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Effect of interrupted endogenous BMP/Smad signaling on growth and steroidogenesis of porcine granulosa cells*

Wei WANG, Li WANG, Xin-xiu LI, Xia CHEN, Hai-yan ZHANG, Yu HE,
Jing-jing WANG, Yong-yan ZHAO, Bao-le ZHANG, Yin-xue XU†‡
(*College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China*)
†E-mail: xuyinxue@njau.edu.cn

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Abstract: Bone morphogenetic proteins (BMPs) play a critical role in the growth and steroidogenesis of granulosa cells (GCs). BMP signals act through membrane-bound heteromeric serine/threonine kinase receptors. Upon ligand binding, BMPs activate intracellular Smad proteins and regulate growth and apoptosis in various cell types. The objective of this study was to demonstrate the effects of BMP/Smad signal on growth and steroidogenesis of porcine GCs. A strategy of RNA interference (RNAi)-mediated 'gene silencing' of Smad4, a core molecule mediating the intracellular BMP/Smad signal transduction pathways, was used to interrupt endogenous BMP/Smad signaling. Results indicate that Smad4-small interfering RNA (siRNA) caused specific inhibition of Smad4 mRNA and protein expression after transfection. Interrupted endogenous BMP/Smad signaling significantly inhibited growth, and induced apoptosis of porcine GCs, while decreasing estradiol production. In addition, interrupted BMP/Smad signaling significantly (P<0.05) changed the expression of *Cyclin D2*, *CDK4*, *Bcl-2*, and *Cyp19a1*. These findings provide new insights into how BMP/Smad signaling regulates the growth and steroidogenesis of porcine GCs.

Key words: Porcine granulosa cells, Bone morphogenetic protein (BMP)/Smad, Smad4, RNA interference (RNAi), Growth, Steroidogenesis
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1 Introduction

Female fertility in mammals is dependent on the growth and development of follicles. Folliculogenesis involves a series of sequential steps in which a growing follicle either develops to the ovulation stage or undergoes atresia. Significant disruption occurring at any of the stages of folliculogenesis impairs fertility (McGee and Hsueh, 2000).

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β (TGF-β) superfamily, and are key factors for normal folliculogenesis. Recent studies have established that BMPs play a crucial role in female fertility in mammals by regulating steroidogenesis as well as mitogenesis in granulosa cells (GCs) (Shimasaki *et al*., 2003; 2004). BMP-6 inhibits follicle-stimulating hormone (FSH)-induced progesterone synthesis through the suppression of cellular cyclic adenosine monophosphate (cAMP) synthesis (Otsuka *et al*., 2001a). BMP-15 also exhibits potent suppression of FSH actions by inhibiting FSH receptor (FSHR) expression (Otsuka *et al*., 2001b). In addition, growth differentiation factor-9 (GDF-9), which is highly homologous with BMP-15, also inhibits FSH-induced steroidogenesis and luteinizing hormone (LH) receptor expression in rat GCs (Vitt *et al*., 2000; Miyoshi *et al*., 2006).

TGF-β activates a cell surface receptor complex, which consists of two different transmembrane serine/threonine kinases that phosphorylate members of the downstream receptor-regulated Smads (R-Smads) upon ligand binding (Schmierer and Hill, 2007). The

‡ Corresponding author
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closely related R-Smads, Smad1, Smad5, and Smad8, transmit BMP and GDF signals, while Smad2 and Smad3 are phosphorylated in response to TGF-β, activin and nodal signals. The phosphorylated R-Smads in combination with the common mediators, Smad and Smad4, recruit appendant cofactors to form complexes that in turn regulate target gene expression (Massagué *et al*., 2005). Smad4, originally discovered to be a tumor suppressor gene, shares structural features with the R-Smads. However, its MH2 domain lacks the C-terminal SXS motif required for receptor-mediated phosphorylation. Smad complexes regulate various kinds of biological processes, including cell proliferation, differentiation, and apoptosis during embryonic development and adult tissue homeostasis (Costello *et al*., 2009).

