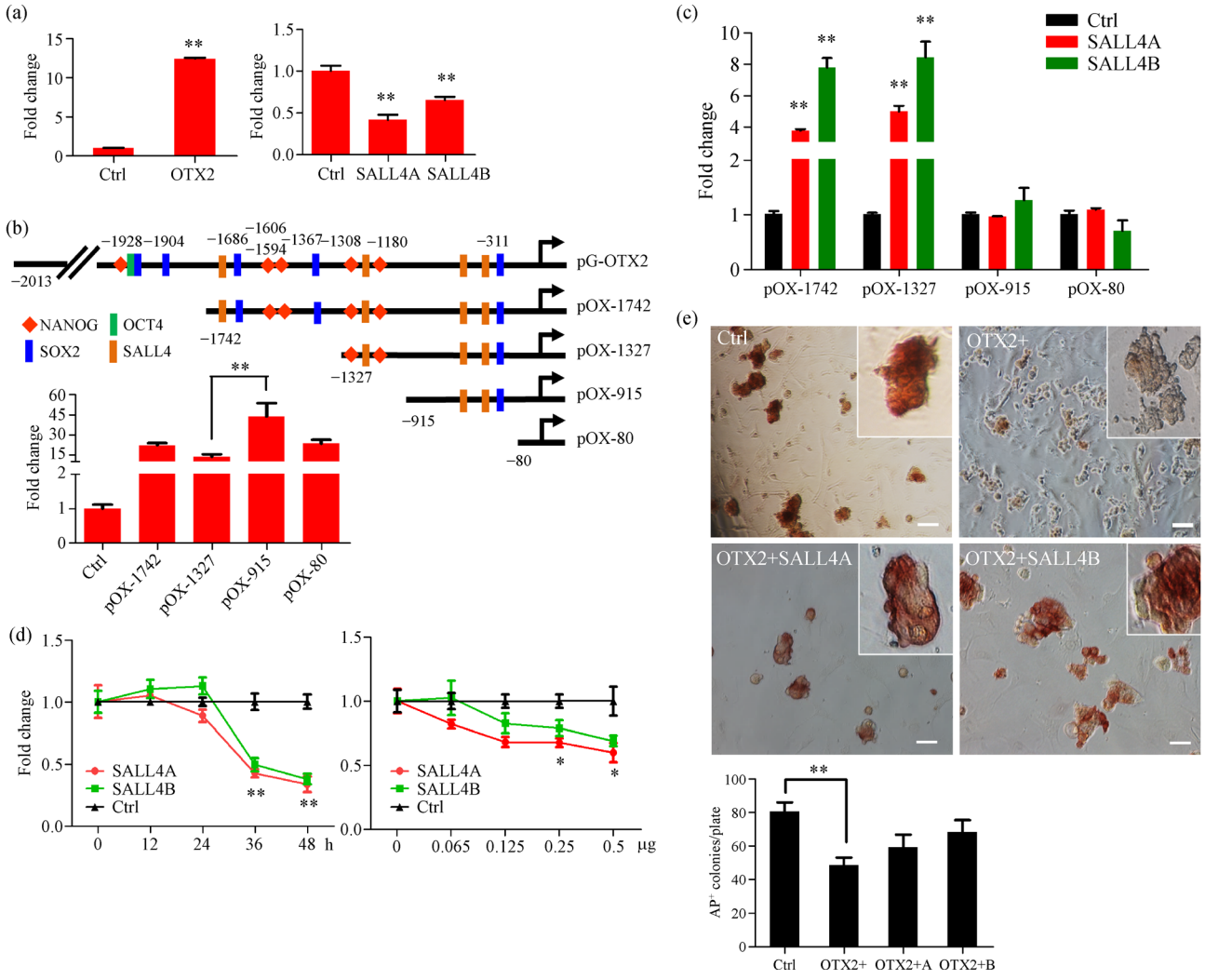

Fig. 4 SALL4 suppresses OTX2 expression. (a) Luciferase assay of *OTX2* promoter activity. The pG-OTX2 only (left) and pG-OTX2 with pSALL4A and pSALL4B (right) were cotransfected into 293T cells for 36 h. Ctrl cells were cotransfected by pG-OTX2 and pGL3-basic; (b) diagram of pG-OTX2 and the truncated *OTX2* promoter constructs with potential transcription binding sites (upper). Luciferase assay of *OTX2* promoter activity in 293T cells (lower). Ctrl, cells were transfected by pGL3-basic; (c) SALL4A and SALL4B regulate *OTX2* promoter activation in 293T cells. Ctrl, cells were transfected by pEGFP-C1; (d) luciferase assays. For time-dependent assay (left), pG-OTX2 with pSALL4 and pSALL4B were cotransfected into 293T cells for 48 h. For dose-dependent assay (right), pG-OTX2 and different amount of SALL4 constructs were cotransfected into 293T cells for 36 h. Ctrl, cells were transfected by pGL3-OTX2; (e) alkaline phosphatase staining of overexpression of OTX2 (pE-OTX2, OTX2 +) and suppression of OTX2 (OTX2 + plus pSALL4A and pSALL4B) in piPSCs. Ctrl, cells were transfected by pEGFP-C1. Number of AP positive clones was counted in 36 h post-transfection. Scale bar, 50 μm. Data are presented as mean±SD, * $P < 0.05$; ** $P < 0.01$; $n = 3$.

