SMAD4 Feedback Activates The Canonical TGF-β Family Signaling Pathways

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Research

Abstract

Background: TGF-β family signaling pathways, including TGF-β and BMP signaling pathways, are widely involved in the regulation of health and disease, which are also regulated by multiple validated mechanisms, such as genetic regulation, epigenetic regulation, and feedback regulation. The objective of this research is to investigate the molecular mechanism and function mode of SMAD4 directly feedback regulation of TGF-β family signaling pathways in porcine granulosa cells (GC).

Results: The transcriptomic alteration of porcine GCs induced by SMAD4 silencing was re-analyzed with the background of *Sus scrofa* RefSeq 11.1 (*Sscrofa* 11.1). A total of 986 differentially expressed mRNAs (DEmRNAs) were identified, including 467 down-regulated and 519 up-regulated genes. Functional assessment showed the impacts of DEmRNAs on the regulation of states and function of GCs, and the oocyte development. As the upstream receptors of SMAD4, *ACVR1B*, *BMPR2*, and *TGFBR2* were selected from down-regulated DEmRNAs for further research. In vitro, qRT-PCR and western blotting were performed and confirmed that SMAD4 significantly induced the expression of *ACVR1B*, *BMPR2*, and *TGFBR2* in porcine GCs. Besides, RACE and luciferase activity assays were carried out to identified the core promoter of porcine *ACVR1B*, *BMPR2*, and *TGFBR2*. Results from ChIP assays showed that SMAD4 directly binds to the SMAD4 binding elements (SBEs) within the core promoter of its upstream receptors by acting as a transcription factor. Furthermore, c-JUN, CREB1, and SP1 were identified as SMAD4-interacted co-activators by IP assays and inhibition of which could dramatically suppress the expression of porcine *ACVR1B*, *BMPR2*, and *TGFBR2* that induced by SMAD4 over-expression. Furthermore, three different interaction modes between SMAD4 and co-activators were identified by reciprocal ChIP assays.

Conclusions: Take together, our findings revealed a novel feedback regulatory mechanism of TGF-β family signaling pathways in porcine GCs, and demonstrated for the first time that SMAD4, the only Co-SMAD, directly feedback activates the transcription of canonical TGF-β family signaling pathway receptors by interacting with three co-activators in different modes, which improves and expands the regulatory network, especially the feedback regulation modes of TGF-β family signaling pathways in the ovary.

Introduction

Transforming growth factor-β (TGF-β) superfamily is a large group of phylogenetically conserved secreted cytokines in eukaryotes, which comprises more than 30 members, such as TGF-βs, activins, inhibins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs) [1]. Members of the TGF-β superfamily are widely expressed in various tissues and cells, which play critical roles in the regulation of health and disease, through activating or inhibiting the TGF-β family signaling pathway [2]. In general, the TGF-β family signaling pathway can be divided into two branches, i.e. TGF-β and BMP signaling pathways, which both signaled in the order of ligands, receptors (including type II and type I receptors), receptor-regulated SMADs (R-SMADs: SMAD2/3 for the TGF-β signaling pathway, and

SMAD1/5/8 for the BMP signaling pathway), and the only common mediator SMAD (Co-SMAD: SMAD4), which finally shuttling into the nucleus to regulate the transcription of target genes [3].

Meanwhile, the TGF-β family signaling pathways have also been shown to be regulated by multiple validated mechanisms, such as genetic regulation, epigenetic regulation, and feedback regulation [4, 5]. Downstream signaling proteins regulate the expression of upstream signaling molecules through regulators or axes, which is the main feedback model of TGF-β family signaling pathways [6]. The regulators involved in this feedback regulation progresses includes (i) miRNAs such as SMAD4-miR-675-TGFBR1 [6], SMAD4-miR-425-TGFBR2 [7], SMAD4-miR-302-BMPR2 [8], and SMAD4-miR-24-3p-SMAD2 [9], (ii) lncRNAs such as SMAD2/3-lncRNA-MALAT1-TGFBR2 [10], (iii) transcription factors such as β-catenin (mediates SMAD4 induction of receptor) [11], and (iv) transcription co-regulators such as Snail [12]. Besides, the experimentally validated axes contain SCF/STAT3 axis (mediates SMAD2 positive feedback regulation of TGF-β1) [13], SLIT2-Gremlin axis (mediates SMAD1/5/9/4 complex regulation of BMP2) [14], and so on. In addition, TGF-β and BMP signals also form feedback loops with regulators, such as PML/PIN1 for TGF-β1 [15], NCX1/TRPC6 complex for SMAD2 [16], lncRNA-Crnde for Smad3 [17], DSPP/DSP for SMAD1/5/8 [18], and HNF4 for SMAD4 [19] to feedback regulate the expression of oneself.

Unlike R-SMADs and Co-SMAD, the inhibitory SMADs (SMAD6 and SMAD7), especially SMAD7, feedback against the TGF-β family signaling pathways through directly interacting with proteins [20] or genes (mainly promoter region) [21] of the members in TGF-β family signaling pathway. However, it is still unknown whether R-SMADs or Co-SMAD can directly feedback regulate the TGF-β family signaling pathway. One of the main reasons for the unclear situation is lacking of the investigation for their effects on the intracellular transcriptomic alteration and without screening their potential target genes. Here, we have focused on characterizing the SMAD4-mediated transcriptomic alteration in porcine GCs since the irreplaceable roles of SMAD4 in maintaining ovary development, as well as the normal states and functions of GCs, and expected to identify the potential functional targets of SMAD4.

In the present study, we have re-analyzed the global transcriptional alteration in SMAD4-inhibited porcine GCs with the background of *Sus scrofa* RefSeq 11.1 and also identified the functional DEmRNAs. Among the down-regulated DEmRNAs, *ACVR1B*, *BMPR2*, and *TGFBR2*, three upstream TGF-β family receptors of SMAD4, were selected for further research and showed to be positive feedback regulated by SMAD4. Intriguingly, the results show that SMAD4, the only Co-SMAD, acts as a transcription factor and positively feedback activates the TGF-β family signaling pathway in porcine GCs through directly binding to the promoter region of its upstream receptors, which is mechanically dependent on three transcription co-activators with different interaction modes.

Materials And Methods

Materials

Dimethyl sulfoxide (DMSO, #2650), Phenylmethylsulfonyl fluoride (PMSF, #P7626), 37% paraformaldehyde (#P6148), Proteinase inhibitor (#P2714), Glycine (#67419), and horseradish peroxidase (HRP) were from Sigma-Aldrich. Non-fat milk (#P0216), RIPA lysis buffer (#P0013B), RNase A (#ST578), and RNase inhibitor (#R0102-2kU) were obtained from Beyotime. T5224 (AP-1 inhibitor) was purchased from Ape×Bio Technology (#B4664). The siRNAs used in this study were designed and synthesized by GenePharma (Shanghai, China). All chemicals and solutions were analytical reagent grade and all buffer components were endotoxin free or low endotoxin from Sigma-Aldrich, as avaliable.

Animals and ethics approval

A total of 85 healthy, non-estrus and sexually mature Duroc-Yorkshire-Landrace sows (*n*=85; average mass 110 kg, average age 180 d) from Zhushun Biological Technology Co. (Nanjing, China) were random selected for bilateral ovaries collection and GC culture. The sows were fed, taken care, and finally slaughtered for ovaries collection according to the corresponding animal welfare strictly. All the animal experiments in this study were approved by the Animal Ethics Committee at Nanjing Agricultural University, Nanjing, P. R. China [(SYXK (Su) 2017-0027)] and performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (No.2 of the State Science and Technology Commission, 11/14/1988).