In mammals, Smad4 is the only co-mediating Smad, translocating to the nucleus with phosphorylated R-Smads, and then modulating transcription of BMP target genes. Smad4 is extensively expressed in embryonic and adult diverse tissues. Smad4 nucleocytoplasmic shuttling is not required for R-Smad phosphorylation or nuclear localization (Biondi *et al*., 2007). R-Smad dephosphorylation and nuclear export are thought to be essential for optimal TGF-β signaling (Lin *et al*., 2006; Dai *et al*., 2009). Loss of Smad4 leads to increased steady state levels of both BMP and TGF-β phosphorylated R-Smads. Enhanced R-Smad phosphorylation levels potentially reflect reduced dephosphorylation and/or nuclear export (Lin *et al*., 2006; Dai *et al*., 2009; Costello *et al*., 2009). Therefore, Smad4 is crucial for BMP/Smad signaling.

The aim of this study was to determine how BMP/Smad signaling regulates the growth and steroidogenesis of porcine GCs using highly potent small interfering RNA (siRNA) interference to suppress Smad4 gene expression.

2 Materials and methods

2.1 Isolation and culture of GCs

Porcine ovaries were obtained from a local slaughterhouse, and transported to the laboratory at 37 °C in saline containing gentamicin and amphotericin. The ovaries were washed three times with pre-warmed phosphate-buffered saline (PBS) supplemented with gentamicin and amphotericin. GCs were collected from follicles (3–5 mm) by needle (20 G) aspiration, and filtered through a stainless steel filter (100 μm) to remove the cumulus-oocytes complexes (COC). The cells were centrifuged at 800×*g* per min and washed twice in DMEM/F12 medium (Dulbecco's modified Eagle medium/Ham's nutrient mixture F-12; Shanghai Invitrogen Biotechnology Co., Ltd., China). GCs were cultured as described previously (Picton *et al.*, 1999). GCs were counted in a hemocytometer, and the viability was determined by trypan blue dye exclusion, and then the GCs were seeded in six-well culture plates (Costar; Corning Inc., NY, USA) at a density of $(3–5)\times10^6$ cells per well in 2 ml of DMEM/F12 medium containing 10% (v/v) fetal calf serum (FCS; Minhai Co., Lanzhou, China), 5 μl/ml of gentamicin, and 10 μl/ml amphotericin. The cells were cultured for 24 h at 37 °C in a 5% (v/v) CO_2 atmosphere and then the wells were washed with PBS to remove unattached cells. Exponentially growing cells were used for the experiments.

2.2 Design and transfection of siRNA

The complementary DNA (cDNA) sequence of porcine Smad4 (GenBank accession No. NM_214072) was examined using Invitrogen's web-based siRNA design software (https://rnaidesigner.invitrogen.com/rnaiexpress/) to select appropriate siRNA target sites. A pair of oligonucleotides corresponding to Smad4 cDNA were designed. A BLAST (basic local alignment search tool) search of these sequences confirmed their specificity to Smad4 only. In addition, a nonsense sequence with no similar match to any known sequence was used as control. The Smad4 small RNA interference (RNAi) was named Smad4-siRNA, and the nonsense sequence to Smad4 was named negative control siRNA (NC-siRNA).

GC cultures were transfected with Smad4-siRNA or NC-siRNA using Lipofectamine™ RNAiMAX Reagent (Shanghai Invitrogen Biotechnology Co., Ltd.). Briefly, RNAi duplex, Lipofectamine™ RNAiMAX, and 250 μl DMEM/F12 medium were combined and incubated for 20 min at room temperature. The transfection complex was added to the cells, and incubated for 48 h at 37 °C.

2.3 Measurement of *Smad4* expression by real-time RT-PCR

Total RNA was extracted from GCs using

TRIzol following the manufacturer's instructions. RNA concentration and purity were determined by measuring optical density (OD) at wavelengths of 260 and 280 nm using a standard spectrophotometer. The OD_{260}/OD_{280} ratios were more than 1.8 for all samples. Aliquots (100 ng) of total RNA from each pool of cultured GCs were independently reverse-transcribed to cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega Corporation, USA) and oligonucleotide primers.

