SMAD3 regulates the diverse functions of rat granulosa cells relating to the FSHR/PKA signaling pathway

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Abstract

The function of Smad3, a downstream signaling protein of the transforming growth factor β (TGFβ) pathway, in ovarian follicle development remains to be elucidated. The effects of Smad3 on ovarian granulosa cells (GCs) in rat were studied. Female rats (21 days of age Sprague–Dawley) received i.p. injections of pregnant mare serum gonadotropin, and GCs were harvested for primary culture 48 h later. These cells were engineered to overexpress or knockdown Smad3, which were validated by immunohistochemistry and western blot. The expression of proliferating cell nuclear antigen (PCNA), cyclin D2, TGFβ receptor II (TGFβRII), protein kinase A (PKA), and FSH receptor (FSHR) was also detected by western blotting. Cell cycle and apoptosis of GCs were assayed by flow cytometry. The level of estrogen secreted by GCs was detected by ELISA. Smad3 overexpression promoted estrogen production and proliferation while inhibiting apoptosis of GCs. Reduction in Smad3 by RNAi resulted in reduced estrogen production and proliferation and increased apoptosis of GCs. Manipulation of Smad3 expression also resulted in changes in FSHR and PKA expression, suggesting that the effects of Smad3 on follicle development are related to FSHR-mediated cAMP signaling.

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Introduction

The important role of growth factors in regulating folliculogenesis has been well characterized over the last several years. One group of growth factors is the transforming growth factor β (TGFβ) superfamily, which has been implicated to play critical roles in follicle growth, development, atresia, oocyte maturation, and steroid hormone production (Elvin et al. 2000, Findlay et al. 2000). Downstream signaling transduction proteins of the TGFβ family include Smads. The Smad family is a group of proteins that transmit TGFβ signals from the cell surface to the nucleus. They are classified into three distinct subclasses based on their intracellular functions: receptor-activated Smads (R-Smads: Smad1, 2, 3, 5, 8, and 9), the common-partner Smad (Co-Smad: Smad4), and inhibitory or antagonistic Smads (anti-Smads: Smad6 and 7). The TGFβ family members initiate their cellular actions via heterodimeric serine–threonine kinase receptor complexes. The ligands bind to their specific type II receptor and then phosphorylate the type I receptor. Activated type I receptor is associated with specific R-Smads, phosphorylating them at the COOH-terminus of the protein. The phosphorylated R-Smad dissociates from the receptor and forms a heteromeric complex with Smad4 that translocates to the nucleus for target gene responses. The SMAD1, 5, and 8 proteins are downstream of activated BMP type I receptors. SMAD2 and SMAD3 proteins are structurally similar and mediate TGFβ and activin signals (Massaous & Hata 1997, Attisano & Wrana 2002, Ten Dijke & Hill 2004). Downstream Smad signaling proteins might be essential determinants of the different actions of TGFβ superfamily members. All Smad proteins are expressed in the mammalian ovary, but little is known about their function and mechanism in female fertility.

The follicle is an essential functional unit of the ovary. SMAD2 and SMAD3 are proteins differentially expressed during the different stages of the estrous cycle and follicular and corpus luteum formation in rat ovaries. This suggests that SMAD2 and SMAD3 are expressed at specific stages during ovarian development. SMAD2 and SMAD3 are found mainly in undifferentiated or poorly differentiated granulosa cells (GCs) of primary follicles and early secondary follicles and are scarcely expressed in secondary follicles and GCs of premature follicles. However, SMAD2 and SMAD3 were significantly expressed in cultured GCs in vitro (Xu et al. 2002). Smad2 null mice are embryonically lethal, while Smad3
knockout mice exhibit abnormal follicular development from preantral to antral follicles and apoptosis of GC in atretic follicles (Tomic et al. 2002). Recent studies have shown that SMAD3 is required for normal follicular FSH responsiveness in mice (Gong & McGee 2009). Overall, SMAD3 plays an important role during follicular development.

