Interference RNA-based silencing of endogenous SMAD4 in porcine granulosa cells resulted in decreased FSH-mediated granulosa cells proliferation and steroidogenesis

Wei Wang, Xia Chen, Xinxu Li, Li Wang, Haiyan Zhang, Yu He, Jingjing Wang, Yongyan Zhao, Baole Zhang and Yinxue Xu

College of Animal Science and Technology, Nanjing Agricultural University, Weigang 1, Nanjing 210095, People's Republic of China

Correspondence should be addressed to Y Xu; Email: xuyinxue@njau.edu.cn

Abstract

FSH plays a critical role in granulosa cell (GC) proliferation and steroidogenesis through modulation by factors including bone morphogenetic proteins family, which belongs to transforming growth factor β (TGFB) superfamily. TGFBs are the key factors in maintaining cell growth and differentiation in ovaries. However, the interaction of FSH and TGFB on the GCs' proliferation and steroidogenesis remains to be elucidated. In this study, we have investigated the role of SMAD4, a core molecule mediating the intracellular TGFB/SMAD signal transduction pathway, in FSH-mediated proliferation and steroidogenesis of porcine GCs. In this study, SMAD4 was knocked down using interference RNA in porcine GCs. Our results showed that SMAD4-siRNA causes specific inhibition of SMAD4 mRNA and protein expression after transfection. Knockdown of SMAD4 significantly inhibited FSH-induced porcine GC proliferation and estradiol production and changed the expression of cyclin D2, CDK2, CDK4, CYP19a1, and CYP11a1. Thus, these observations establish an important role of SMAD4 in the regulation of the response of porcine GCs to FSH.

Reproduction (2011) **141** 643–651

Introduction

FSH is the major regulator of folliculogenesis, particularly in regulating granulosa cell (GC) proliferation and steroidogenesis. Accumulating evidence highlights the intraovarian transforming growth factor β (TGFB) superfamily signaling cascades as key regulatory pathways of fundamental female reproductive events, including folliculogenesis and ovulation (Findlay *et al.* 2002, Juengel & McNatty 2005, Kaivo-oja *et al.* 2006, Knight & Glister 2006, Pangas 2007). Recent studies have established the concept that TGFBs, particularly bone morphogenetic proteins (BMPs), play a crucial role in female fertility in mammals by regulating steroidogenesis as well as mitogenesis in GCs (Shimasaki *et al.* 2003, 2004). Notably, in GCs, a major regulatory process governed by BMPs involves the control of FSH signaling. For example, BMP6 inhibits FSH-induced progesterone (P_4) synthesis through suppression of cellular cAMP synthesis (Otsuka *et al.* 2001a). BMP15 also exhibits potent suppression of FSH actions by inhibiting FSH receptor (FSHR) expression (Otsuka *et al.* 2001b). In addition, growth and differentiation factor-9 (GDF9), which is highly homologous with BMP15, also inhibits FSH-induced steroidogenesis and LH receptor expression

in rat GCs (Vitt *et al.* 2000). Using a human granulosa KGN cell line, Miyoshi & Otsuka found that FSH preferentially upregulates the expression of type I receptors (BMPR1A (ALK-3) and BMPR1B (ALK-6)) as well as type II receptors (ACVR2A (ActRII) and BMPR2). In addition, FSH increased the expression levels of SMAD1/5 and decreased the expression of inhibitory SMADs, SMAD6/7. Thus, it is most likely that FSH augments the bioavailability of BMP signaling in human granulosa (Miyoshi *et al.* 2006).

SMADs are the downstream signaling molecules mediating the intracellular TGFB/SMAD signal transduction pathways and are divided into three functional groups: common SMAD (co-SMAD), receptor-regulated SMADs (R-SMADs), and inhibitory SMADs. Classical TGFB signaling pathway is initiated when the ligands bind their serine/threonine kinase type 2 and type 1 (activin receptor-like kinase) receptors to form an oligomeric receptor complex (Kaivo-oja *et al.* 2006, Knight & Glister 2006, Schmierer & Hill 2007). Subsequently, R-SMADs are phosphorylated and activated by type 1 receptor. The R-SMADs then form heteromeric complexes with co-SMAD and accumulate in the nucleus to regulate ligand-specific gene

expression via recruitment of distinct transcription factors, coactivators, and corepressors (Massague 1992, 2000). Activation of SMADs can be modulated at multiple levels by numerous molecules including the ligand-induced inhibitory SMADs, SMAD6 and SMAD7, the function of which is to prevent R-SMADs from phosphorylation and signaling activation by their binding to type 1 receptors (Kawabata *et al.* 1998, Massague 2000, Moustakas *et al.* 2001, Mehra & Wrana 2002, ten Dijke & Hill 2004).