Target genes and the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were quantified by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) with an ABI 7300 using a commercial kit (SYBR® Premix Ex Taq™, TaKaRa, Dalian, China). The gene-specific primers were designed on the basis of porcine mRNA sequences (Table 1). The cDNA generated was used as a template for PCR using a 25-μl reaction mixture for 40–45 cycles. Relative concentrations of mRNA were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). A water control was included to identify possible contamination in all reactions. After amplification, each sample was applied to a 2% (w/v) agarose/ethidium bromide gel for electrophoresis.

2.4 Measurement of Smad4 expression by immunofluorescence

GCs were grown in glass chamber slides. After treatments were applied, cells were washed with PBS three times, fixed with 4% (w/v) paraformaldehyde, washed in PBS, permeabilized with 0.3% (v/v) Triton X-100 in PBS, and blocked in 0.5% (w/v) bovine serum albumin (BSA) and 0.3% (v/v) Triton X-100 in PBS for 30 min at room temperature. Slides were then incubated with primary antibodies as follows: rabbit monoclonal antibody against Smad4 (1:200 dilution in PBS; Abcam; ab40759) for 2 h at room temperature. Slides were washed three times with cold PBS and incubated with biotinylated anti-rabbit immunoglobulin G (IgG; 1:200 dilution in PBS; Abcam; ab6939) for 1 h at room temperature, and then mounted with Vectashield mountant containing the nuclear counter-stain 4′,6-diamidino-2-phenylindole (DAPI; Sigma, USA). Fluorescent images were captured with a Leica DMLB2 fluorescent microscope. Morphometric analysis was carried out using the ImageJ program.

2.5 Measurement of Smad4 expression by Western blotting

Total proteins from GCs were extracted in a cell lysis buffer for Western and immunol precipitation (Beyotime, China) containing 10 mmol/L phenylmethyl sulfonylfluoride (PMSF; Beyotime, China), and the resultant cell suspension was centrifuged at 12000 r/min for 5 min. The supernatant was removed and the amount of protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Beyotime, China). The homogenized samples (15 μg crude proteins per lane) were subjected to electrophoresis on 12% (w/v) poly-acrylamide-sodium dodecyl sulfate (SDS) gels under standard reducing conditions with precision protein molecular weight marked (MBI Fermentas, China), and then transferred to

Table 1 Details of primers for target gene amplification

Target gene (accession number)	Primer sequence	Product size (bp)	Annealing temperature (°C)
Smad4 (NM_214072)	Forward: TTTGCGTCAGTGTCATCG Reverse: TGCTCTGCCTTGGGTAAT	236	59.5
Cyclin D2 (NM-214088)	Forward: TTACCTGGACCGCTTCTTG Reverse: GAGGCTTGATGGAGTTGTCG	155	55.0
CDK4 (NM-001123097)	Forward: GCATCCCAATGTTGTCCG Reverse: GGGGTGCCTTGTCCAGATA	125	60.5
Bcl-2 (AB271960)	Forward: TTCTTTGAGTTCGGTGGGG Reverse: CCAGGAGAAATCAAATAGAGGC	195	62.0
Bax (AJ606301)	Forward: CCGAAATGTTTGCTGACG Reverse: AGCCGATCTCGAAGGAAGT	154	58.0
Cyp19a1 (SSU92246)	Forward: GCTGCTCATTGGCTTAC Reverse: TCCACCTATCCAGACCC	187	60.5
Cyp11a1 (Bx674071)	Forward: AGACACTGAGACTCCACCCCA Reverse: GACGGCCACTTGTACCAATGT	110	69.5
GAPDH (AF017079)	Forward: GGACTCATGACCACGGTCCAT Reverse: TCAGATCCACAACCGACACGT	220	57.2

polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) at 2.5 mA/cm^2 for 45 min. Then they were blocked with 5% (w/v) BSA in PBS (blocking buffer) for 1 h at room temperature. Immunoblotting was carried out by incubating the membranes overnight at 4 °C with antibodies against Smad4 (Abcam; 1:2000 dilution) or GAPDH (Sigma, 1:2000 dilution). The membranes were washed three times with 1% (v/v) Tween-20 in PBS, then hybridized with horse radish peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG antibody (Santa Cruz Biotechnology Inc., California, USA) diluted at 1:5000 for 2 h. After three washes with 1% (v/v) Tween-20 in PBS, the signals were detected as chemical luminescene by X-ray film with BeyoECL Plus kit (Beyotime, China).