GCs are the major cell type found in the follicles and essential for proper oocyte and follicular development and steroidogenesis in the ovary. TGFβ superfamily members are critical in regulating GC growth, differentiation, and apoptosis. However, the precise function of SMAD3 in GCs is still unclear. In this study, SMAD3 expression was manipulated by overexpression or reduction by RNAi to determine the effects on proliferation, differentiation, and apoptosis of GCs in vitro.

**Materials and methods**

**Animals**

Female Sprague–Dawley rats (21 days old) were obtained from the Department of Experimental Animals in Peking University Health Science Center and housed under standard conditions. Each rat received an i.p. injection of 20 IU pregnant mare serum gonadotropin and 48 h later was killed by cervical dislocation (Ray et al. 2005). The ovaries were removed for GCs culture. All animal experiments were performed in accordance with Peking University H.S.C. guidelines with institutional approval.

**GCs culture**

Ovaries were placed in serum-free high-glucose DMEM; adipose and connective tissue were dissected under an anatomical microscope; and ovaries were then washed and suspended in DMEM containing 5% fetal bovine serum (FBS). Follicle puncture was performed with fine needles under microscopic visualization. GCs were gently pelleted and rinsed in medium and then cells were collected and centrifuged at 1000  g for 10 min. The supernatant was removed and cells were resuspended in 10% FBS–DMEM. Cells were counted using a hemocytometer, and viability was determined by trypan blue staining. Cells were then cultured in fresh 10% FBS–DMEM at 37 °C in a humid atmosphere containing 5% CO2. All culture media were supplemented with penicillin (100 IU/ml) and streptomycin (100 mg/ml).

**Plasmid transfection**

Cells were cultured at a density of 2 × 105/ml, and after 24 h, the medium was changed to 5% FBS–DMEM for 24 h and then cells were divided into four groups. (a) Control, GCs were cultured without any treatment; (b) GCs were treated with non-lipid transfection reagent FuGENE-6 (Roche); (c) GCs were transfected with p3×FLAG-CMV empty vector; and (d) GCs were transfected with p3×FLAG-CMV-Smad3. Procedures of transfection were performed according to the manufacturer’s guidelines. Transfection reagent (3 μl) and DNA (1 μg) were added for each 1 ml medium. Twelve hours after transfection, 5% FBS-DMEM medium was refreshed. Cells were collected 48 h later.

**siRNA transfection**

Cells were cultured at a density of 2 × 105/ml and after 24 h, cells were divided into four groups. (A) Control, GCs were cultured without any treatment; (B) GCs were treated with transfection reagent Lipofectamine RNAiMix; (C) GCs were transfected with non-silencing siRNA; and (D) GCs were transfected with specific anti-Smad3 siRNA.

The specific sequence of anti-Smad3 siRNA was 5′-AAUG-GUGCGAGAAGCGGUCAdTdT-3′. siRNA were synthesized by Shanghai Jikai Gene Chemical Technology Limited Corporation (Shanghai, China). The transfection reagent Lipofectamine RNAiMix (Invitrogen) was used, and the transfection procedures were performed according to the manufacturer’s guidelines. Cells were transfected with 60 nM siRNA for 48 h and then cells were transfected again, and 24 h later, cells were harvested.

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde and washed with PBS, treated with 3% H2O2 for 30 min, and incubated with 10% goat serum for 30 min, and then specific mouse MAB against FLAG (3 μg/ml respectively. Zymed Lab, South San Francisco, CA, USA) was applied and cells were incubated overnight at 4 °C. After washing with PBS, specific binding of antibody was detected with an HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 30 min. Cells were washed with PBS and then staining was performed using the DAB Substrate Kit (Santa Cruz). The cells were washed, counterstained lightly with hematoxylin, and dehydrated before mounting with Permount (eBioscience, San Diego, CA, USA). For negative controls, cells were incubated with goat serum instead of primary antibody. Photomicroscopy was performed at 200× magnification. Brown staining was taken as positive cell.