Cell culture and treatment

Fresh porcine bilateral ovaries were collected and placed in a thermos flask with 37°C PBS and transported back to the laboratory within 1 h. The collected ovaries were washed with PBS 5 times and porcine GCs were harvested from 2-5 mm non-atretic ovarian follicles by using syringe with 22-gauge needle and cultured as previously described [22]. Briefly, the isolated porcine GCs were washed with 37°C PBS containing 1% penicillin and streptomycin three times, and then seeded in cell culture plates with DEME/F12 containing 10% fetal bovine serum (FBS) in a 37°C humid atmosphere with 5% CO_2. KGN cells were purchased from American Type Culture Collection (ATCC, Manassas, USA) and cultured in RPMI-1640 with 10% FBS under the same condition mentioned above. All cells used in this study were tested and found to be uncontaminated and mycoplasma-negative. For cell tranfection, Lipofectamine® 3000 transfection reagent (#L3000015, Life Technologies, Carlsbad, CA 92008 USA) was used according to the manufacturer's instruction to transiently transfect the oligonucleotides or plasmids into the cells cultured in vitro. For T-5224 (c-JUN inhibitor) treatment, the cell culture medium was replaced with fresh medium without FBS for 8 h and T-5224 was subsequently added to a final concentration of 75 and 150 μM.

RNA isolation and qRT-PCR

After treatment with indicated times, the total RNA from porcine GCs was isolated and purified by using TRIzol regent (#15596026, ThermoFisher Scientific). The quantity and quality of the purified RNA were detected by NanoDrop 3000 spectrophotometer (Agilent Technologies, USA). The degradation and contamination of the total RNA was estimated by running on a 1.0% agarose gel, and an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) was used to detect the integrity of each sample. For qRT-PCR

assays, 1 µg RNA was first reverse-transcribed into cDNA by using HiScript$^{®}$ II Q-RT SuperMix (#R223-01, Vazyme Biotech Co., Ltd, Nanjing, China). qRT-PCR was performed as we described in our previous study [23] and the relative expression levels of interested genes were calculated through the $2^{-\Delta\Delta Ct}$ approach. Data from qRT-PCR assays were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Each group has three different samples and the experiments were performed with three independent replicates. The primers used for qRT-PCR were listed in Table S1.

Rapid amplification of cDNA ends (RACE)

The transcription start sites (TSS) of porcine *ACVR1B*, *BMPR2*, and *TGFBR2* were obtained by using the Rapid Amplification of cDNA end (RACE) with SMARTer RACE 5'/3' Kit (#634858, Clontech Laboratories, Inc, CA94043, USA) according to the manufacturer's instruction. Briefly, 4 µg total RNA from porcine GCs was used for RACE-Ready cDNA synthesis, and the 5'-end of porcine *ACVR1B*, *BMPR2*, and *TGFBR2* was amplified with gene specific primers. The gene specific anti-sense primers designed for RACE were listed as following: *ACVR1B*-GSP: CCAGGTCGAGAGAGGGCTCTGATGC; *BMPR2*-GSP: CCGACCCCGACGTGGAGAGGTCGT; *TGFBR2*-GSP: ATGGCCAGGTGCTCACTGAACTCCA. Then, PCR products were analyzed by electrophoresis on a 2.0% agarose gel, and the clear DNA bands were collected and purified. Finally, the purified DNA fragments were cloned into pClone007 vector and the corresponding TSSs were verified by Sanger sequencing.

Re-mapping on the pig reference genome and data re-analysis

For re-analysis of the potential targets of SMAD4, the total clean tags were checked and genome mapping was re-performed with the background of pig reference genome (*Sus Scrofa* RefSeq 11.1) by Top Hat v2.0. Then, the information of sequence data were converted into gene expression level. For gene expression level normalization, reads per kilobase transcriptome per million mapped reads (RPKM) method was used and RPKM ≥ 1 was settled as the threshold to determine the gene expression. The differentially expressed mRNAs (DEmRNAs) were identified with the criterion below: (i) |-log$_2$(fold change)| ≥ 0.59 (|fold change| ≥ 1.5), (ii) *P*-value ≤ 0.05, and (iii) FPKM ≥ 1 in all samples.

To further evaluate the potential functions, roles, and biological processes of DEmRNAs, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) were performed by using DAVID v6.8. The GO and KEGG terms with *P*-value ≤ 0.05 were considered as the significant ones. For SMAD4-mediated gene-function and protein-protein interaction (PPI) network construction, the function-known DEmRNAs and interactions between them were analyzed by STRING v11.0 database (https://string-db.org/) with the basic settings: interaction degree ≥ 1 and the minimum required interaction score ≥ 0.9 [0-1]. Then, the network was visualized using Cytoscape v3.7.2 software.

Bioinformatics analysis

The potential promoters of pig *ACVR1B*, *BMPR2* and *TGFBR2* were predicted by using Softberry (http://linux1.softberry.com/all.html) and PromoterScan (http://www-bimas.cit.nih.gov/molbio/proscan/)

online software. The candidate transcription factors which potentially target *ACVR1B*, *BMPR2* and *TGFBR2* promoter and their corresponding binding motifs were analyzed by JASPAR (http://jaspar.genereg.net/) and GCBI online database (https://www.gcbi.com.cn/). The SMAD4-associated proteins (including transcription factors) were also obtained from STRING v11.0 database.

siRNAs and inhibitors

To inhibit the endogenous expression of *CREB1* and *SP1* in porcine GCs, three gene specific interfering RNAs (siRNAs) for each target gene were designed and synthesized by GenePharma (Shanghai, China). The siRNAs used in this study were listed in Table S2. For c-JUN inhibition, T-5224 was used and the treatment concentration (75 and 150 μM) was arranged according to the manufacturer's instructions. The inhibitory efficiency of the siRNAs and inhibitor were detected at both mRNA and protein levels.

Plasmids construction and luciferase activity assay

To identify the core promoter of porcine *ACVR1B*, *BMPR2*, and *TGFBR2*, the different fragments of *ACVR1B*, *BMPR2* and *TGFBR2* promoters were amplified and cloned into pGL3-Basic reporter vector between *Kpn*I and *Xho*I. To detect the effects of SMAD4 on the transcription activity of porcine *ACVR1B*, *BMPR2*, and *TGFBR2*, their promoters containing the wild-type SMAD4 binding sites (SBEs) were amplified and cloned into pGL3-Basic reporter vector between *Kpn*I and *Xho*I. Besides, the SBEs mutant type vectors were generated by using Trelief™ SoSoo Cloning Kit (#TSV-S1, Beijing TsingKe Biotech Co., Ltd) with the wild-type plasmids as templates according to the manufacturer's instruction. To analyze the target sites of transcription co-activators (c-JUN, CREB1, and SP1) within the promoter of porcine *ACVR1B*, *BMPR2*, and *TGFBR2*, their promoters containing the wild or mutant type binding elements were synthesized and inserted into pGL3-Basic reporter vector. All the recombination plasmids were verified by Sanger sequencing.

For the luciferase activity detection, cells were collected after treatment for 24 h and a Dual-Luciferase Reporter Assay System (#E1910, Pormega, Madison, USA) was performed to measure the firefly luciferase and Renilla luciferase activities following the kit's manual. The relative luciferase activity of each sample was calculated as the activity of firefly luciferase realtive to Renilla luciferase.