2.6 Measurement of cell growth by MTT assay

GCs were counted in a hemocytometer, and the viability was determined by trypan blue dye exclusion, and then the GCs were seeded in 96-well culture plates (Costar; Corning Inc., NY, USA) at a density of (3–5)×10^4 cells per well. GCs were treated with or without siRNA. The number of live cells at the end of the process of culture was determined with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) kit (KeyGEN Biotechnology Co., Ltd., Nanjing, China), as recommended by the manufacturer. The optical density at 550 nm (OD_{550}) was determined using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-TEK Instruments, USA).

2.7 Measurement of cell cycle and apoptosis by flow cytometry

After culture, GCs were digested and prepared by washing twice with PBS, and then fixed in 70% (v/v) ethanol and stained with 100 μg/ml propidium iodide (PI) at 4 °C for 1 h. DNA content was monitored by FACScan (COULTESR, Elite, USA).

Also after culture, GCs were digested and prepared by washing twice with PBS, and then 5 μl annexin V-rhodamine isothiocyanate (RITC) and PI were added to the cells, combined, and incubated for 15 min at room temperature. The rate of apoptosis was monitored by FACScan (COULTESR, Elite, USA).

2.8 Measurement of estradiol and progesterone

GCs (2×10^5 viable cells) were cultured in 24-well plates. After treatment, the culture medium was collected and stored at −80 °C until assay. The levels of estradiol (E2) and progesterone (P4) in the medium were measured by chemiluminescent immunoassay (CLIA; ACCESS, Beckman Coulter Co., USA). Steroid contents were negligible (P4 <0.1 ng/ml and E2 <10 pg/ml). The intra- and inter-assay coefficients of variation were 15% and 20% for E2, and 5% and 10% for P4, respectively.

2.9 Statistical analysis

All results are shown as mean±standard error of the mean (SEM) from at least three separate experiments, each performed with triplicate samples. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Fisher's least significant difference (LSD) test for multiple comparisons using SPSS 16.0 software. Differences were considered to be statistically significant at the 95% confidence level (P<0.05).

3 Results

3.1 Effect of siRNA on *Smad4* expression

We knocked down Smad4 in porcine GCs by siRNA. The expression of Smad4 mRNA and protein was analyzed by real-time PCR, immunofluorescence, and Western blotting at post-transfection, as described in Fig. 1. The results in Fig. 1a show that the expression of *Smad4* mRNA was inhibited after transfection with Smad4-siRNA, the *Smad4* transcript level being reduced by approximately 89% compared with that in the control cultures (blank and NC-siRNA). Western blotting and immunofluorescence staining analyses of Smad4 expression clearly revealed the suppressive effect of Smad4-siRNA on the expression of the Smad4 protein in the GCs (Figs. 1c and 1d). The results of the analyses demonstrate that the level of Smad4 protein also markedly decreased 48 h post-transfection with the Smad4-siRNA, by approximately 56.6% (Fig. 1b).

3.2 Effect of interrupted BMP/Smad signaling on growth of GCs

MTT analysis was performed to test the effect of interrupted BMP/Smad signaling on the growth of GCs. As expected, interrupted BMP/Smad signaling

significantly inhibited the growth of cells, as compared with the control cultures (blank and NC-siRNA) cells (Table 2).

3.3 Effect of interrupted BMP/Smad signaling on cell cycle of GCs

To further determine the effect of interrupted BMP/Smad signaling on the growth of GCs, flow cytometry was used to analyze the cell cycle changes of GCs. The results in Table 3 show that interrupted BMP/Smad signaling can significantly increase the proportion of cells in G1-phase, and decrease the cells in S-phase, and there are no significantly differences in the proportion of cells in G2-phase.