**Western blotting**

GCs were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.1% SDS, and 1 mM phenylmethylsulphonyl fluoride) and then centrifuged for 20 min, and the supernatant was collected. Protein content was quantitated with the BCA analysis kit (Applygen Technologies, Inc., Beijing, China). Twenty microliters of protein lysate from each sample were separated by 10% SDS–PAGE and transferred to PVDF membranes. The resultant membranes were treated with 5% (w/ml) fat-free dry milk/TBS at room temperature for 2 h, then washed with TBST (a mixture of TBS and 0.05% Tween 20), three times for 10 min each, and then incubated at 4 °C overnight with 0.8, 0.8, 0.9, 0.5, 0.8, 2, 0.25, 0.8, and 1 μg/ml primary antibody against Smad3 and P-Smad3 (Zymed Lab), FLAG (Zymed Lab), TGFβRII (Cell Signaling Technology, Danvers, MA, USA), PCNA (Zymed Lab), FSH-R.
(Bioworld Technology, Louis Park, MN, USA), PKA (Santa Cruz Biotechnology, Inc.), Smad2 (Zymed Lab), and cyclin D2 (Cell Signaling Technology, Inc.) respectively. After washing three times in TBST for 10 min, the membranes were incubated with an HRP-conjugated affinipure goat anti-rabbit IgG diluted 1:2000 in 5% (w/ml) fat-free dry milk/TBS for 1 h at room temperature. They were then washed with TBST three times for 10 min each and imaged with the ECL Plus Western

**Figure 1** Overexpression of Smad3. (a) Control, GCs cultured without any treatment; (b) GCs treated with non-lipid transfection reagent FuGENE-6; (c) GCs transfected with p3 × FLAG-CMV empty vector; and (d) GCs transfected with p3 × FLAG-CMV-Smad3. (a, b, c, and d in all figures are the same). (I) Immunocytochemical staining for FLAG in rat ovarian GCs. Brown staining was interpreted as positive (II) Western blotting analysis for detecting the expression of FLAG protein in rat ovarian GCs. In (a and b) there was no band detected, in (c) the band for FLAG was detected, and in (d) Smad3 was detected using an antibody detecting by FLAG/SMAD3 fusion protein. (III) Western blotting analysis for detecting the expression of SMAD3 protein in rat ovarian GCs. Transfected Smad3 (d) increased its expression significantly compared with the control group (a). *P<0.05 vs control.

**Figure 2** Knockdown of Smad3. (A) Control, GCs cultured without any treatment; (B) GCs treated with transfection reagent Lipofectamine RNAiMIX; (C) GCs transfected with non-silencing siRNA; and (D) GCs transfected with specific anti-Smad3 siRNA. (A, B, C, and D in all figures are the same). (I) RT-PCR for detecting the mRNA level of Smad3. Smad3 mRNA was decreased significantly in cells transfected with siRNA (D) compared with the control group (A). *P<0.05 vs control. (II and III) Western blotting analysis for detecting the expression of SMAD3 (II) and P-SMAD3 (III) proteins in rat ovarian GCs. Smad3 knockdown cells (D) exhibited decreases in both total Smad3 and P-Smad3 expression compared with the control group (A). *P<0.05 vs control.
Figure 3 Proliferation of GCs. (I) Western blotting analysis for detecting the expression of PCNA protein in rat ovarian GCs. The PCNA expression was significantly increased in the Smad 3-overexpressing cells (d) compared with the control (a) and was significantly reduced in the group transfected with siSmad3 (D) compared with control group (A), *P<0.05 vs control. (II) Western blotting analysis for detecting the expression of cyclin D2 protein in rat ovarian GCs. The level of cyclin D2 protein did not show significant increase in the Smad 3-overexpressing cells (d) compared with the control (a) (P>0.05). The cyclin D2 expression was significantly reduced in the group transfected with siSmad3 (D) compared with control group (A), *P<0.05 vs control. (III) Cell cycle and PI of GCs by flow cytometry. The ratio of S phase and the value of PI increased significantly in Smad 3-overexpressing GCs (d) compared with the control group (a) (Table 1). Knockdown of Smad3 (D) decreased the number of S phase cells and value of PI compared with the control group (A) (Table 2). *P<0.05 vs control.
The changes of cell cycle and proliferative index in GCs (n = 5). (a) Control, GCs cultured without any treatment; (b) GCs treated with non-lipid transfection reagent FuGENE-6; (c) GCs transfected with p3 × FLAG-CMV empty vector; and (d) GCs transfected with p3 × FLAG-CMV-Smad3.