Western blotting

After treatment for the indicated time, porcine GCs were collected and western blotting assays were performed as we previously described [24]. In brief, the total protein from porcine GCs were extracted by ice-cold RIPA lysis buffer with 1% PMSF, and the concentration of total protein were measured by BCA method. Then, equal amount (~15 μg) of total protein was separated on an 4-20% SDS-PAGE gel and subsequently transferred into PVDF membrane (Merck Millipore, Germany) after electrophoresis. After incubation with 5% non-fat milk at room termperature for 90 min, the membranes were incubated with primary antibodies at 4°C overnight and then with corresponding secondary antibodies for 1 h. The images were obtained after incubation with ECL reagent. The primary antibodies used here were as

follows: anti-SMAD4 (1:1000, #10231-1-AP, Proteintech, Jiangsu, China), anti-ACVR1B (1:1000, #D120045, Sangon Biotech, Shanghai, China), anti-BMPR2 (1:1000, #D221406, Sangon Biotech, Shanghai, China), anti-TGFBR2 (1:800, #sc-400, Santa Cruz, USA), anti-CREB1 (1:1000, #9197, Cell Signaling Technology, USA), anti-c-JUN (1:2000, #9165, Cell Signaling Technology, USA), anti-SP1 (1:1000, #2250, Cell Signaling Technology, USA) and anti-GAPDH (1:3000, #TA802519, ORIGENE, Jiangsu, China).

Immunoprecipitation (IP) and chromatin immunoprecipitation (ChIP)

To analyze the interaction between SMAD4 and co-regulators, immunoprecipitation (IP) assays were performed. In brief, the total protein extracted from porcine GCs were incubated with 4 µg anti-SMAD4 antibody at 4°C overnight to form antibody/protein-protein complex, and then incubated with the Protein A/G magnetic beads (#LSKMAGT02, Merck Millipore) for 4 h at room temperature. The pretreatment of the magnetic beads was performed according to the manufacturer's instructions. Then, the magnetic beads with antibody/protein-protein complex was isolated and the interested proteins were obtained after elution, and further detected by western blotting assays. Anti against IgG (Biogot, #BD0051) was used here as a negative control and 100 µL total protein were used as input.

To identify the binding elements of the interested transcription factors within the promoter of target genes, chromatin immunoprecipitation (ChIP) and ChIP-qPCR assays were conducted as previously described [25, 26]. Briefly, protein-DNA were crosslinked by 37% paraformaldehyde and 2.5 M glycine at 37°C incubator for 20 min. Then, the complexes were ultrasonic with the following settings: 40% output for 130 s (10s on and 30s off) at 4°C, and subsequently pulled down with corresponding antibodies. After de-crosslinking, the enrichment of DNA fragments were analyzed by semi-quantitative PCR or qPCR. Same like IP, IgG was used as internal control for normalization of the specific antibody ChIP signals, and the unprocessed chromatin served as the input for fold enrichment from the same sample.

Statistical analysis

Statistical analyses were performed by using GraphPad Prism v7.0 software (GraphPad software) and SPSS v20.0. All data were presented as mean±SD of three independent experiments. Comparison between two groups was performed by using a two-tailed Student's t-test. $*P<0.05$ and $**P<0.01$ were considered as statistically significant, and the significance levels were stated in the corresponding figure legend.

Results

Re-analysis of the transcriptomic alteration in SMAD4-inhibited porcine GCs

In this study, with the background of *Sus Scrofa* RefSeq 11.1 (*Sscrofa* 11.1), we have re-analyzed our previous RNA-seq data (GSE65696) obtained from porcine GCs after SMAD4 silencing which was originally mapped to the *Sscorfa* RefSeq 10.2 [27]. After examination, a total of 11804 genes were

mapped and 986 DEmRNAs were identified with the criterion |-log$_2$(fold change)| ≥ 0.59, *P*-value ≤ 0.05, and FPKM ≥ 1 in all samples (Fig. 1a). Among them, 519 DEmRNAs were significantly up-regulated and the other 467 were dramatically down-regulated (Fig. 1b). All the DEmRNAs identified here are presented in Table S3.

To further explore the biological functions and physiological processes associated with the SMAD4-induced DEmRNAs, Gene Ontology (GO) and KEGG pathway enrichment analyses were performed. As shown in Table S4, a total of 83 significantly enriched GO terms were identified in three categories, including 18 (21.7%) in cellular components (CC), 53 (63.9%) in biological processes (BP), and 12 (14.4%) in molecule function (MF). Functional analysis showed that these identified DEmRNAs were mainly associated with the functional groups which are essential for transcription regulation, such as chromatin binding, protein kinase binding, and gene expression regulation (Fig. 1c). Furthermore, the results obtained from KEGG pathway analyses demonstrated that the SMAD4-induced DEmRNAs were significantly enriched in 13 pathways involved in the regulation of cell states, functions, and stimulation response, including TGF-β, FOXO, and p53 signaling pathways (Fig. 1d, Table S5).

In order to establish the SMAD4-mediated gene-function interaction network, the identified DEmRNAs were analyzed by STRING protein-protein interaction (PPI) database and the nodes were clustered based on their known functions obtained from GO analysis (Fig. S1). Among them, a highly interacted network with 78 nodes and 171 edges was mapped (Fig. 1e). After function assessment, seven major sub-clusters were identified within the network, including cell apoptosis, cycle, and oocyte development, which provides a systematic view of the biological functions of SMAD4 in diverse biological processes.

SMAD4 is a strong inducer of its upstream receptors

Next, we have analyzed the differentially expressed unigenes in porcine GCs after SMAD4 silencing, and interestingly noticed that six receptors of TGF-β family signaling pathways were down-regulated (Fig. 2a). Among them, three receptors, including ACVR1B (type I receptor for BMP signaling pathways), BMPR2 (type II receptor for BMP signaling pathways), and TGFBR2 (type II receptor for TGF-β signaling pathways), were significantly down-regulated after SMAD4 inhibition (Fig. 2b), which were chosen for further research. Our previous study has demonstrated that SMAD4 could feedback enhances *TGFBR2* expression through miR-425 [7]. To further evaluate the identification results, we have examined the effects of SMAD4 on the expression levels of *ACVR1B* and *BMPR2* in porcine GCs. With the results obtained from gain- or loss-of-function, we have confirmed that SMAD4 positively feedback regulates the expression of *ACVR1B* and *BMPR2*, the other two receptors of TGF-β family signaling pathways, at both mRNA and protein levels in porcine GCs (Fig. 2c, d). Furthermore, we have analyzed the expression levels of *ACVR1B*, *BMPR2*, and *TGFBR2* in porcine GCs treated with pcDNA3.1-SMAD4 or SMAD4-siRNA with different concentrations and times as indicated. The results demonstrated that over-expression of SMAD4 significantly induced, but knockdown of SMAD4 dramatically inhibited the mRNA levels of *ACVR1B*, *BMPR2*, and *TGFBR2* in a dose- and time-dependent manner (Fig. 2e-g, Fig. S2). Taken together, the results demonstrate that SMAD4 is a strong inducer for the transcription of its upstream receptors,

further indicates the feedback regulation roles of SMAD4 in TGF-β family signaling pathways in porcine GCs.

SMAD4 feedback regulates its upstream receptors by acting as a transcription factor

It has been proved that SMAD4 regulates the target genes expression by acting as a transcription factor. We therefore speculated that SMAD4 feedback regulates the transcription of upstream receptors (*ACVR1B*, *BMPR2*, and *TGFBR2*) with its transcription factor activity. To validate this, we first identified the transcription start site (TSS) of pig *ACVR1B*, *BMPR2*, and *TGFBR2* gene by RACE assays (Fig. 3a-c). Furthermore, their core promoters were also identified by using bioinformatics analysis and dual-luciferase reporter system. As shown in Fig. S3, the core promoter of *ACVR1B*, *BMPR2*, and *TGFBR2* were located at -1236/-952, -487/-195, and -2128/-1890 (TSS was considered as +1), respectively. After analysis, three, one, and two putative SMAD4 binding elements (SBE) were located within their core promoter regions (Fig. S4), suggesting that SMAD4 may bind to the core promoter of its upstream receptors and further regulates their transcription.