Table 2 Effect of interrupted BMP/Smad signaling on cell growth of GCs

Group	OD_{550}*
Blank	0.2360±0.01[a]
NC-siRNA	0.2616±0.02[a]
Smad4-siRNA	0.1510±0.01[b]

* Values are presented as mean±SEM. Statistically significant differences (P<0.05) among the groups are indicated by different letters

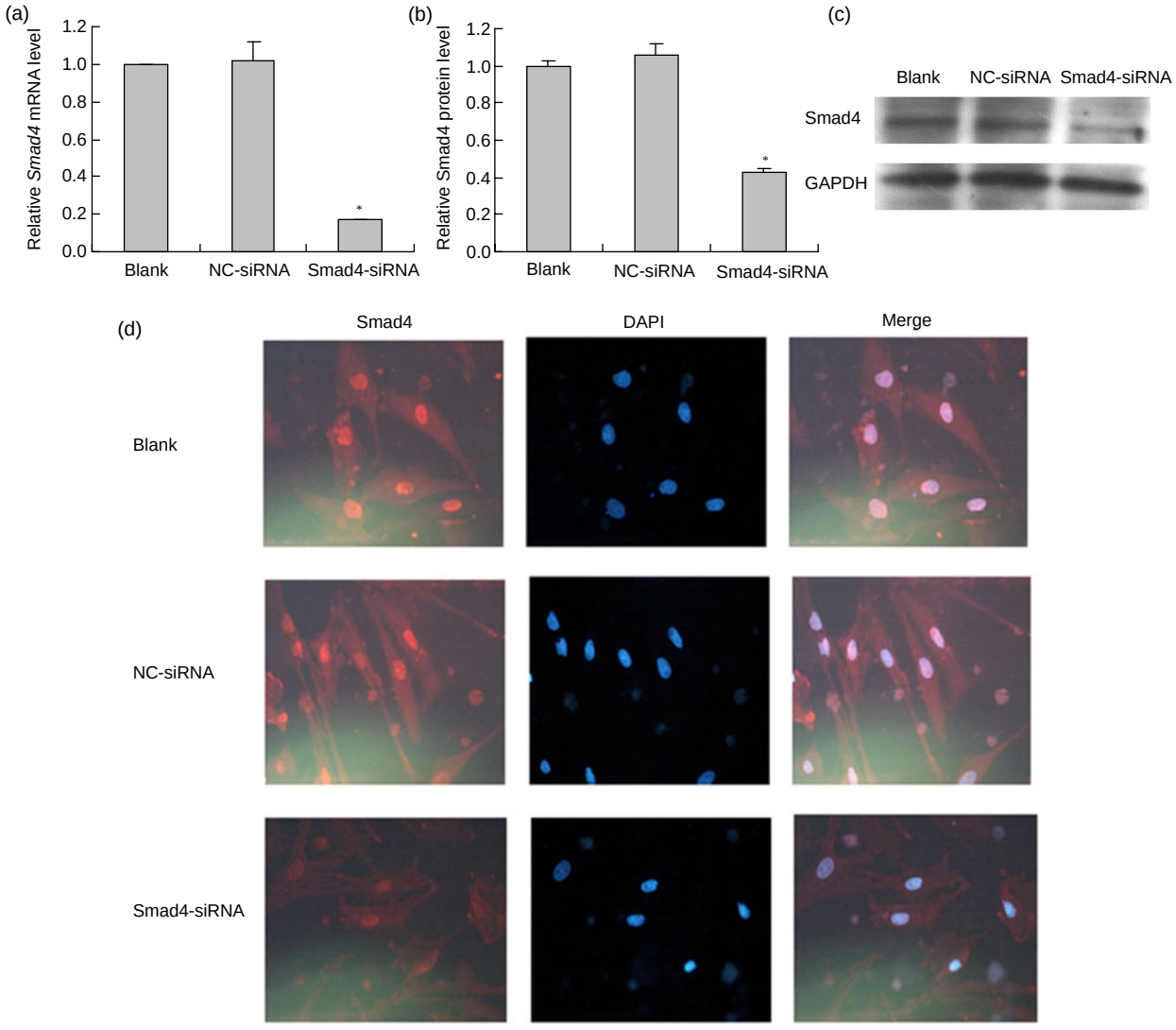

Fig. 1 Analyses of the expression of Smad4 mRNA and protein by real-time PCR, Western blotting, and immunofluorescence at post-transfection