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<tr>
<th>Percentage of G0/G1</th>
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<td>Percentage of S</td>
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<td>Percentage of G2/M</td>
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<td>PI</td>
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<td>7.68 ± 1.81</td>
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<td>18.05 ± 0.16</td>
<td>19.63 ± 0.25</td>
<td>18.81 ± 1.11</td>
<td>30.03 ± 1.38*</td>
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*P < 0.05 vs control (a).

Flow cytometry

Cultured cells were washed with PBS, digested with 0.01 mol/l trypsin and 0.02% EDTA solution, then digestion was terminated with 5% FBS–DMEM, and cells were centrifuged at 1000 g for 5 min. The supernatant was discarded and cells were resuspended in 0.01 mol/l PBS. Cells were counted and fixed in 70% cold alcohol at a quantity of 1 × 10^7 per tube for 24 h and then centrifuged for 10 min at 1000 g. The alcohol was then discarded and cells were washed with PBS twice. After addition of 100 μg/ml RNase, cells were incubated for 30 min at 37 °C. Cells were then filtered with a 300-mesh nylon net and placed in pyridine iodination staining solution, and both the cell cycle and percentage of apoptotic cells were evaluated with flow cytometry using DNA analysis software. The percentage of apoptotic GCs and PI were calculated, the latter as PI = ([S + G2M]/[S + G2M + G0G1]) × 100%.

ELISA

ELISA was used for determination of the E2 level. Culture medium was collected and all procedures were performed according to the manufacturer’s guidelines. With the ELISA kit (RapidBio Lab., West Hills, CA, USA) at room temperature, 25 μl standard preparation and 25 μl culture medium were placed in corresponding wells, and then 200 μl conjugate were added to each well, mixed slightly, and the optical density (OD) was read 60 min later at 450 nm.

Statistical analysis

Experiments for each group were repeated at least four times. Data are represented as X ± S.D. Statistical analyses were carried out with SAS Software (SAS Institute, Cary, NC, USA). P values of < 0.05 compared with control were regarded as statistically significant. P values of < 0.01 were regarded as highly statistically significant.

Results

Transfection efficiency

SMAD3 expression was detected by FLAG using immunocytochemistry (ICC) and western blotting. As shown in Fig. 1I, FLAG was detected in the cytoplasm in 69.89 ± 2.04% and 70.45 ± 2.17% of ovarian GCs in the groups transfected with empty vector and vector carrying Smad3 respectively. Western blotting results showed that there were no detectable bands in the control group, and the group transfected with reagent. The band for FLAG was detected in the group transfected with empty vector. On the other hand, in the group transfected with Smad3, Smad3 was detected using an antibody for the FLAG/SMAD3 fusion protein (Fig. 1II).

The overexpression of SMAD3 protein was determined by western blotting. There were no significant differences in its expression in three (a, b, and c) groups. However, the expression of SMAD3 protein in cells transfected with Smad3 plasmid (d) was significantly increased (P < 0.05) compared with the control group (a) (Fig. 1III).

RT-PCR was used to detect the knockdown efficiency of Smad3. As shown in Fig. 2I, Smad3 mRNA was...
decreased significantly in cells transfected with siRNA compared with the control group (P<0.05).

Total SMAD3 protein and phosphorylated SMAD3 (P-SMAD3) protein levels were also examined. The results showed that cells transfected with siRNA (D) exhibited decreases in both total SMAD3 and P-SMAD3 expression compared with the control group (P<0.05; Fig. 2II and III).

The expression of cyclin D2 protein was determined by western blotting. The results showed that Smad3 knockdown cells exhibited a significant decrease in cyclin D2 expression compared with the control groups (P<0.05). However, SMAD3 overexpression in cells did not effect cyclin D2 levels significantly (P>0.05; Fig. 3II).