All TGFβs can transduce their signals *in vitro* through SMAD2 and SMAD3, which share more than 90% identity in their amino acid sequences (Brown *et al.* 2007), whereas BMPs activate their signaling pathways through SMAD1, SMAD5, and SMAD8 (Chang *et al.* 2001, Brown *et al.* 2007). However, in both cases, TGFβ superfamily signaling cascades transmit their signals through co-mediating SMAD. In mammals, SMAD4 is the only co-mediating SMAD, which translocates to the nucleus with phosphorylated receptor-activated SMADs and then modulates transcription of TGFβ target genes. SMAD4 is broadly expressed in embryonic and adult 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 required for optimal TGFβ signaling (Lin *et al.* 2006, Dai *et al.* 2009). SMAD4 loss leads to increased steady-state levels of both BMP and TGFβ phosphorylated R-SMADs. Enhanced R-SMAD phosphorylation levels potentially reflect decreased dephosphorylation and/or nuclear export (Lin *et al.* 2006, Dai *et al.* 2009). SMAD4 is thus crucial for TGFβ/SMAD signaling.

In oocytes, GCs, and theca interna cells (TIC) of both immature and cycling rats, SMAD4 (Guéripel *et al.* 2004) has been detected. However, the regulation of SMAD4, as the only co-mediating SMAD, in FSH stimulation of porcine ovarian GCs remains poorly understood. In this study, we applied siRNA approach to knockdown SMAD4 expression and investigated the role of SMAD4 in FSH-induced proliferation and steroidogenesis of porcine GCs.

Results

Inhibition of SMAD4 expression by RNA interference

We knocked down SMAD4 expression in porcine GCs by RNA interference (RNAi). Cells transfected with negative control siRNA (NC-siRNA) and SMAD4-siRNA were analyzed for the expression of SMAD4 mRNA and protein 48 h post-transfection by real-time PCR, immunofluorescence, and western blotting, as shown in Fig. 1. The results as in Fig. 1A showed that SMAD4 mRNA expression was inhibited after transfection with SMAD4-siRNA, the SMAD4 transcript level being reduced by ~89.0% compared with that in the

control cultures (blank). The expression of SMAD4 protein clearly revealed the suppressive effect of SMAD4-siRNA in the GCs by immunofluorescence staining and western blotting (Fig. 1B and C), with the level of SMAD4 protein being decreased by ~56.6% 48 h post-transfection with the SMAD4-siRNA (Fig. 1D).

Effect of SMAD4 knockdown on SMAD1, 3, and 5 expressions

To test whether other SMADs were affected by SMAD4-siRNA in GCs, cells were collected after treatment, and SMAD1, 3, and 5 mRNA levels were determined

Figure 1 (A) Expression of SMAD4 mRNA after transfection for 48 h. (B) Silencing effect of expression SMAD4 protein measured by immunofluorescence staining. (C) Silencing effect of expression SMAD4 protein measured by western blotting. (D) Expression of SMAD4 protein after transfection for 48 h. Statistically significant differences from the blank group are indicated by * ($P < 0.05$).

Figure 2 (A) Expression of *SMAD1* mRNA after transfection for 48 h. (B) Expression of *SMAD3* mRNA after transfection for 48 h. (C) Expression of *SMAD5* mRNA after transfection for 48 h.

by quantitative real-time PCR. The results showed that the *SMAD1*, 3, and 5 mRNA levels were not affected by *SMAD4* silencing in porcine GCs (Fig. 2A–C).

Effect of *SMAD4* knockdown on FSH-induced FSHR expression

To better understand the effects of *SMAD4* knockdown on the role of FSH in GCs, we verified the expression of FSHRs. GCs were collected after treatment, and mRNA levels were determined by quantitative real-time PCR. The results in Fig. 3 showed that FSHR is significantly downregulated in *SMAD4*-siRNA cells without FSH, and in FSH + *SMAD4*-siRNA treatment, the expression of *FSHR* mRNA significantly decreased as compared with that of FSH.

Effect of *SMAD4* knockdown on the number of living cells in FSH-induced GCs

MTT (methyl thiazolyl tetrazolium) analysis was performed to test the effect of *SMAD4* knockdown on the number of living cells in FSH-induced GCs. As expected, FSH induced an increase in the number of living cells in untransfected cells, compared with blank group (Fig. 4). In contrast, the increase of living cells was downregulated for cells transfected with *SMAD4*-siRNA, comparing FSH + *SMAD4*-siRNA group with FSH group.

Figure 3 Effect of knockdown of endogenous *SMAD4* on FSH-induced *FSHR* mRNA levels. Significant differences among the groups are indicated by different letters ($P < 0.05$).

Effect of *SMAD4* knockdown on FSH-induced cell cycle

To further determine the effect of knockdown of *SMAD4* expression on FSH-induced cell growth, flow cytometry was used to analyze cells for the changes of cell cycle. Cells were labeled with propidium iodide (PI) after treatment. The effects of FSH on the cell cycle were investigated in untransfected cells as well as cells transfected with the *SMAD4*-siRNA.

The results in Table 1 show that knockdown of *SMAD4* expression significantly increases the proportion of cells in G_0/G_1 phase. And with FSH + *SMAD4*-siRNA treatment, the number of G_0/G_1 phase cells significantly increased compared with FSH. Silencing *SMAD4* expression can significantly decrease the cells of S phase in both the presence and the absence of FSH. No significant differences were observed in the proportion of cells in G_2/M phase between groups.