(a) Expression of *Smad4* mRNA after transfection for 48 h; (b) Expression of Smad4 protein after transfection for 48 h; (c) Silencing effect of Smad4 protein expression measured by Western blotting; (d) Silencing effect of Smad4 protein expression measured by immunofluorescence staining. After treatment, cells were fixed and stained with the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI; blue). The data are presented as mean±SEM of three independent experiments. * Statistically significant differences from the blank group (P<0.05)

Table 3 Effect of interrupted BMP/Smad signaling on cell cycle of GCs

Group	Cell cycle		
	G0/G1	S	G2/M
Blank	86.52±0.53[a]	5.65±0.32[a]	6.50±0.16
NC-siRNA	87.31±0.61[a]	5.95±0.56[a]	6.74±0.06
Smad4-siRNA	92.44±0.16[b]	1.66±0.08[b]	5.89±0.16

Values are presented as mean±SEM. Statistically significant differences (P<0.05) among the groups are indicated by different letters

3.4 Effect of interrupted BMP/Smad signaling on cell cycle marker genes

To investigate the mechanism by which interrupted BMP/Smad signaling regulates the cell cycle of GCs, we measured the expression of *Cyclin D2* and *CDK4*, functional markers of cell proliferation (Fig. 2). In Smad4 knockdown cells, *Cyclin D2* and *CDK4* mRNA levels were lower than those in the controls.

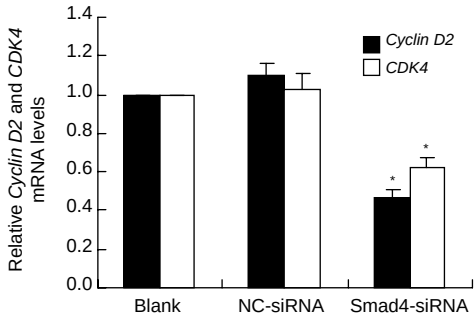

Fig. 2 Effect of interrupted BMP/Smad signaling on cell cycle marker gene mRNA levels
Data are presented as mean±SEM of three independent experiments. * Statistically significant differences (P<0.05) among the groups

3.5 Effect of interrupted BMP/Smad signaling on apoptosis of GCs

Flow cytometry analysis was performed to test the effect of interrupted BMP/Smad signaling on the apoptosis of GCs. Table 4 shows that interrupted BMP/Smad signaling increased the rate of apoptotic cells, as compared with the control cultures (blank and NC-siRNA).

3.6 Effect of interrupted BMP/Smad signaling on cell apoptosis related genes

To investigate the mechanism by which interrupted BMP/Smad signaling encourages the apoptosis of GCs, we measured the mRNA expression of *Bcl-2* and *Bax*, related markers of apoptosis cell (Fig. 3).

Table 4 Effect of interrupted BMP/Smad signaling on apoptosis of GCs

Group	Rate (%)		
	Living cells	Apoptotic cells	Dead cells
Blank	83.12±0.73	15.98±0.62[a]	0.90±0.06
NC-siRNA	81.89±0.95	17.26±0.46[a]	0.85±0.10
Smad4-siRNA	75.16±0.56	23.95±0.88[b]	0.89±0.08

Values are presented as mean±SEM. Statistically significant differences (P<0.05) among the groups are indicated by different letters

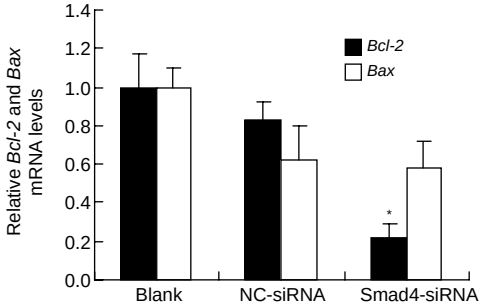

Fig. 3 Effect of interrupted BMP/Smad signaling on apoptosis related gene mRNA levels
Data are presented as mean±SEM of three independent experiments. * Statistically significant differences (P<0.05) among the groups

In Smad4 knockdown cells, *Bcl-2* mRNA levels were significantly lower than those in controls. In contrast, the *Bax* mRNA levels were not affected by interrupted BMP/Smad signaling.