The cell cycle and proliferative index of GCs

To further evaluate the proliferation of GCs, a cell cycle analysis was used for FCM, and proliferative index (PI) was calculated as PI = (S+G2M)/(S+G2M+G0-G1)×100%. As shown in Fig. 3III, changes in SMAD3 expression altered the cell cycle distribution of GCs. When SMAD3 protein was overexpressed, the ratio of S phase and the value of PI significantly increased (P<0.05), and the ratio of G0/G1 significantly decreased (P<0.05) compared with the control group (Fig. 3III and Table 1). Moreover, knockdown of Smad3 decreased the number of S phase cells and PI (P<0.05), and increased the ratio of G0/G1 (P<0.05) compared with the control group (Fig. 3III and Table 2).

Apoptosis of GCs

As shown in Fig. 4, the results of FCM demonstrated that Smad3 regulates the apoptosis of GCs. In the overexpression experiments, the percentage of apoptotic cells was (a) 21±0.5% in the control group, (b) 20.2±0.41% in the group transfected with reagent alone, and (c) 21.7±0.55% in the group transfected with empty vector. No significant differences were observed among these groups (P>0.05). However, SMAD3 overexpression (d) decreased the percentage of apoptotic cells to 13.8±0.38% (P<0.05) compared with the control group (a) (Fig. 4I and Table 3). On the other hand, Smad3 knockdown (D) increased the percentage of apoptotic cells to 17.68±1.22% (P<0.05, compared with the control group A). The percentage of apoptotic cells in the A, B, and C groups was 8.05±0.83, 7.59±0.47, and 8.29±0.43% respectively (Fig. 4II and Table 4).

Secretion of estradiol

The ELISA results are shown in Table 5. GCs did not show any significant differences in estradiol (E2) production among the control groups (P>0.05). However, E2 levels significantly increased and decreased upon Smad3 overexpression (P<0.01) and knockdown (P<0.05) respectively.

Table 3 The apoptotic rate of GCs by FCM (n=5). (a) Control, GCs cultured without any treatment; (b) GCs treated with non-lipid transfection reagent FuGENE-6; (c) GCs transfected with p3×FLAG-CMV empty vector; and (d) GCs transfected with p3×FLAG-CMV-Smad3.

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<td>Apoptosis (%)</td>
<td>21.0±0.5</td>
<td>20.2±0.41</td>
<td>21.7±0.55</td>
<td>13.8±0.38*</td>
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*P<0.05 vs control (a).
Table 4  The apoptotic rate of GCs by FCM (n = 5). (A) Control, GCs cultured without any treatment; (B) GCs treated with transfection reagent Lipofectamine RNAiMIX; (C) GCs transfected with non-silencing siRNA; and (D) GCs transfected with specific anti-Smad3 siRNA.

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<tr>
<td>Apoptosis (%)</td>
<td>8.05±0.83</td>
<td>7.59±0.47</td>
<td>8.29±0.34</td>
<td>17.68±1.22*</td>
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*P<0.05 vs control (A).

The expression of FSH receptor protein
As shown in Fig. 5, SMAD3 overexpression significantly enhanced FSH receptor (FSHR) levels (P<0.05, Fig. 5I). Furthermore, Smad3 knockdown significantly decreased FSHR protein levels compared with the control group (P<0.05, Fig. 5II).

The expression of protein kinase A protein
SMAD3 overexpression increased levels of protein kinase A (PKA) protein (Fig. 6I, P<0.05). Smad3 knockdown led to decreased PKA expression compared with the control group (P<0.05, Fig. 6II).

The expression of TGFβ receptor II protein
Increased TGFβ receptor II (TGFβRII) was detected in GCs upon SMAD3 overexpression (P<0.05, Fig. 7I), and its knockdown significantly reduced TGFβRII levels (P<0.05, Fig. 7II) compared with the control group.

The expression of SMAD2 protein
SMAD2 protein was detected in GCs in all groups. SMAD3 overexpression (d) reduced SMAD2 levels, but the differences were not significant compared with the control group (a) (Fig. 8I). However, Smad3 knockdown (D) did significantly increased SMAD2 expression compared with the control group (A) (P<0.05, Fig. 8II).