To address this, the reporter vectors containing the promoter of *ACVR1B*, *BMPR2*, and *TGFBR2* with the wild-type or mutant type SBEs were constructed and co-transfected with pcDNA3.1-SMAD4 or SMAD4-siRNA into porcine GCs and KGN cells, respectively. After 24 h, luciferase activity assays were performed and showed that over-expression of SMAD4 (SMAD4^{OE}) significantly increased, but knockdown of SMAD4 (siSMAD4) decreased the activity of wild-type reporters, while had on effect on the activity of reporters containing *ACVR1B* promoter with SBE3 mutation, *BMPR2* promoter with SBE1 mutation, and *TGFBR2* promoter with SBE2-mutation (Fig. 3d-f and fig. S3). Besides, chromatin immunoprecipitation (ChIP) assays were performed and proved that SMAD4 directly interacts with the core promoters of pig *ACVR1B*, *BMPR2*, and *TGFBR2* gene by recognizing the corresponding SBE (Fig. 3g-i). Based on the observations above, we proved that SMAD4 functions as a transcription factor and positively feedback regulates the transcription of its upstream receptors (*ACVR1B*, *BMPR2*, and *TGFBR2*) in TGF-β family signaling pathways by directly binding to their core promoters.

Three co-activators (CREB1, c-JUN, and SP1) are essential for SMAD4-mediated feedback regulation of TGF-β family signaling pathways

It has been well documented that transcription factors and co-regulators (including activators and inhibitors) are essential for R-SMAD/SMAD4 complexes-mediated regulation of target genes expression in the nucleus. Thus, we focused on investigating whether the transcription factors or co-regulators are involved in SMAD4-mediated positive feedback regulation to the upstream receptors in the following study. First, we explored the interaction between SMAD4 and the potential functional proteins by using online programs STRING v11.0 database. Several transcription co-regulators, including c-JUN, CREB1, FOS, FOXO3, HIF-1α, p53, p300, and SP1 have been identified as SMAD4-interacted regulators (Fig. 4a and fig. S5a). Besides, the interaction between the candidate co-regulators mentioned above and the upstream receptor genes (*ACVR1B*, *BMPR2*, and *TGFBR2*) of SMAD4 was analyzed using JASPAR and

GCBI online database. Interestingly, analysis results showed that the binding motifs of c-JUN (the main subunit of AP-1 (activator protein 1)), CREB1 (cAMP responsive element binding protein 1) and SP1 (Sp1 transcription factor) are all located near the validated SBEs (Fig. 4b and fig. S5b-d), indicating that c-JUN, CREB1, and SP1 may serve as co-regulators during SMAD4-mediated feedback regulation process.

To address this, we first detected the physical interaction between SMAD4 and c-JUN, CREB1, and SP1 in porcine GCs. IP was performed and proved that SMAD4 directly interacts with c-JUN, CREB1, and SP1 in porcine GCs (Fig. 4c). Next, we examined the effects of three co-regulators on the expression and promoter activity of *ACVR1B*, *BMPR2*, and *TGFBR2* in porcine GCs with the indicated treatment. Results showed that inhibition of these co-regulators dramatically suppressed the expression of related receptors at both mRNA and protein levels in the absence or presence of SMAD4 stimulation (Fig. 4d-i and fig. S6a-c), and also significantly inhibited the promoter activity induced by SMAD4 over-expression (Fig. 4j). Notably, we also found that the mutation of binding sites for c-JUN, CREB1, and SP1 strongly influences the transcription activity of receptor genes elevated by SMAD4 stimulation (Fig. 4l). Together, our findings demonstrate that c-JUN, CREB1, and SP1 are three co-activators and necessary for the SMAD4-mediated feedback regulation of its upstream receptors through forming regulatory complexes with SMAD4 in porcine GCs.

SMAD4 interacts with co-activators in different modes during feedback regulation processes.

ChIP assays were performed to detect the interaction between these co-activators and the promoters of pig *ACVR1B*, *BMPR2*, and *TGFBR2*, and the results showed that c-JUN and CREB1 could bind to the promoter of *ACVR1B* and *TGFBR2*, while SP1 could bind to *TGFBR2* promoter by recognizing the corresponding response elements (Fig. 5a-c). Based on the findings above, we expected to explain whether SMAD4 interacts with these three identified co-activators in the same or different modes in porcine GCs. To address this, reciprocal ChIP-qPCR assays were performed in the following study and three different interaction patterns were identified between SMAD4 and co-activators during SMAD4-mediated feedback regulation of upstream receptors, (i) the enrichment of CREB1 and c-JUN on *ACVR1B* promoter were remarkably reduced after SMAD4 inhibition, while c-JUN inhibition rather than CREB1 silencing impaired SMAD4 binding to *ACVR1B* promoter, indicating that SMAD4 and c-JUN forms a complex which further plays a positioning role in recruiting CREB1 to *ACVR1B* promoter (Fig. 5d); (ii) the enrichment of SP1 on *BMPR2* promoter was dramatically reduced after SMAD4 silencing, but SP1 inhibition had no effect on SMAD4 binding enrichment, indicating that SMAD4 is also an anchor protein and plays a positioning role in recruiting SP1 to *BMPR2* promoter (Fig. 5e); (iii) knockdown of SMAD4 did not change the enrichment of CREB1 and c-JUN on *TGFBR2* promoter, while the enrichment of SMAD4 was notably impaired after CREB1 silencing or c-JUN inhibition, indicating that CREB1 and c-JUN play a positioning role in recruiting SMAD4 to *TGFBR2* promoter (Fig. 5e). Taken together, our data deeply revealed that SMAD4 acts as a transcription factor and positively feedback regulates upstream receptors of TGF-β family signaling pathways by interacting with three co-activators (CREB1, c-JUN, and SP1) in different modes (Fig. 6).

Discussion

In this study, we have attempted to investigate the biological functions of SMAD4 in mammalian ovarian GCs by screening for its target genes and interacting proteins. Bioinformatics analyses indicate that SMAD4 is crucial for the normal states and functions of GCs, as well as oocyte development and maturation, which is consistent with the previous researches [22, 28]. Besides, the differentially expressed genes were identified and interestingly found multiple members of TGF-β family signaling pathways including three important receptors (ACVR1B, BMPR2, and TGFBR2), which highlights the feedback regulation functions of SMAD4. In TGF-β family signaling pathways, SMAD4 is the main feedback regulators that control ligands, receptors, and SMADs in various cells, for instance, TGF-β1 in hepatic stellate cells [29], BMP2 in myoblasts [14], TGFBR1 in cardiac fibroblasts [6], BMPR2 in neuron [8], SMAD2 in C2C12 cells [9], and SMAD4 in Caco-2 cells [19]. In the present study, we have proved that *ACVR1B*, *BMPR2*, and *TGFBR2* are three direct targets of SMAD4 and further demonstrated for the first time that SMAD4 can feedback activate the whole TGF-β family signaling pathways through receptors in a certain cell type, the porcine GCs. Meanwhile, our findings have accumulated more evidences for feedback regulation in TGF-β family signaling pathways.

It has been well established that TGF-β and BMP signaling pathways are closely related in multiple biological processes [30-32]. The crosstalk between these two pathways has been widely studied and their members are well known to be regulated by each other [33, 34]. To be noticed, the core members belonging to TGF-β and BMP signaling pathways are quite different, except for SMAD4, the only common downstream molecule which is necessary for the TGF-β and BMP signals shuttling into the nucleus. Therefore, it is rare to identify the regulators which could mediate the downstream molecules simultaneously feedback regulation of TGF-β and BMP signaling pathways in the same cell type, tissue or during the same biological process. A recent study reported that Endoglin, a common non-canonical receptor of TGF-β and BMP signaling pathways, could regulate both of two signaling pathways [11]. However, little is known whether the core members feedback regulate these two signaling pathways. Our current study demonstrate for the first time that SMAD4, a core common component, directly feedback regulates both TGF-β and BMP signaling pathways in porcine GCs by acting as a transcription factor and directly interacting with the promoter region of upstream receptors, which further inducing their transcription. Notably, it is obviously different from previous studies which have shown that SMAD4 achieve feedback regulation of the TGF-β family signaling pathways via one or more mediators [6, 7, 35].