3.7 Effect of interrupted BMP/Smad signaling on estradiol and progesterone production

We examined interrupted BMP/Smad signaling effects on steroidogenesis. As shown in Table 5, interrupted BMP/Smad signaling decreased E2 production, and had no significant effect on P4 production.

Table 5 Effect of interrupted BMP/Smad signaling on estradiol (E2) and progesterone (P4) production

Group	E2 (pmol/L)	P4 (nmol/L)
Blank	117.25±2.75[a]	3.075±0.19
NC-siRNA	120.86±6.15[a]	3.642±0.14
Smad4-siRNA	102.65±3.25[b]	2.943±0.08

Values are presented as mean±SEM. Statistically significant differences (P<0.05) among the groups are indicated by different letters

3.8 Effect of interrupted BMP/Smad signaling on steroidogenic enzyme genes

To confirm whether the effects of interrupted BMP/Smad signaling were involved in the expression

changes of steroidogenic enzyme genes, levels of mRNA encoding *Cyp19a1* and *Cyp11a1*, key enzymes regulating E2 and P4 production in GCs (Lee *et al*., 2001), were examined by quantitative real-time PCR. Interrupted BMP/Smad signaling decreased *Cyp19a1* mRNA expression (Fig. 4).

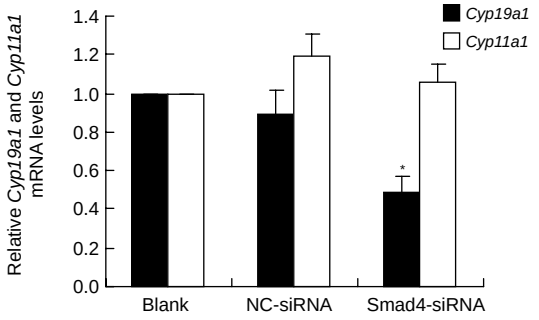

Fig. 4 Effect of interrupted BMP/Smad signaling on steroidogenic enzyme gene mRNA levels
Data are presented as mean±SEM of three independent experiments. * Statistically significant differences (P<0.05) among the groups

4 Discussion

The BMP family controls a variety of intraovarian reproductive processes (Shimasaki *et al*., 2004). To further understand the effect of endogenous BMP/Smad signaling on porcine GCs, in the present study, RNAi was employed to silence the *Smad4* gene, the key mediator of BMP/Smad signaling. It was observed that the knockdown of endogenous *Smad4* gene blocked BMP/Smad signaling, inhibited the growth of GCs, induced cell apoptosis, and decreased E2 production, suggesting that the endogenous BMP/Smad signaling is important to porcine GCs.

Pangas *et al*. (2006)'s study on the Smad4 conditional knockout (cKO) ovary suggested that one of the primary defects of Smad4 loss is premature luteinization of GCs. We silenced endogenous Smad4, as an alternative to block BMP/Smad signaling, in porcine GCs to characterize a potential role of BMP/Smad signaling. We selected RNAi technique in part because the sequence-specific knockdown of target mRNA is more efficient than that of antisense oligonucleotides or ribozymes (Dorsett and Tuschl, 2004; Mittal, 2004). Our results show that Smad4-siRNA can significantly inhibit the mRNA and protein expression of Smad4, as demonstrated by real-time RT-PCR, Western blotting, and immunofluorescence staining. We established a strategy for identifying efficient siRNA sequence for gene knockdown by transfecting porcine GCs.