Effect of knockdown of *SMAD4* on the expression of cell cycle marker genes of FSH-induced GCs

To investigate the mechanism by which *SMAD4* knockdown represses FSH-induced growth of GCs, we measured the expression of cyclin B, cyclin D2, cyclin-dependent kinase 2 (CDK2), and CDK4, the functional markers of cell cycle (Fig. 5). FSH increased levels of mRNA encoding cyclin B and cyclin D2 in untransfected cells. In *SMAD4* knockdown cells, cyclin D2 mRNA levels were lower than that in the blank group. In FSH + *SMAD4*-siRNA group, FSH-induced increase in the expression of cyclin D2 was significantly inhibited, as compared with that of FSH. However, the expression of cyclin B did not significantly change in the FSH and FSH + *SMAD4*-siRNA groups. The *CDK2* and *CDK4* mRNA levels were decreased by *SMAD4* silencing based on whether cells were stimulated by FSH or not.

Effect of *SMAD4* knockdown on FSH-induced E_2 and P_4 production

We examined the effects of *SMAD4* on steroidogenesis regulated by FSH. As shown in Table 2, FSH increased the levels of E_2 , and *SMAD4*-siRNA decreased the levels of E_2 , and *SMAD4* knockdown attenuated FSH-induced augmentation of E_2 . FSH raised the production of P_4 but *SMAD4*-siRNA did not, and *SMAD4* knockdown did not

Figure 4 Effect of knockdown of endogenous *SMAD4* expression on the number of living cells in FSH-induced GCs. Significant differences among the groups are indicated by different letters ($P < 0.05$).

affect FSH-induced secretion of P_4 . *SMAD4* interference decreased FSH-induced E_2 production and had no significant effect on FSH-induced P_4 , comparing FSH group with FSH + *SMAD4*-siRNA group.

Effect of *SMAD4* knockdown on FSH-induced steroidogenic enzyme genes

To confirm whether the effects of *SMAD4* were involved in the expression changes of steroidogenic enzyme genes, levels of mRNA encoding *CYP19a1*, *CYP11a1*, *STAR*, and *HSD 3B*, the key enzymes regulating E_2 and P_4 production in GCs, were examined by quantitative real-time PCR. Knockdown of *SMAD4* decreased *CYP19a1* and *CYP11a1* mRNA expression induced by FSH as compared between the FSH and FSH + *SMAD4*-siRNA groups. In contrast, the *STAR* and *HSD 3B* mRNA levels induced by FSH were not affected by *SMAD4* silencing (Fig. 6).

Discussion

The TGF β superfamily has been implicated in multiple GC processes, including proliferation, differentiation, and regulation of steroidogenesis. SMADs are downstream signaling molecules mediating the intracellular TGF β /SMAD signal transduction pathways. *SMAD4* is the only co-SMAD in mammals. This study aimed to determine the role of *SMAD4* in growth and steroidogenesis of porcine GCs *in vitro*. We have uncovered a cellular mechanism of regulation of GC growth and steroidogenesis involved in the interaction between FSH and *SMAD4* in porcine GCs.

SMADs are a class of very important factors regulating ovarian function in mammals, but they are also mediated by TGF β /SMAD signal transduction pathways. The *SMAD2* cKO mouse model supplied evidence that *SMAD2* is not requisite in transducing TGF β signals in

GCs (Li *et al.* 2008). The results from *SMAD3* cKO mice support the concept that *SMAD3* is capable of activating essential target genes downstream of TGF β -related ligands (Gong & McGee 2009). Furthermore, results from *SMAD2* and *SMAD3* double cKO mice showed that the loss of *SMAD2/3* signaling in GCs led to disrupted follicular development and reduced ovulation efficacy (Li *et al.* 2008). Recently, *SMAD1/5/8* cKO mice have been generated by Pangas' group, and these mice developed metastatic tumors with increased signaling of *SMAD2/3* in GCs (Pangas *et al.* 2008). Moreover, ablation of all TGF β signaling in the ovarian GCs by conditionally inactivating *SMAD4* resulted in premature luteinization of GCs, leading to the predisposition of premature ovarian failure (Pangas *et al.* 2006). The R-SMADs form heteromeric complexes with *SMAD4*, which translocates to the nucleus and then modulates transcription of TGF β target genes. We chose to knock down endogenous *SMAD4*, as an alternative, in porcine GCs to characterize a potential interaction between FSH and *SMAD4*. We adopted an RNAi technique in part because the sequence-specific knockdown of target mRNA is more efficient than antisense oligonucleotides or ribozymes (Dorsett & Tuschl 2004, Mittal 2004). Our results showed that our *SMAD4*-siRNA significantly inhibits the mRNA and protein expression of *SMAD4*, as demonstrated by real-time RT-PCR, immunofluorescence, and western blotting. The role of *SMAD4* as a co-SMAD is not required for R-SMAD phosphorylation or nuclear localization (Biondi *et al.* 2007) with which our results are consistent. Therefore, we detected the expression of *SMAD1*, *3*, and *5* mRNA, with their mRNA levels being not affected by *SMAD4* loss in porcine GCs.