In the nucleus, R-SMAD/SMAD4 transcription complex regulates the spatial and temporal expression of target genes, usually along with other modulators and co-regulators such as transcription factors (e.g., β-catenin [11]), transcription co-factors including co-activators (e.g., p300/CBP [36]), and co-repressors (e.g., Ski and SnoN [37]). However, the roles of SMAD4 in the interaction with these co-regulators are quite different. For example, SMAD4 regulates the transcription *SMIF*, *PAI-1*, *MMP2*, and *Rorc* by recruiting the p300/CBP complex, SNIP-1, and SKI, respectively [38]. SMAD4 just acts as a positioner and plays a protein-recruiter role during these processes. But in other cases, SMAD4 functions as a recruited transcription factor or directly binds to the promoter of target genes by specifically recognizing SBE

motifs [39]. Based on the observations in the current study, we propose a molecular mechanism model that depicts the SMAD4-mediated feedback regulation of TGF-β family signaling pathway (Fig. 6). SMAD4 acts as a transcription factor and directly binds to the promoter of TGF-β family receptors (ACVR1B, BMPR2, and TGFBR2) by interacting with three co-activators (CREB1, c-JUN, and SP1) in different modes, which further elevates their expression levels in porcine GCs. Our findings not only prove that co-regulators are necessary for SMAD4 regulation the transcription of target genes, but also demonstrate that SMAD4 can regulate the target genes with different modes, even for those members from the same family or signaling pathways.

To be honest, one issue will be important to resolve but we still do not understand is the precise manipulation mechanism of SMAD4 to regulate the expression pattern (up- or down-regulation) of target genes. As we all known, SMAD4 and R-SMADs function as transcription factors by recognizing the SMAD binding elements (SBEs) within the promoter region of target genes [19, 40, 41]. However, the SBE motifs are highly conserved among mammals which we do not believe is the crucial factor for the different expression pattern of SMAD4-targeted genes. According to the findings here, we hypothesize that the co-regulators which form transcription complexes with SMAD4 might be responsible for altering the expression pattern of SMAD4-targeted genes, but now we still lack strong evidence for this hypothesis, which needs further investigation. Once it is resolved, the mechanisms of not only SMAD4, but also all transcription factors-mediated difference in expression patterns of their target genes may be figured out.

Conclusion

In summary, we have detected the effects of SMAD4 inhibition on the transcriptome of porcine GCs and identified 986 DEGs with the RNA-seq data, which were further analyzed to be involved in the regulation of cell states, functions, and gene expression regulation. Among these DEGs, three crucial TGF-β family signaling pathway upstream receptors (ACVR1B, BMPR2, and TGFBR2) were significantly inhibited in SMAD4-silenced porcine GCs. Furthermore, the results demonstrate for the first time that SMAD4 forms transcription complexes with co-activators (c-JUN, CREB1, and SP1) in different interaction modes, which further feedback activates TGF-β family signaling pathways by directly binding to the promoter of its upstream receptors and activating their transcription in porcine GCs. Our findings provides a theoretical basis and experimental evidence for the mechanism of SMAD4-mediated feedback regulation, which improves and expands the regulatory network, especially the feedback regulation modes of TGF-β family signaling pathways in the ovary.

Abbreviations

ChIP: Chromatin immunoprecipitation; DEGs: Differentially expressed unigenes; FBS: Foetal bovine serum; FC: Fold change; GC: Granulosa cell; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; IP: Immunoprecipitation; PPI: Protein-protein interaction; RACE: Rapid Amplification of cDNA Ends; RPKM: Reads per kilobase transcriptome per million mapped reads; RNA-seq: High-throughput

sequencing of RNA; SBE: SMAD4 binding element; siRNA: Specific interfering RNA; TF: Transcription factor; TSS: Transcription start site;

Declarations

Acknowledgements

Authors' contributions

XD and QFL conceived the project and designed experiments. XD, QQL, and LY performed experiments. XD and QQL analyzed the data. XD and QFL wrote the manuscript.

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Competing interests

The authors declare that they have no competing interests.

Ethics approval

All the animal experiments in this study were approved by the Animal Ethics Committee at Nanjing Agricultural University, Nanjing, P. R. China [(SYXK (Su) 2017-0027)] and performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (No.2 of the State Science and Technology Commission, 11/14/1988).

Consent for publication

Not applicable

Availability of data and materials

All data needed to evaluate the conclusions are presented in the paper or Supplementary Materials which are available from the corresponding author on request.

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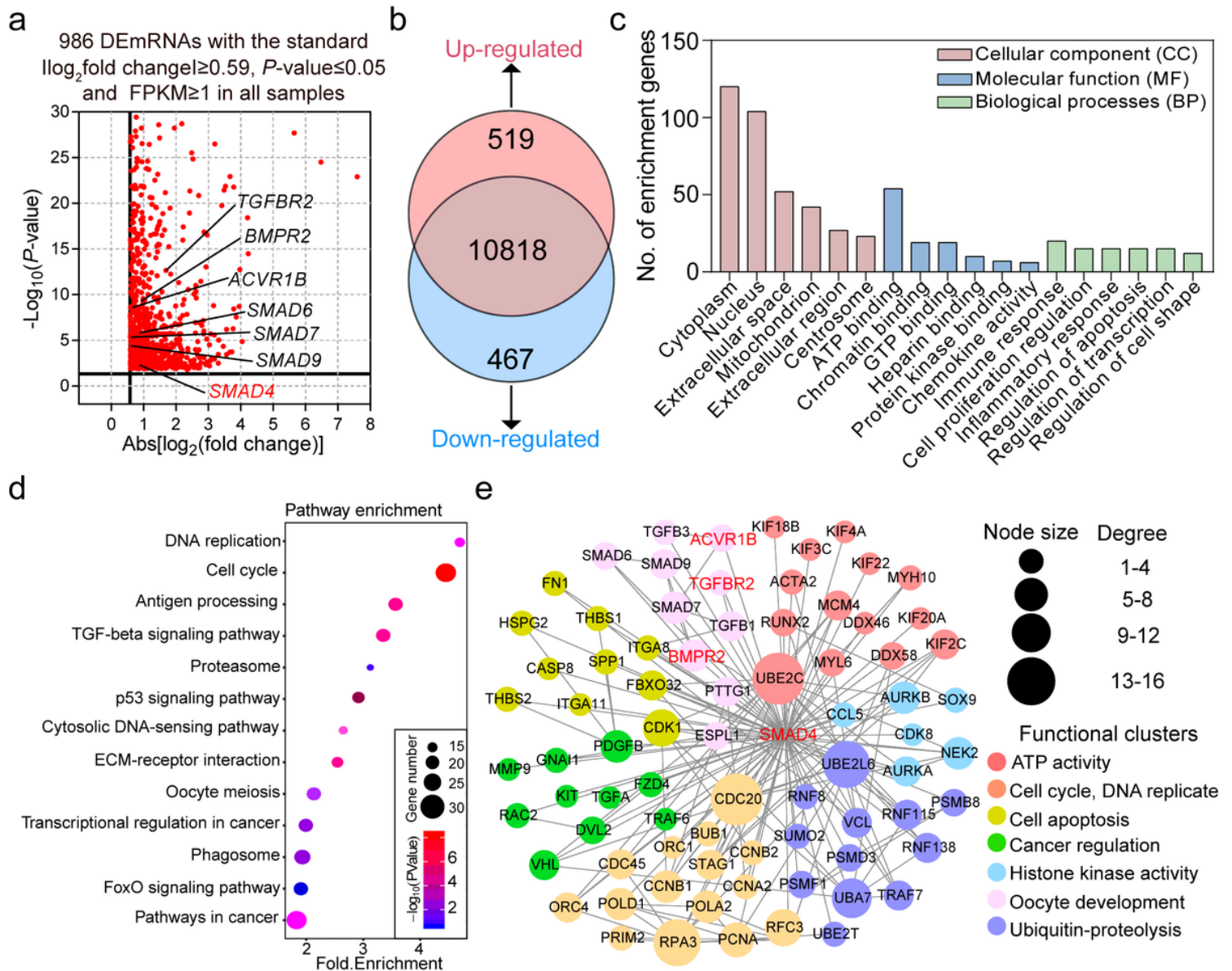
Figures