It has been well documented that cell proliferation depends on the cell cycle. Therefore, we investigated the effects of blocked BMP/Smad signaling on cell cycle of porcine GCs. Blocked BMP/Smad signaling significantly increased and decreased the cells of G1 and S phase stages, respectively. We also investigated the mechanism by which blocked BMP/Smad signaling suppresses cell growth in GCs. The G1 checkpoint can be viewed as a master checkpoint of the mammalian cell cycle (Paterson *et al*., 2002; Matsuura *et al*., 2004). Regulation of the G1 phase of the cell cycle involves many different families of cyclins, Cyclin D and cyclin-dependent kinases (CDKs). Inhibition of growth of GCs by blocked BMP/Smad signaling was associated with a decrease in *Cyclin D2* and *CDK4* expression. *Cyclin D2* and *CDK4* act as a downstream gene of *Smad4*, participating in the regulation of BMPs in porcine GCs, and they may be involved in BMPs-induced cell growth in a Smad4-dependent manner. Thus, we postulate that at least part of blocked BMP/Smad signaling effect on GCs growth is mediated by the decrease in *Cyclin D2* and *CDK4* expression. RNAi-mediated Smad4 silencing resulted in decreased proliferation of the cells and G1 cell cycle arrest, indicating that BMP/Smad signaling plays a key role in the normal growth of porcine GCs.

Follicular development in mammals is dependent on many factors, such as gonadotropin hormones, FSH and LH, and the steroid hormones, E2, and P4, as well as ovarian local factors including TGF-β superfamily. Yamashita *et al*. (2007) and Kawashima *et al*. (2008) believed that the pituitary gonadotropin FSH is essential for GCs' E2 production and increases estrogen concentrations in follicular fluid during the development of mammalian ovarian follicles. Changes in the expression of mRNA encoding steroidogenic genes exhibit patterns of expression that coordinated with steroid production. In vivo, the growth and differentiation of GCs are tightly regulated by gonadotropins and ovarian local factors such as estrogen, GDF-9/BMP-15, and insulin-like growth factor 1 (IGF-1). However, in vitro, people cannot mimic

these conditions. We investigated the effects of interrupted endogenous BMP/Smad signaling on steroidogenesis of porcine GCs. After transfection, the E2 level of Smad4-siRNA group significantly decreased, while transfection had no significant effect on P4. A possible reason is that we measured the basal levels of E2 and P4 in cultured GCs; thus, unknown factors in the serum may have affected GC steroidogenesis. These require further elucidation.

A widely accepted opinion is that the Ras-MAPK (mitogen-activated protein kinase) pathway (activated after LH surge) plays a major role in regulating GC steroidogenesis (Fan *et al*., 2008; 2009). The TGF-β superfamily members are critical in maintaining cell growth and differentiation in the ovary (Pangas *et al*., 2008). BMPs play a key role in regulating GC steroidogenesis. In particular, they change the expression of steroidogenic enzymes such as *Cyp19a1* (aromatase) and *Cyp11a1* (p450scc) (Winters *et al*., 1998), and these actions are important, and are modulated by various growth factors, acting in a paracrine or autocrine way (Khamsi and Roberge, 2001). In vitro, BMP-4 and BMP-7 enhanced FSH-dependent E2 production (Shimasaki *et al*., 1999), whereas BMP-4, BMP-6, BMP-7, BMP-15, and GDF-9 inhibited FSH-induced P4 secretion by rat GCs in culture (Shimasaki *et al*., 1999; Otsuka *et al*., 2000; Vitt *et al*., 2000; Fraser *et al*., 2001; Lee *et al*., 2001), suggesting that BMP factors regulate GC differentiation, particularly in delaying luteinization. In the present study, blocked BMP/Smad signaling inhibited E2 secretion, but did not affect P4 secretion. In order to evaluate the regulation of E2 and P4 production, we examined the steady-state level of mRNA for *Cyp19a1* and *Cyp11a1* genes, two of the key regulators in the steroidogenesis implicated in the E2 and P4 synthesis pathways. Our results show that blocked BMP/Smad signaling significantly inhibited *Cyp19a1* expression.

In conclusion, the interruption of endogenous BMP/Smad signaling by RNAi-mediated Smad4 gene silencing not only inhibits the growth but also induces the apoptosis of porcine GCs. In addition, interrupted BMP/Smad signaling changes steroidogenesis. Our findings provide a new insight that interrupted BMP/Smad signaling plays an important and specific role in mediating porcine GC growth and steroidogenesis.

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