We demonstrated that *SMAD4*, the only co-mediating SMAD, plays a key role in FSHR expression *in vitro*. Our findings showed that FSHR is significantly down-regulated in *SMAD4*-siRNA-treated cells, which is in agreement with a previous study in which FSHR was slightly downregulated in GCs from pregnant mare's serum gonadotrophin (PMSG)-stimulated *SMAD4* cKO ovaries (Pangas *et al.* 2006). Knockdown of *SMAD4* also diminished the ability of FSH to upregulate its own receptor. Genes regulating FSHR activity may be of great functional importance. *SMAD4* deficiency does not turn off the FSHR, but it does reduce the ability of the

Table 1 Knockdown of endogenous expression of *SMAD4* alters FSH-induced cell cycle progression.

Group	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
Blank	92.08 ± 0.66 ^{bc}	3.25 ± 0.21 ^a	4.67 ± 0.87 ^a
FSH	90.64 ± 0.27 ^c	4.12 ± 0.18 ^a	5.24 ± 0.09 ^a
<i>SMAD4</i> -siRNA	94.41 ± 0.35 ^a	0.95 ± 0.69 ^c	4.65 ± 0.34 ^a
FSH + <i>SMAD4</i> -siRNA	92.38 ± 0.36 ^b	2.15 ± 1.25 ^b	5.48 ± 0.90 ^a

Significant differences among the groups are indicated by different letters ($P < 0.05$).

Figure 5 Knockdown of endogenous expression of SMAD4 effect FSH-induced cell cyclin B, cyclin D2, *CDK2*, and *CDK4* mRNA levels. Significant differences among the groups are indicated by different letters ($P < 0.05$).

GCs to respond to gonadotropins. Further studies are necessary to clarify the levels of regulation and the role of signaling cross talk in determining FSHR regulation and downstream function.

FSH stimulates porcine GC proliferation, but SMAD4 loss inhibits cell proliferation in this study. It has been well documented that cell proliferation depends on progression through the cell cycle, and in this study, we showed that SMAD4 knockdown resulted in G_0/G_1 arrest and a decrease in the proportion of cells in S phase. This study also investigated the mechanism(s) whereby SMAD4-siRNA suppresses FSH-induced cell proliferation in GCs. FSH potentially increases cell cycle progression at the G_0/G_1 phase (Paterson *et al.* 2002, Matsuura *et al.* 2004). The G_0/G_1 checkpoint can be viewed as a master checkpoint of the mammalian cell cycle (Paterson *et al.* 2002, Matsuura *et al.* 2004). Regulation of the G_0/G_1 phase of the cell cycle involves many different families of cyclins: cyclin B, cyclin D, and CDKs. Inhibition of FSH-induced proliferation of GCs by SMAD4-siRNA was associated with decrease in cyclin D2 expression, instead of cyclin B. Cyclin D2 acts as a downstream gene of SMAD4, participating in the regulation of FSH in porcine GCs, and it may be involved in FSH-induced cell growth in a SMAD4-dependent manner. So we postulate that at least part of the SMAD4-siRNA effect on FSH-induced GC proliferation is mediated by a decrease in cyclin D2 expression. This hypothesis needs further studies to be verified.

FSH plays a central role in regulating GC steroidogenesis. In particular, FSH enhances the expression of steroidogenic enzymes such as CYP11a1 and CYP19a1. *In vitro*, FSH-stimulated steroidogenesis is modulated by growth factors, including members of the BMP family. Recombinant BMP4, BMP6, BMP7, BMP15, and GDF9

inhibited FSH-induced P_4 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), whereas BMP4 and BMP7 enhanced FSH-dependent E_2 production, suggesting that BMP factors may delay luteinization (Shimasaki *et al.* 1999). In this study, SMAD4 knockdown inhibited FSH-induced E_2 secretion but did not affect FSH-induced P_4 secretion. These data led us to analyze the expression of *CYP19a1*, *CYP11a1*, *STAR*, and *HSD 3B* genes, the four of the key regulators in the steroidogenesis implicated in the E_2 and P_4 synthesis pathway. Our results showed that SMAD4-siRNA significantly inhibits FSH-induced CYP19a1 and CYP11a1 expression. There is a conflicting report regarding the effect of SMAD4 on steroidogenesis in other species. Studies by Pangas *et al.* (2006) showed that SMAD4 loss did not alter CYP19a1 mRNA levels in mouse GCs, but upregulated CYP11a1 mRNA levels. These results demonstrate the difficulty in extrapolating data between species and also between different conditions (*in vivo* and *in vitro*). The mRNA expression of *STAR* and *HSD 3B* induced by FSH were not affected by SMAD4 silencing. Our results are consistent with the studies by Pangas *et al.* Their results showed that *STAR* was significantly upregulated by PMSG, even in SMAD4 cKO mice. FSH signaling pathway is a complex regulatory network, in which related gene expression is affected by many factors. Our results showed that the *STAR* and *HSD3B* mRNA levels induced by FSH were not affected by SMAD4 silencing. However, the results are inconsistent with our expected ones. We expected that FSH-responsive genes would be regulated by FSH in the presence of SMAD4 silencing. This needs further investigation.