Discussion
GCs are the closest somatic cells to the germ cells. During follicular development, GCs mediate the regulation of gonadotropins and maintain a favorable microenvironment for oocyte maturation via autocrine and paracrine growth factors. The proliferation and differentiation of GCs are needed for follicular maturation and luteinization, and the apoptosis of GCs is indispensable for follicular atresia.

Gonadotropin and autocrine/paracrine growth factors can regulate the function of GCs. TGFβ is an important growth factor, which regulates follicular growth, atresia, and maturation of the ovum (Liu et al. 1999, Saraguet et al. 2002). TGFβ is considered to play a dual regulatory role in the function of GCs (Juneja et al. 1996). As an important TGFβ-activated downstream signaling transcription protein, SMAD3 is found in cultured GCs (Xu et al. 2002). In this study, we show that the effects of SMAD3 are diverse in rat GCs.

PCNA is a nuclear protein that is part of the DNA polymerase complex in eukaryotic cells and is a reliable marker for mitosis (Xiong et al. 1992). Tomic et al. (2004) illustrated that Smad3+/− mice had lower levels of PCNA compared with WT mice. We analyzed the expression of PCNA protein in GCs by western blotting. The results showed that the expression level of this protein was significantly upregulated upon Smad3 overexpression and downregulated upon its knockdown in GCs. However, the expression of PCNA is not completely associated with the proliferation of GCs. The increased expression of PCNA is the earliest marker for growth of GCs during early-stage follicular development. PCNA is negatively correlated with the growth of GCs as previously reported as its expression is tightly associated with the cell cycle. During the cell cycle, PCNA binds to cyclin D2, a marker of cell division, and promotes proliferation during S phase. PCNA expression was lower during M and G0 phase than S phase (Liu et al. 1989). Thus, we further analyzed the cell cycle of GCs and the PI by FCM and found that SMAD3 significantly altered the cell cycle of GCs. SMAD3 stimulated more GCs to exit the G-phase and enter S phase.

FSH is a vital gonadotropin for the biological function of GCs. It is well documented that FSH accelerates the growth of GCs and initiates a network of signaling pathways to regulate the physiology of GCs, leading to follicular development, oocyte maturation, and ovulation (Tedeschi et al. 1994, Richards et al. 2002). The interaction between FSH and TGFβ/Smad signaling pathways is of great importance and fundamental to the understanding of the physiology and pathology of the ovary.

Table 5  ELISA results showed the level of estradiol secreted by GCs (pg/ml, n = 8). (a) Control, GCs cultured without any treatment; (b) GCs treated with non-lipid transfection reagent FuGENE-6; (c) GCs transfected with p3×FLAG-CMV empty vector; and (d) GCs transfected with p3×FLAG-CMV-Smad3. (A) Control, GCs cultured without any treatment; (B) GCs treated with transfection reagent Lipofectamine RNAiMIX; (C) GCs transfected with non-silencing siRNA; and (D) GCs transfected with specific anti-Smad3 siRNA.