Figure 1

Re-analysis of the transcriptomic alteration in SMAD4-silenced porcine GCs. a The volcano plot of the identified DEmiRNAs. The DEmRNAs are shown as red dots. x-axis and y-axis are described according to |log2(fold change)| and -log10(P-value), respectively. b The expression pattern distribution of DEmRNAs under SMAD4-silencing. c Gene Ontology (GO) analyses of the DEmRNAs in SMAD4-inhibited porcine GCs. Top 6 significantly enriched function terms in CC, MF, and BP categories were represented. d KEGG pathway analyses of the DEmRNAs in porcine GCs under SMAD4 inhibition and 13 significantly enriched pathways were presented. e SMAD4-mediated gene-function network. The function-known DEmRNAs are shown as circles. Edges indicate the potential interaction between different nodes. The size of nodes reflects their degree in the network. Nodes are clustered according to their biological functions as indicated.
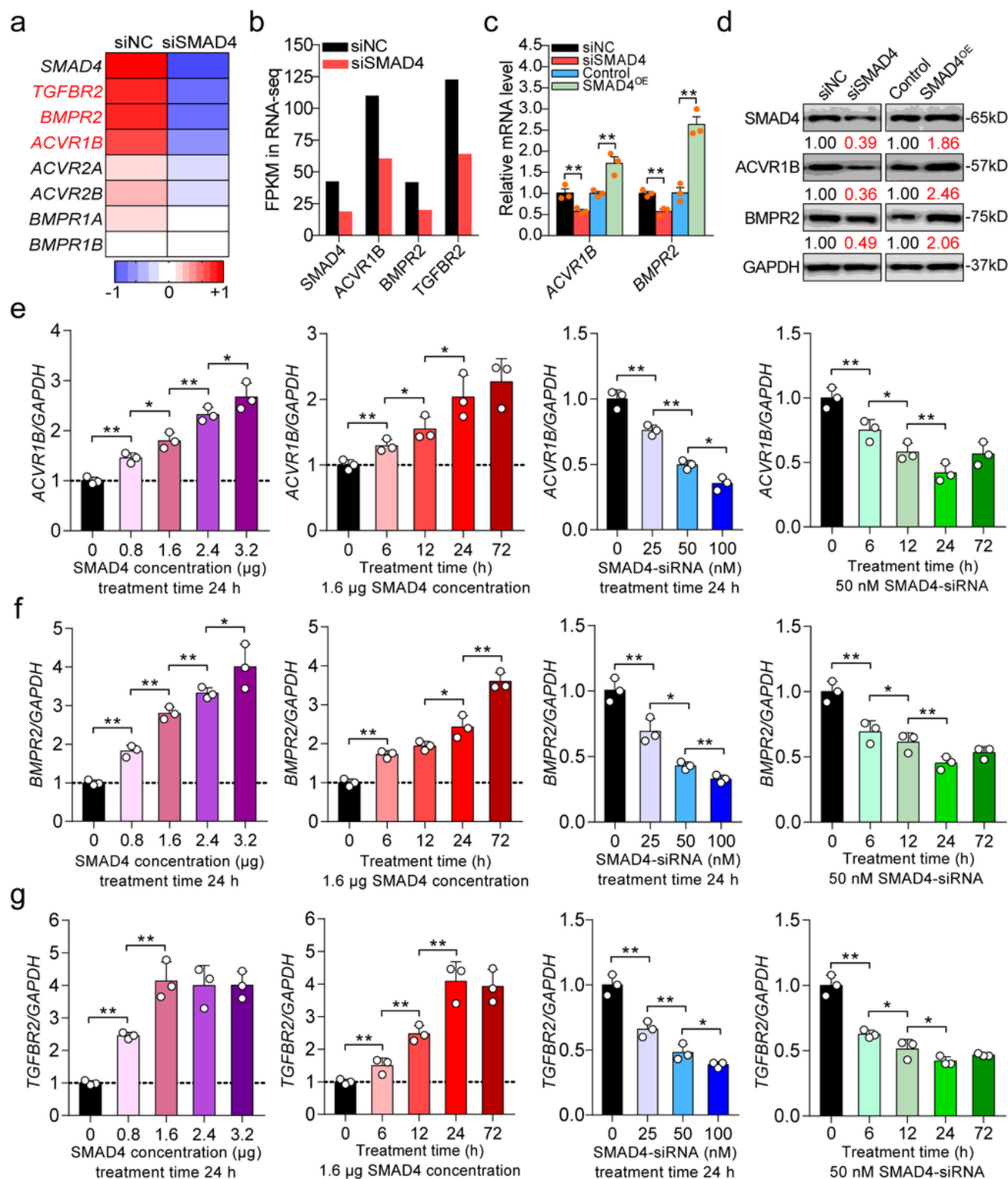
Figure 2

SMAD4 induces the transcription of its upstream receptors in porcine GCs. a Heatmap showing the expression pattern of TGF-β family signaling receptors in porcine GCs after SMAD4 silencing (siSMAD4). The color scale indicates the expression degree, increase (red) and decrease (blue). b The expression levels (FPKM) of SMAD4, ACVR1B, BMPR2, and TGFBR2 in SMAD4-silenced porcine GCs according to RNA-seq. c, d The mRNA (c) and protein (d) levels of ACVR1B and BMPR2 in SMAD4-overexpressed or -