In summary, we used siRNA to knock down SMAD4 expression in porcine GCs and provided evidence that SMAD4 plays an important and specific role in mediating FSH-induced GCs proliferation and steroidogenesis.

Materials and Methods

Isolation and culture of GCs

Porcine ovaries were obtained at a local slaughterhouse and transported to the laboratory at 37 °C in saline containing gentamicin and amphotericin. The ovaries were washed three

Table 2 Effects of knockdown of endogenous expression of SMAD4 on FSH-induced estradiol (E_2) and progesterone (P_4) production.

	E_2 (pmol/l)	P_4 (nmol/l)
Blank	117.25 ± 2.75 ^b	3.075 ± 0.19 ^b
FSH	154.75 ± 8.75 ^a	4.688 ± 0.04 ^a
si-SMAD4	104.25 ± 5.25 ^c	2.703 ± 0.02 ^b
FSH + siSMAD4	124.00 ± 6.50 ^b	4.145 ± 0.07 ^a

Significant differences among the groups are indicated by different letters ($P < 0.05$).

Figure 6 Effects of knockdown of endogenous *SMAD4* expression on FSH-induced steroidogenic enzyme genes. Significant differences among the groups are indicated by different letters ($P < 0.05$).

times with pre-warmed PBS supplemented with gentamicin and amphotericin. GCs were collected from follicles (3–5 mm) by aspiration using a syringe with needle (20 G) and filtered through a stainless steel filter (100 μ m) to remove the cumulus–oocytes complexes. The cells were centrifuged at 800 g /min and washed twice in DMEM/F12 medium (Shanghai Invitrogen Biotechnology Co., Ltd). 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, then seeded in six-well culture plates (Costar, Corning, Inc., New York, NY, USA) at a density of $(3\text{--}5) \times 10^6$ cells per well in 2 ml of DMEM/F12 containing 10% FCS (MinHai Co., LanZhou, People's Republic of China), gentamicin (5 μ l/ml) and amphotericin (10 μ l/ml). The cells were cultured for 24 h at 37 $^{\circ}$ C in a 5% CO_2 atmosphere and then the wells were washed with PBS to remove unattached cells. Exponentially growing cells were used for the experiments.

Design and transfection of siRNA

The cDNA sequence of *SMAD4* (GenBank accession number, NM_214072) was examined using Invitrogen's web-based siRNA design software (<https://rnaidesigner.invitrogen.com/rnaexpress/>) to select appropriate siRNA target sites. A pair of oligonucleotides corresponding to *SMAD4* cDNA at the start position 925 bp was designed. The siRNA sequence of *SMAD4* is as follows: sense, CAC CAG GAA UUG AUC UCU CAG GAU U; antisense, AAU CCU GAG AGA UCA AUU CCU GGU G. A Blast search of these sequences confirmed their specificity to *SMAD4* only. In addition, a nonsense sequence with no similar match to any known sequence was designed as control. The siRNA sequence of nonsense sequence is as follows: sense, CAC AAG GAG UUC UCU GAC UGA CAU U; antisense, AAU GUC AGU CAG AGA ACU CCU UGU G. The *SMAD4* small RNAi was named *SMAD4*-siRNA, the nonsense sequence to *SMAD4* was named NC-siRNA.

Cultures of GCs were transfected with *SMAD4*-siRNA or NC-siRNA using Lipofectamine RNAiMAX Reagent (Shanghai

Table 3 Details of primers for target gene amplification.

Target gene (accession number)	Primer sequence	Product size (bp)	Annealing temperature ($^{\circ}$ C)
<i>SMAD4</i> (NM_214072)	Forward: TTTGCGTCAGTGTCATCG Reverse: TGCTCTGCCTTGGGTAAT	236	59.5
<i>SMAD1</i> (NM_213965)	Forward: CTGCGAGTTTCCTTTGGT Reverse: GAGGCTGTGCTGAGGGTTA	133	63.5
<i>SMAD3</i> (NM_214137)	Forward: GACTACAGCCATTCCATCCC Reverse: CTGTGGTTCATCTGGTGGTC	134	55.5
<i>SMAD5</i> (NM_001001419.1)	Forward: TGTTGCCATGAAGAGCCTAA Reverse: CACCTTTTCCAATATGTCGC	215	57.0
<i>FSHR</i> (NM_214386)	Forward: TCCCTCGGTTCTTATGT Reverse: ATGGCGGACTTGCACTTT	269	53.5
Cyclin B (NM_001170768)	Forward: TGGCTAGTGCAAGTTTCAG Reverse: CAGTCACAAAGGCAAAGT	199	54.5
Cyclin D2 (NM_214088)	Forward: TTACCTGGACCGCTTCTTG Reverse: GAGGCTTGATGGAGTTGTCCG	155	55.0
<i>CDK2</i> (XM-001928965)	Forward: AAACAAGTTGACGGGAGA Reverse: GTGAGAATGGCAGAAAGC	298	56.0
<i>CDK4</i> (NM_001123097)	Forward: GCATCCCAATGTTGTCCG Reverse: GGGGTGCCTTGTCAGATA	125	60.5
<i>Cyp19a1</i> (SSU92246)	Forward: GCTGCTCATTGGCTTAC Reverse: TCCACCTATCCAGACCC	187	60.5
<i>Cyp11a1</i> (Bx674071)	Forward: AGACACTGAGACTCCACCCCA Reverse: GACGGCCACTTGTAACCAATGT	110	69.5
<i>STAR</i> (NM_213755)	Forward: GGAGAGCCCGCAGGAGAATG Reverse: CTTCTGCAGGATCTTGATCTTCTTG	191	54.5
<i>HSD 3B</i> (NM_001004049)	Forward: AGGGTTTCTGGGTGAGAGGATC Reverse: CGTTGACCACGTCGATGATAGAG	107	56.5
<i>GAPDH</i> (AF017079)	Forward: GGACT CATGACCAG GTCCA T Reverse: TCAGATCCAC AACCG ACACG T	220	57.2