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<td>E2 (pg/ml)</td>
<td>401.964±6.41</td>
<td>405.377±4.09</td>
<td>361.979±4.81</td>
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<tr>
<td>E2 (pg/ml)</td>
<td>435.890±28.20</td>
<td>417.178±25.67</td>
<td>401.284±30.50</td>
<td>223.112±32.20*</td>
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*P<0.05 vs control (a/A).
has also been investigated over the last several years (Mulheron & Schomberg 1990, Magoffin & Magarelli 1995, Zachow et al. 1999, Woods & Johnson 2005). It has been shown that FSH could enhance the stimulatory effect of TGFβ on Smad2 and Smad3 in cultured GCs (Xu et al. 2002). Using immature mice exposed to sequential gonadotropin treatment, Gueripel et al. (2004) observed that FSH and LH could increase TGFβ2, TβRI, Smad2, and that Smad4 expression is further stabilized upon decreasing Smad6 expression. FSH serum levels were elevated in mature Smad3−/− mice, although there was no or only a modest decrease in the mRNA expression of FSHR from whole ovary lysates of cycling animals (Looyenga & Hammer 2007). In a different study, FSHR expression was decreased in Smad3−/− mice relative to controls in ovarian lysates from juveniles and isolated GCs (Gong & McGee 2009). The FSHR is expressed mainly on the GCs of the ovarian follicle (Grieshaber et al. 2000). A study from McGee and colleagues showed a decrease in FSHR mRNA levels from freshly isolated GCs in Smad3−/− mice, and that the responsiveness of GCs to FSH stimulation depends on Smad3 (Gong & McGee 2009). FSH functions by binding to G protein-coupled FSHR on GC surface that activates downstream PKA signaling. However, the effects on the signaling molecule PKA in these Smad3 knockout mice were not assessed. To further investigate why increasing Smad3 expression enhanced the growth of GCs, we evaluated the expression of FSHR protein and its downstream signaling protein PKA in GCs. Western blotting results showed that Smad3 significantly stimulated FSHR expression in GCs, which suggests that Smad3 facilitates the utilization of FSH by GCs. This was also associated with increased cell cycle entry and PI levels. PKA is a key kinase for cell growth that could be blocked by Smad2, suggesting that PKA is critical for Smad2 signaling (Grieshaber et al. 2000). In this study, we showed that Smad3 could promote the expression of PKA in GCs, which suggests that the two are also closely related.

TGFβ can stimulate DNA synthesis in rat GCs (Dorrington et al. 1988) and hamster GCs within preantral follicles (Roy 1993) in vitro. Our results demonstrated that Smad3 significantly enhanced the expression of TGFBRII. The upregulation of TGFBRII facilitated the effects of TGFβ on GCs that promoted the proliferation of GCs. However, unexpectedly, our data

**Figure 5** (I and II) Western blotting analysis for detecting the expression of FSHR protein in rat ovarian GCs. The expression of FSHR was significantly increased in the Smad 3-overexpressing cells (d) compared with the control (a) and was significantly reduced in the group transfected with siSmad3 (D) compared with control group (A). *P<0.05 vs control.

**Figure 6** (I and II) Western blotting analysis for detecting the expression of PKA protein in rat ovarian GCs. The PKA expression was significantly increased in the Smad 3-overexpressing cells (d) compared with the control (a) and was significantly reduced in the group transfected with siSmad3 (D) compared with control group (A). *P<0.05 vs control.
did not show an increase in cyclin D2 protein upon Smad3 overexpression. Roy and colleagues reported that FSH-induced DNA synthesis in the hamster GCs involved the activation of CDK4 rather than cyclin D2 synthesis (Yang & Roy 2004). TGFβ, as an important positive regulatory molecule for DNA synthesis in rat GCs, acting via Smad2 and Smad3, can antagonize the degradation of cyclin D2 protein by blocking its phosphorylation. MAPK14 (p38 Mapk) and PKC can degrade CDKN1B, leading to CDK4 activation and DNA synthesis (Yang & Roy 2006). The results of Looyenga’s recent study demonstrated that compared with WT mice, cyclin D2 showed lower expression in isolated GCs from Smad3 knockout mice (Looyenga & Hammer 2007). In our study, the level of cyclin D2 protein decreased apparently upon the knockdown of Smad3. The proliferation of GCs was promoted by Smad3, one of the reasons for this might be that the degradation of cyclin D2 is inhibited by Smad3 rather than cyclin D2 synthesis.

During follicle development, 99% of the follicles are gradually degraded during various developmental stages known as atresia. Follicular atresia is attributed to the apoptosis of GC cells. Using flow cytometry, our results showed that Smad3 inhibited the apoptosis of GCs. This apoptotic effect was mediated by both gonadotropins and growth factors. It has been well documented that FSH inhibits the apoptosis of GCs (Tilly et al. 1991). Tomic et al. (2002) reported that compared with WT mice, apoptotic GC was more frequently observed in Smad3−/− mice. We showed that SMAD3 overexpression led to increased FSHR levels, which may contribute to the decreased apoptosis of GCs.