cells followed by luciferase assays. The *SALL4* promoter was significantly activated in the construct pL0.7 versus pL0.5 (Fig. 5e), suggesting that this region retains the crucial regulatory sequence that regulates *SALL4* promoter activity (Fig. 5e). *SALL4* reporter pL2.1 and five truncated constructs pL0.1, pL0.5, pL0.7, pL1.0, and pL1.9 were transiently cotransfected with pE-OTX2 into 293T cells. The promoter activity was significantly repressed in cells transfected with pL0.7 vs. pL0.5 (Fig. 5f), which was further confirmed the observation seen in Fig. 5e. These results indicated that OTX2 could block activation of the *SALL4* promoter by binding to the distal region of *SALL4* promoter. In a future study, gel shift assay or ChIP-seq experiments might be needed to reveal whether OTX2 directly binds to the *SALL4* promoter.

4 Discussion

4.1 Identification of porcine SALL4 splicing variants

Pluripotent stem cells exhibit two features distinguishing that from somatic cells, which are pluripotency and self-renewal and they also possess a number of unique

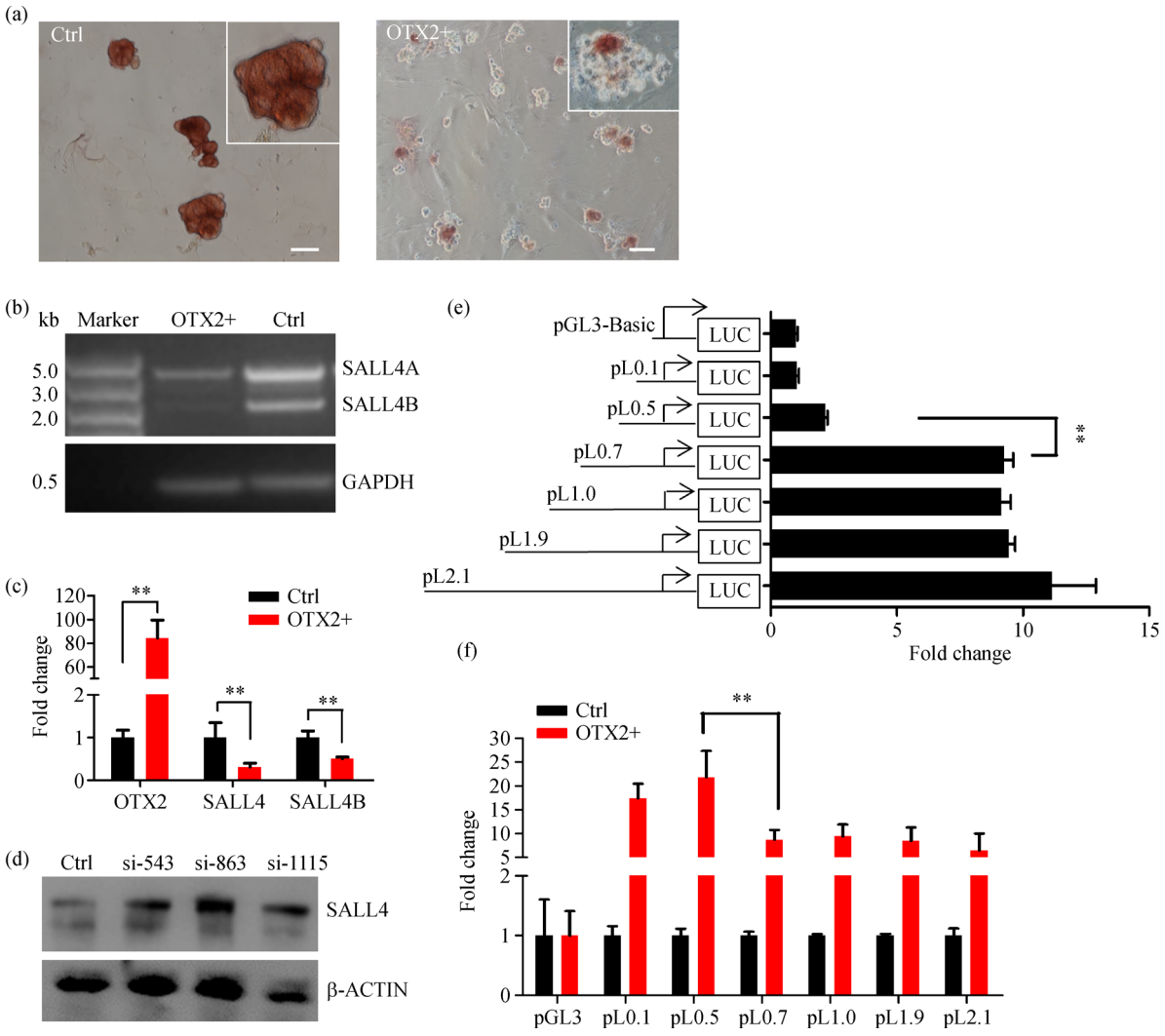

Fig. 5 OTX2 regulates SALL4 expression. (a) Morphology and AP staining of piPSCs that were transfected with pE-OTX2 (OTX2+). Ctrl, cells without transfection of pE-OTX2. Scale bar, 50 μm; (b, c) semiquantitative (b) and quantitative (c) RT-PCR analyses of *SALL4A* and *SALL4B* in piPSCs transfected by pE-OTX2 (OTX2+). Ctrl, cells were transfected by pEGFP-C1; (d) western blot analysis of SALL4 expression in piPSCs that were treated by OTX2 siRNAs. Ctrl, cells were treated by an unspecific siRNA; (e) constructs (left) and luciferase assay (right) of the full and truncated *SALL4* promoter; (f) luciferase assay of *SALL4* promoter activity. The *SALL4* constructs with pE-OTX2 were cotransfected into 293T cells, respectively, for 36 h. Ctrl, cells without transfection of pE-OTX2. Data are presented as mean±SD. * $P < 0.05$; ** $P < 0.01$; $n = 3$.

properties, including a diversity of splice variants. These variants can form protein–protein interactions that could lead to developmental state-specific regulatory networks, thereby increasing the biological complexity compare to a single locus. For instance, Oct4 has more than two splice variants, in which Oct4A is expressed in ESCs and iPSCs, while Oct4B is expressed in differentiated cells and somatic tissues[32–34]. In this study, we found that porcine *SALL4B* was globally expressed in somatic tissues and cells, however, *SALL4A* was only detected in piPSCs, but was undetectable in muscle and liver tissues. We also identified the splice site mutations (AA → CG) in porcine *SALL4* (Fig. S2). This indicates that porcine *SALL4A* and *SALL4B* may retain the diverse function to regulate self-renewal and pluripotency of piPSCs.

4.2 SALL4 splicing variants regulate the expression of pluripotent genes in piPSCs

Sall4 is a type of C2H2 zinc finger protein essential for establishment of pluripotent stem cells and maintaining pluripotency[35–38]. Previous studies have shown that Sall4, Oct4, Sox2 and Nanog can form a robust and integrated network to govern pluripotency in ESCs[10,39] Sall4a and Sall4b can collaborate in maintenance of the pluripotency in murine ESCs, since Sall4a and Sall4b are