Invitrogen Biotechnology Co., Ltd). Briefly, 8 pmol RNAi duplex, 4 μ l Lipofectamine RNAiMAX, and 250 μ l DMEM/F12 were combined and incubated for 20 min at room temperature. The transfection complex was added to the cells and incubated for 8 h at 37 °C. Then the medium was drawn, and the new DMEM/F12 containing 1% FCS (MinHai Co.), gentamicin (5 μ l/ml), and amphotericin (10 μ l/ml) was added. At the same time, the cells in FSH group and FSH+SMAD4-siRNA group were treated with FSH (NingBo Hormone Products Co., Ltd, NingBo, People's Republic of China) at the concentration of 1 IU/ml for 48 h.

Real-time quantitative RT-PCR

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

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

Immunofluorescence

GCs were grown in glass chamber slides. After treatments, cells were washed with PBS three times, fixed in 4% paraformaldehyde for 15 min, washed three times with PBS, and then pre-incubated with PBS supplemented with 0.5% BSA for 30 min at room temperature, to permeabilize the cells in order to allow antibody penetration for the detection of intracellular as well as cell surface antigens. Cells were incubated with rabbit MAB against SMAD4 (1:200 dilution in PBS; Abcam, Shanghai, People's Republic of China; ab40759) for 2 h at room temperature. Slides were washed three times with cold PBS and incubated with biotinylated anti-rabbit IgG (1:200 in PBS; Abcam; ab6939) for 1 h at room temperature. Then, the slides were washed three times with cold PBS and were mounted with Vectashield mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI, Sigma). Cells were counterstained with DAPI. The immunofluorescent signals were examined with a Leica DMLB2 fluorescence microscope. All images were captured using the same settings and saved in the same format. The gray-scale signal of 20 fields was analyzed for each replicate using the ImageJ Software (<http://rsbweb.nih.gov/ij/>).

Measurement of SMAD4 expression by western blotting

Protein from GCs was extracted in a cell lysis buffer for western and IP (Beyotime, Shanghai, People's Republic of China) containing 10 mM phenylmethylsulphonyl fluoride (Beyotime), and the resultant cell suspension was centrifuged at 13 800 *g* (12 000 r.p.m.) for 5 min. The supernatant was removed and the amount of protein was quantified using the BCA Protein Assay kit (Beyotime). The homogenized samples (15 μ g crude proteins per lane) were subjected to electrophoresis on 12% polyacrylamide-SDS gels and then transferred to PVDF membranes (Millipore, Bedford, MA, USA) at 2.5 mA/cm² for 45 min. Then they were blocked with 5% 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; 1:200) or GAPDH (Sigma; 1:2000). The membranes were washed three times with 1% Tween-20 in PBS and then hybridized with HRP-conjugated secondary goat anti-rabbit IgG antibody (Santa Cruz Biotechnology Shanghai, People's Republic of China) diluted at 1:5000 for 2 h. After three washes with 1% Tween-20 in PBS, the signals were detected as chemical luminescence by X-ray film with BeyoECL Plus kit (Beyotime).

Cell proliferation assay

GCs were counted in a hemocytometer, and the viability was determined by trypan blue dye exclusion and then seeded in 96-well culture plates (Costar, Corning, Inc.) at a density of $(3-5) \times 10^4$ cells per well, and treated with or without siRNA and FSH. The number of living cells at the end of culture was determined with the MTT kit (KeyGEN Biotechnology Co., Ltd, Nanjing, Jiangsu, People's Republic of China) as recommended by the manufacturer. After treatment, MTT (50 μ l) was added into each well. After 4 h, the liquid in the wells was drawn out, 150 μ l DMSO was added into each well, and then incubated for 10 min at 37 °C. The OD at 550 nm was determined using an ELISA reader (Bio-TEK Instruments, Winooski, VT, USA).