Furthermore, it has been reported that estrogen can inhibit the apoptosis of GCs by blocking Ca2+ activation and Mg2+-dependent endonuclease (Boone & Tsang 1997). It has also been reported that TGFβ induces the secretion of estrogen and enhances the promotion of E2 secretion by FSH (Bendell & Dorrington 1991). In this study, Smad3 overexpression also significantly increased E2 secretion in GCs, which may also contribute to decreased apoptosis.

GCs in preovulatory follicles are highly differentiated and the peak concentration of estrogen is seen before ovulation. E2 is one of the most active estrogens secreted

![Figure 7](image1.png)  ![Figure 8](image2.png)
by GCs, and its level is a primary indicator of GC differentiation. Using ELISA, we showed that Smad3 significantly increased secretion of E\textsubscript{2} in GCs.

In GCs, estrogen is regulated primarily by FSH. Upon binding to FSHR, FSH activates P-450 aromatase (Cyp19) to produce estrogen (Hillier \textit{et al}. 1995). It was reported that the expression of Cyp19 was decreased in GCs of Smad3\textsuperscript{−/−} mice, and the responsiveness of these cells to gonadotropins was reduced (Gong \& McGee 2009). In our study, FSHR was upregulated upon Smad3 overexpression, which led to aromatization increased E\textsubscript{2} secretion. On the other hand, TGF\textbeta is a co-mediator for FSH induction of E\textsubscript{2}. TGF\textbeta has the ability to enhance FSH-induced aromatase activity and increase the generation of cAMP in the GCs and increase estrogen production (Zachow \textit{et al}. 1999). When Smad3 was overexpressed, the expression levels of both TGF\textbetaRII and PKA were increased, which enhanced the responsiveness of GCs to FSH to produce more E\textsubscript{2}.

Smad2 and Smad3 are highly homologous R-Smads that have been associated with both TGF\textbeta and activin signaling. Because of the structural differences, Smad3 could bind to DNA but Smad2 could not (Watanabe \& Whitman 1999). Smad2 null mice are embryonically lethal, while Smad3 knockout mice demonstrate immune defects with reduced life span. In the ovary, both proteins are expressed in the oocytes of primordial and primary follicles and in GC cells of preantral follicles (Xu \textit{et al}. 2002). A conditional knockout strategy was formulated to selectively inactivate Smad2, Smad3, or both Smad2 and Smad3 in ovarian GCs. While GC ablation of Smad2 or Smad3 singly caused insignificant changes in female fertility, deletion of both Smad2 and Smad3 led to dramatic reductions of female fertility and fecundity (Li \textit{et al}. 2008). These results suggested that the Smad2 and Smad3 can compensate one another in regulating ovarian function. However, in our experiments, silencing of Smad3 alone significantly increased SMAD2 expression that affected the function of GCs. In other words, Smad2 cannot completely compensate for Smad3 in the regulatory function of GCs. Smad3 null female mice (exon 8) also demonstrated severe fertility defects (Tomic \textit{et al}. 2002). In vivo, Smad2 and Smad3 show differential expression in rat estrous cycle, Smad2 expression, but not Smad3, is observed in luteal cells, the relative effectiveness of the growth factors suggests a functional preference of Smad2 for activation by activin and Smad3 for activation by TGF\textbeta in GCs (Xu \textit{et al}. 2002). Schmierer \textit{et al}. (2003) reported that activin A signaling induces Smad2 but not Smad3 in GCs from the avian ovary. Consistent with differential expression of Smad2 and Smad3 in the ovary, there also seems to be functional differences in their regulation of key events related to follicle development. Further characterization of the specific functions of Smad2 and Smad3 may reveal their unique roles in regulating folliculogenesis.

Conclusions

Overall, our results demonstrated that Smad3 could promote the proliferation and differentiation of rat GCs and inhibit their apoptosis \textit{in vitro}. These effects were mediated by the FSHR/PKA signaling pathway. Future studies are needed to determine other molecular mechanisms of Smad3 regulating the function of GCs.

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.

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