inhibited porcine GCs were determined by qRT-PCR and western blot assays (n=3). e-f The mRNA level of ACVR1B, BMPR2, and TGFBR2 in porcine GCs treated with pcDNA3.1-SMAD4 or SMAD4-siRNA with different concentration and treatment time as indicated (n=3). The data in c-f were normalized by GAPDH and shown as mean ± SD of three independent experiments. t-test, *P < 0.05, **P < 0.01.
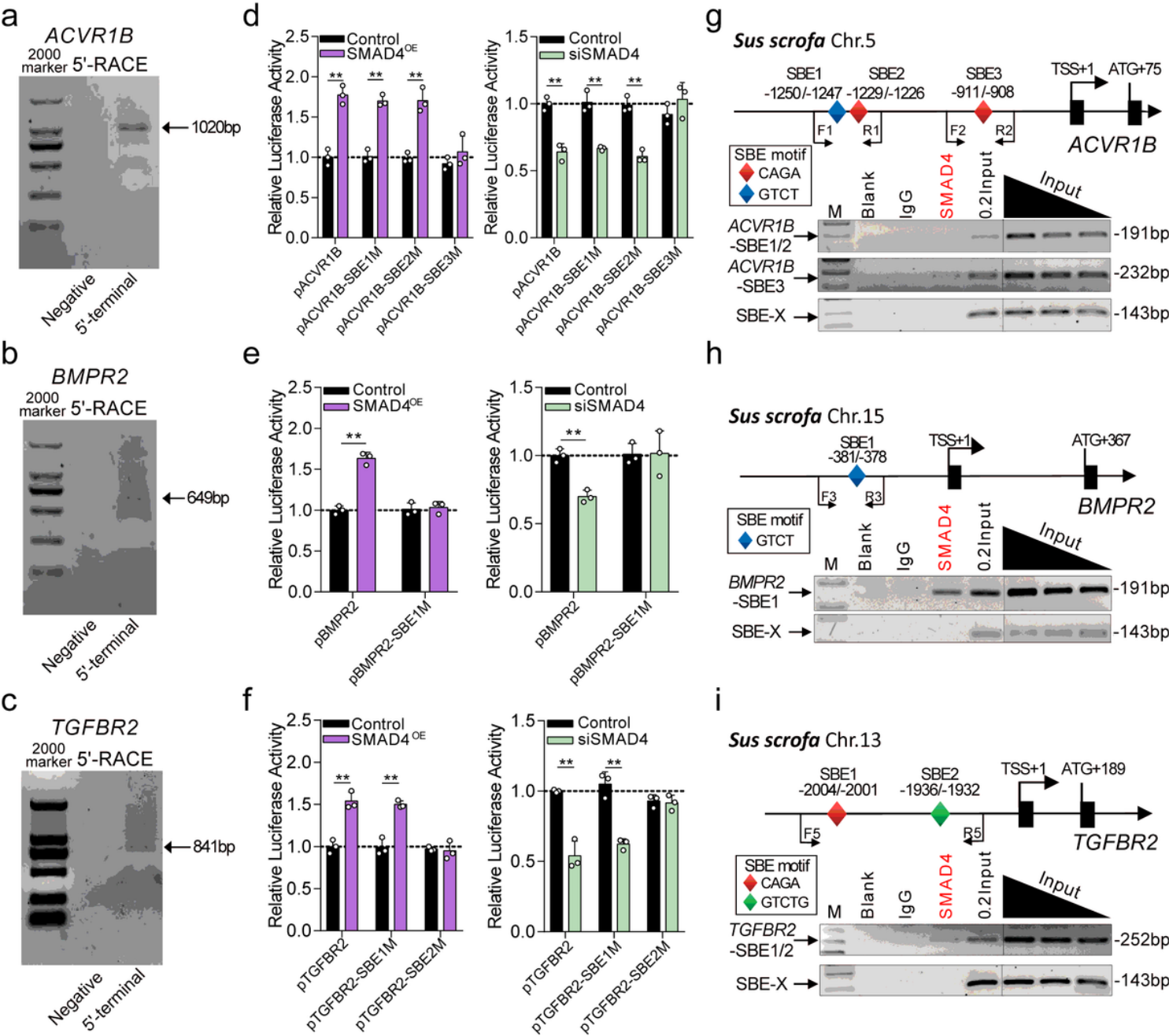

Figure 3

SMAD4 directly binds to the core promoter of its upstream receptors. a-c Identification of the transcription start sites (TSS) of SMAD4 upstream receptors. Gel images depicting the 5'-RACE results of porcine ACVR1B (A), BMPR2 (B) and TGFBR2 (C). 5'-terminal indicates GSP (Gene-Specific Primer) addition and negative means no GSP primer. The size of each band was indicated by arrow. d-f The effects of SMAD4 on the promoter activity of porcine ACVR1B (d), BMPR2 (e) and TGFBR2 (f) with wild-type or mutant type of SBEs were detected by luciferase activity assays (n=3). g-i The binding motifs of SMAD4 within the

core promoter of porcine ACVR1B (g), BMPR2 (h), and TGFBR2 (i) were identified by ChIP assays. Different SBE motifs were indicated by diamonds with different colors. The data in d-f were shown as mean ± SD of three independent experiments. P-values were calculated by a two-tailed Student's t-test, **P < 0.01.
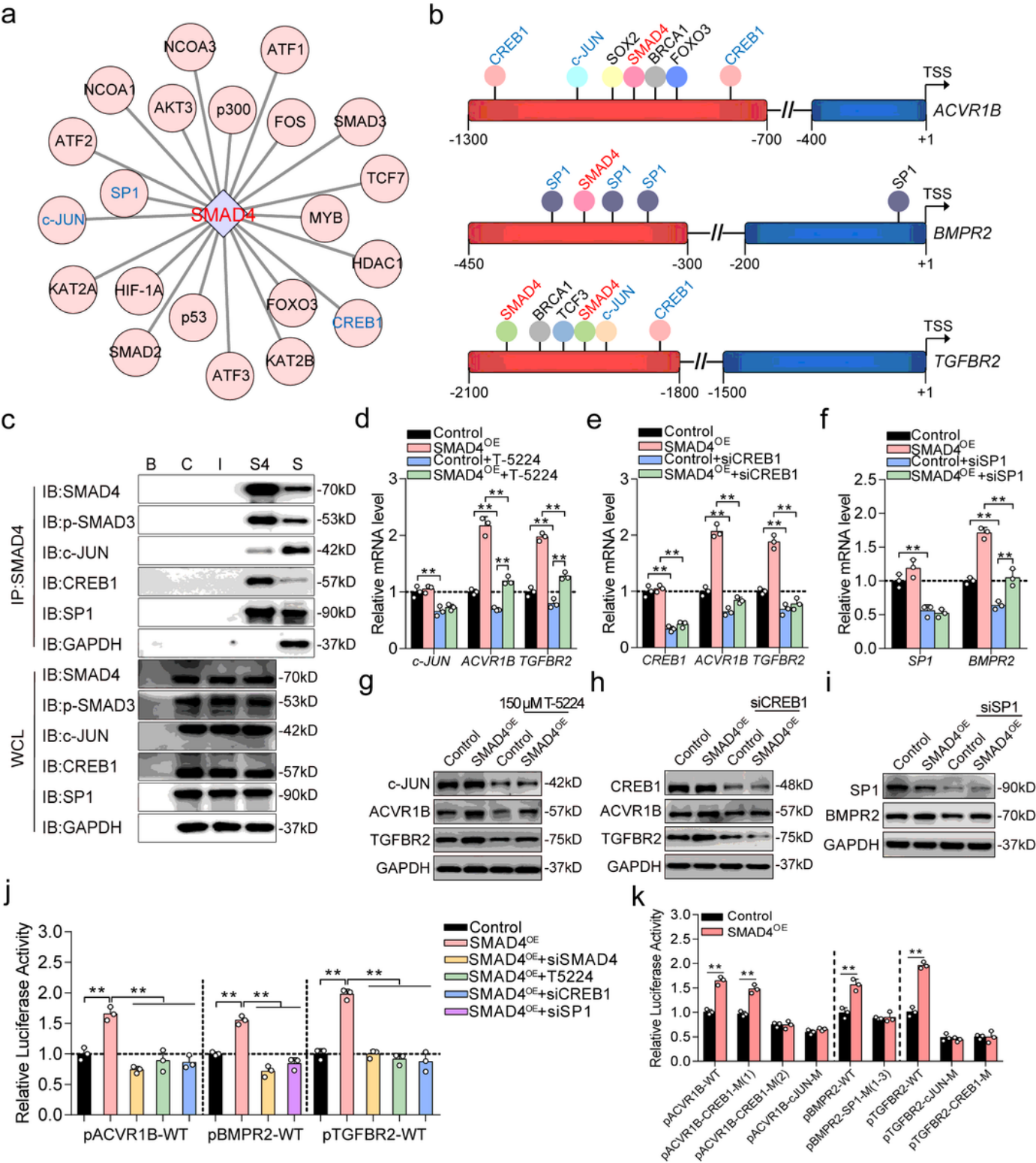