Flow cytometry

In order to do flow cytometry, we seeded GCs in 25 cm² culture flask at a density of $(5-8) \times 10^6$ cells per flask. After treatment, GCs were digested using 0.25% pancreatic enzyme without EDTA, washed twice with PBS, fixed in 70% ethanol, and stained with 100 μ g/ml propidium iodide at 4 °C for 1 h. The DNA content was monitored by FACScan FACS Calibur (Becton Dickinson, Franklin, NJ, USA).

Measurement of P_4 and E_2

GCs (2×10^5 viable cells) were cultured in 24-well plates. After treatment, the culture media was collected and stored at -80 °C. The levels of P_4 and E_2 in the media were measured by chemiluminescent immunoassay (ACCESS, Beckman Coulter Co., Pasadena, CA, USA). Steroid contents were negligible ($P_4 < 0.1$ ng/ml and $E_2 < 10$ pg/ml). The intra- and interassay coefficients of variation were 15 and 20% for E_2 respectively and were 5 and 10% for P_4 respectively.

Statistical analysis

All results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. Data were analyzed by one-way ANOVA, followed by Fisher's least significant difference test for multiple comparisons using the SPSS 16.0 software (SPSS Inc., Shanghai, People's Republic of China). Statistical significance was accepted at $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by Hi-Tech Research and Development Program of China (2006AA10Z136).