able to form a homodimer or a heterodimer with each other, and to interact with Nanog[40]. Additionally, through analysis of genome wide location of Sall4a and Sall4b, Sall4b, but not Sall4a, preferentially binds to highly expressed loci in ESCs[40], in which Sall4b alone can maintain the pluripotency of embryonic stem cells. Similar observations were made in this study. We discovered that knocking down *SALL4* in piPSCs led to piPSCs differentiation. On the other hand, overexpression of SALL4 could activate the expression of pluripotency genes. Though both SALL4A and SALL4B significantly enhance expression of pluripotency genes including ESRRB, and stabilize the self-renewal of piPSCs, SALL4B compared to SALL4A can specially and significantly activate *OCT4* expression, yet, SALL4A versus SALL4B can significantly activate *KLF4* expression. These results indicate that the function of SALL4A and SALL4B is crucial for the pluripotent state of piPSCs, however, each individual may play a distinct role in regulating its downstream target genes in piPSCs.

4.3 SALL4 and OTX2 form a negative feedback regulatory network

An abundance and lack of SALL4A or SALL4B in piPSCs repressed and activated the *OTX2* promoter, respectively, in a time and dose dependent manner (Fig. 4). The transcription factor Otx2, acts as a negative switch in the regulation of transition from the naive state to primed pluripotency in ESCs. In mouse, the *Otx2* knockout ESCs showed a sphere-like colony, with uniform AP staining[41–43]. In our previous studies, we have identified that porcine OTX2 is an important cell-state-specific regulator for determining the pluripotency of piPSCs. Overexpression of OTX2 alone could repress the expression of pluripotent genes in piPSCs, and change piPSC colonies to a flattened and incompact morphology with uneven AP staining[22]. In this study, we found that overexpression of SALL4 in OTX2^{+}piPSCs can rescue cells and recover the morphology and AP activity. The dose and time dependent assays confirmed that SALL4 repressed *OTX2* activation (Fig. 4). Conversely, OTX2 can block activation of *SALL4* promoter by binding to the distal region of *SALL4* promoter and might have a role in regulating SALL4 expression negatively. Our findings suggest that SALL4 and OTX2 form a negative feedback regulatory network to maintain pluripotent states in piPSCs.

5 Conclusions

It was found that porcine SALL4, an essential factor for the maintenance of the pluripotency of piPSCs, contains two alternative splicing variants *SALL4A* and *SALL4B*, which have differential regulatory effects on the downstream target genes. We also showed that SALL4 and OTX2 provide negative feedback to balance the pluripotent state of piPSCs.

Supplementary materials The online version of this article at https://doi.org/10.15302/J-FASE-2017180 contains supplementary materials (Tables S1–S2; Figs. S1–S4).

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This article does not contain any studies with human or animal subjects performed by any of the authors.

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