Figure 4

CREB1, c-JUN, and SP1 are essential co-activators for SMAD4 feedback regulation of its upstream receptors. a SMAD4-mediated protein interaction network. The interactions between SMAD4 and transcription factors and co-regulators were obtained from STRING v11.0 and visualized by Cytoscape v3.7.2 software. b Diagram showing the location of the binding motifs of candidate transcription co-regulators within the promoter of pig ACVR1B, BMPR2, and TGFBR2. The SBEs identified in this study were labeled in red font and the binding motifs of c-JUN, CREB1, and SP1 were indicated in blue. c The physical interactions between SMAD4 and candidate co-regulators (c-JUN, CREB1, and SP1) in porcine GCs were identified using IP assay. B, blank group; C, control group; I, IgG group; S4, anti-SMAD4 group; S, supernatant. d-i Porcine GCs were treated with 150 μM T-5224 (d, g), siCREB1 (e, h), or siSP1 (f, i) in the absence or present of SMAD4 stimulation for 24 h and 48 h, and the mRNA and protein levels of ACVR1B, BMPR2, and TGFBR2 were detected by qRT-PCR and western blotting, respectively (n=3). j The co-activators were inhibited and the promoter activities of ACVR1B, BMPR2, and TGFBR2 in SMAD4-overexpressed porcine GCs were detected by luciferase activity assays (n=3). k The effects of SMAD4 over-expression on the activity of reporters containing ACVR1B, BMPR2 and TGFBR2 promoter with the wild-type or mutant type binding elements of CREB1, SP1, and c-JUN were detected by luciferase activity assay (n=3). The data in d-k were shown as mean ± SD of three independent experiments. P-values were calculated by a two-tailed Student's t-test, *P < 0.05, **P < 0.01.
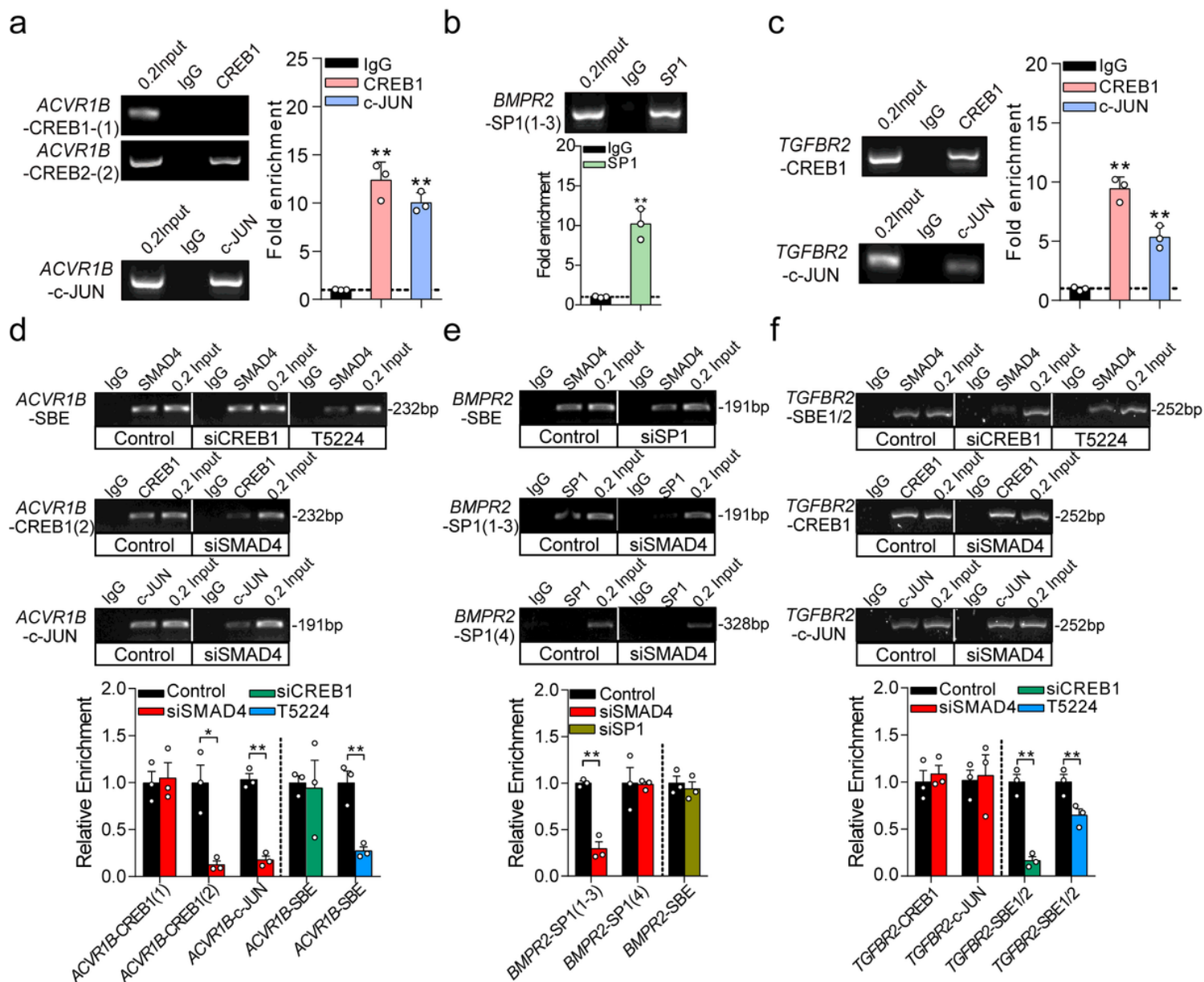
Figure 5

Three interaction modes are identified between SMAD4 and co-activators in porcine GCs. a-c ChIP assays were performed to identify the interaction between co-activators (c-JUN, CREB1, and SP1) and the promoter of ACVR1B (a), BMPR2 (b), and TGFBR2 (c) (n=3). d-f The interaction modes between SMAD4 and co-activators during the SMAD4-meidated feedback regulation the transcription of ACVR1B (a), BMPR2 (b), and TGFBR2 (c) were identified by reciprocal ChIP assay. Data were shown as mean ± SD (n=3). t-test, *P < 0.05, **P < 0.01.
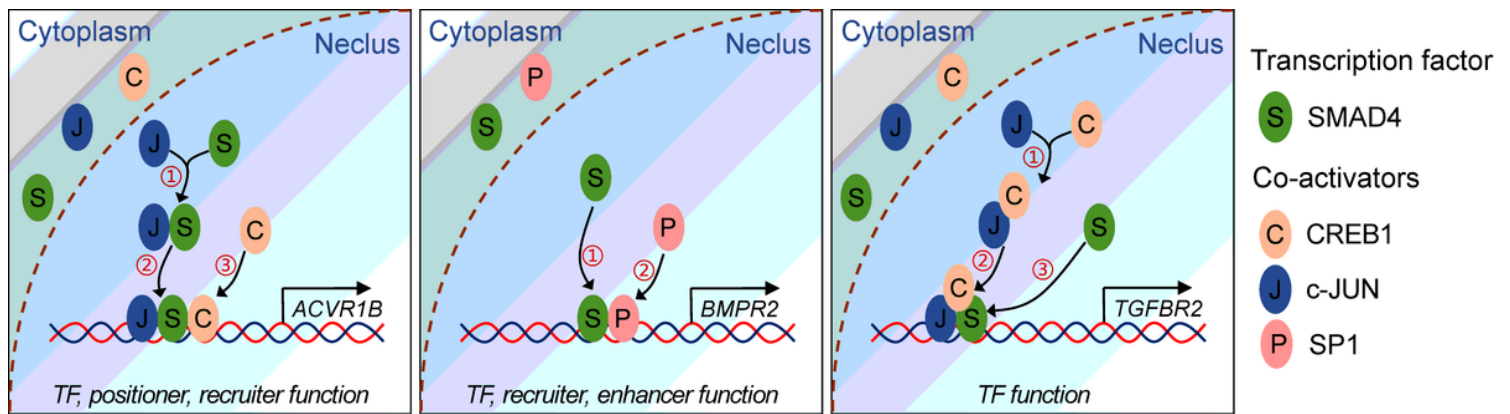
Figure 6

Schematic illustration showing the interaction modes between SMAD4 and co-activators during SMAD4-mediated feedback regulation of TGF-β family signaling pathway.

Supplementary Files

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