References

- Biondi CA, Das D, Howell M, Islam A, Bikoff EK, Hill CS & Robertson EJ 2007 Mice develop normally in the absence of SMAD4 nucleocytoplasmic shuttling. *Biochemical Journal* **404** 235–245. (doi:10.1042/BJ20061830)
- Brown KA, Pietenpol JA & Moses HL 2007 A tale of two proteins: differential roles and regulation of SMAD2 and SMAD3 in TGF- β signaling. *Journal of Cellular Biochemistry* **101** 9–33. (doi:10.1002/jcb.21255)
- Chang H, Lau AL & Matzuk MM 2001 Studying TGF-beta superfamily signaling by knockouts and knockins. *Molecular and Cellular Endocrinology* **180** 39–46. (doi:10.1016/S0303-7207(01)00513-5)
- Dai F, Lin X, Chang C & Feng XH 2009 Nuclear export of SMAD2 and SMAD3 by RanBP3 facilitates termination of TGF-beta signaling. *Developmental Cell* **16** 345–357. (doi:10.1016/j.devcel.2009.01.022)
- ten Dijke P & Hill CS 2004 New insights into TGF-beta-Smad signalling. *Trends in Biochemical Sciences* **29** 265–273. (doi:10.1016/j.tibs.2004.03.008)
- Dorsett Y & Tuschl T 2004 siRNAs: applications in functional genomics and potential as therapeutics. *Nature Reviews. Drug Discovery* **3** 318–329. (doi:10.1038/nrd1345)
- Findlay JK, Drummond AE, Dyson ML, Baillie AJ, Robertson DM & Ethier JF 2002 Recruitment and development of the follicle; the roles of the transforming growth factor-beta superfamily. *Molecular and Cellular Endocrinology* **191** 35–43. (doi:10.1016/S0303-7207(02)00053-9)
- Fraser M, Braems GA & Challis JR 2001 Developmental regulation of corticotrophin receptor gene expression in the adrenal gland of the ovine fetus and newborn lamb: effects of hypoxia during late pregnancy. *Journal of Endocrinology* **169** 1–10. (doi:10.1677/joe.0.1690001)
- Gong X & McGee EA 2009 Smad3 is required for normal follicular follicle-stimulating hormone responsiveness in the mouse. *Biology of Reproduction* **81** 730–738. (doi:10.1095/biolreprod.108.070086)
- Guéripel X, Benahmed M & Gougeon A 2004 Sequential gonadotropin treatment of immature mice leads to amplification of transforming growth factor beta action, via upregulation of receptor-type 1, Smad2 and 4, and downregulation of Smad6. *Biology of Reproduction* **70** 640–648. (doi:10.1095/biolreprod.103.021162)
- Juengel JL & McNatty KP 2005 The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. *Human Reproduction Update* **11** 143–160.
- Kaivo-oja N, Jeffery LA, Ritvos O & Mottershead DG 2006 Smad signalling in the ovary. *Reproductive Biology and Endocrinology* **4** 21. (doi:10.1186/1477-7827-4-21)
- Kawabata M, Imamura T & Miyazono K 1998 Signal transduction by bone morphogenetic proteins. *Cytokine and Growth Factor Reviews* **9** 49–61. (doi:10.1016/S1359-6101(97)00036-1)
- Knight PG & Glistner C 2006 TGF-beta superfamily members and ovarian follicle development. *Reproduction* **132** 191–206. (doi:10.1530/rep.1.01074)
- Lee WS, Otsuka F, Moore RK & Shimasaki S 2001 Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. *Biology of Reproduction* **65** 994–999. (doi:10.1095/biolreprod65.4.994)
- Li Q, Pangas SA, Jorgez CJ, Graff JM, Weinstein M & Matzuk MM 2008 Redundant roles of Smad2 and Smad3 in ovarian granulosa cells *in vivo*. *Molecular and Cellular Biology* **28** 7001–7011. (doi:10.1128/MCB.00732-08)
- Lin X, Duan X, Liang YY, Su Y, Wrighton KH, Long J, Hu M, Davis CM, Wang J, Brunicardi FC *et al.* 2006 PPM1A functions as a Smad phosphatase to terminate TGF-beta signaling. *Cell* **125** 915–928. (doi:10.1016/j.cell.2006.03.044)
- Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25** 402–408. (doi:10.1006/meth.2001.1262)
- Massague J 1992 Receptors for the TGF-beta family. *Cell* **69** 1067–1070. (doi:10.1016/0092-8674(92)90627-O)
- Massague J 2000 How cells read TGF-beta signals. *Nature Reviews. Molecular Cell Biology* **1** 169–178. (doi:10.1038/35043051)
- Matsuura I, Denisova NG, Wang G, He D, Long J & Liu F 2004 Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* **430** 226–231. (doi:10.1038/nature02650)
- Mehra A & Wrana JL 2002 TGF-beta and the Smad signal transduction pathway. *Biochemistry and Cell Biology* **80** 605–622. (doi:10.1139/o02-161)
- Mittal V 2004 Improving the efficiency of RNA interference in mammals. *Nature Reviews. Genetics* **5** 355–365. (doi:10.1038/nrg1323)
- Miyoshi T, Otsuka F, Suzuki J, Takeda M, Inagaki K, Kano Y, Otani H, Mimura Y, Ogura T & Makino H 2006 Mutual regulation of follicle-stimulating hormone signaling and bone morphogenetic protein system in human granulosa cells. *Biology of Reproduction* **74** 1073–1082. (doi:10.1095/biolreprod.105.047969)
- Moustakas A, Soucheinytskyi S & Heldin CH 2001 Smad regulation in TGF-beta signal transduction. *Journal of Cell Science* **114** 4359–4369.
- Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF & Shimasaki S 2000 Bone morphogenetic protein-15. Identification of target cells and biological functions. *Journal of Biological Chemistry* **275** 39523–39528. (doi:10.1074/jbc.M007428200)
- Otsuka F, Moore RK & Shimasaki S 2001a Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. *Journal of Biological Chemistry* **276** 32889–32895. (doi:10.1074/jbc.M103212200)
- Otsuka F, Yamamoto S, Erickson GF & Shimasaki S 2001b Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. *Journal of Biological Chemistry* **276** 11387–11392. (doi:10.1074/jbc.M010043200)
- Pangas SA 2007 Growth factors in ovarian development. *Seminars in Reproductive Medicine* **25** 225–234. (doi:10.1055/s-2007-980216)
- Pangas SA, Li X, Robertson EJ & Matzuk MM 2006 Premature luteinization and cumulus cell defects in ovarian-specific Smad4 knockout mice. *Molecular Endocrinology* **20** 1406–1422. (doi:10.1210/me.2005-0462)
- Pangas SA, Li X, Umans L, Zwijsen A, Huylebroeck D, Gutierrez C, Wang D, Martin JF, Jamin SP, Behringer RR *et al.* 2008 Conditional deletion of Smad1 and Smad5 in somatic cells of male and female gonads leads to metastatic tumor development in mice. *Molecular and Cellular Biology* **28** 248–257. (doi:10.1128/MCB.01404-07)
- Paterson IC, Davies M, Stone A, Huntley S, Smith E, Pring M, Eveson JW, Robinson CM, Parkinson EK & Prime SS 2002 TGF-beta1 acts as a tumor suppressor of human malignant tumor keratinocytes independently of Smad4 expression and ligand-induced G(1) arrest. *Oncogene* **21** 1616–1624. (doi:10.1038/sj.onc.1205217)
- Picton HM, Campbell BK & Hunter MG 1999 Maintenance of oestradiol production and expression of cytochrome P450 aromatase enzyme mRNA in long-term serum-free cultures of pig granulosa cells. *Journal of Reproduction and Fertility* **115** 67–77. (doi:10.1530/jrf.0.1150067)

- Schmierer B & Hill CS** 2007 TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nature Reviews. Molecular Cell Biology* **8** 970–982. (doi:10.1038/nrm2297)
- Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S, Ueno N & Sampath K** 1999 A functional bone morphogenetic protein system in the ovary. *PNAS* **96** 7282–7287. (doi:10.1073/pnas.96.13.7282)
- Shimasaki S, Moore RK, Erickson GF & Otsuka F** 2003 The role of bone morphogenetic proteins in ovarian function. *Reproduction Supplement* **61** 323–337.
- Shimasaki S, Moore RK, Otsuka F & Erickson GF** 2004 The bone morphogenetic protein system in mammalian reproduction. *Endocrine Reviews* **25** 72–101. (doi:10.1210/er.2003-0007)
- Vitt UA, Hayashi M, Klein C & Hsueh AJ** 2000 Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. *Biology of Reproduction* **62** 370–377. (doi:10.1095/biolreprod62.2.370)

Received 1 February 2010

First decision 16 April 2010

Revised manuscript received 26 January 2011

Accepted 3 February 2011