Smad3 Is Required for Normal Follicular Follicle-Stimulating Hormone Responsiveness in the Mouse

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ABSTRACT

Follicle-stimulating hormone (FSH) is the major regulator of folliculogenesis, but other factors modulate its action, including members of the transforming growth factor (TGF) beta family. The intersection of signal transduction pathways that integrate the follicular response to FSH remains to be elucidated. Herein, we investigated the role of Smad3, a critical molecule mediating the intracellular TGF beta family proteins, in follicle development and the expression of FSH receptors. We found that gonadotropin stimulation could not induce normal ovulation in Smad3-deficient mice. Moreover, FSH could not stimulate early follicle growth in Smad3-deficient mice in vivo or in vitro systems. Cultured granulosa cells from Smad3-deficient animals had reduced cell division rates following FSH treatment compared with granulosa cells derived from the ovaries of wild-type (WT) mice. Whole ovaries and isolated granulosa cells from Smad3-deficient animals had lower basal expression of FSH receptor (Fshr), aromatase (Cyp19a1), and cyclin D2 (Ccn2) mRNA compared with WT mice. Follicle-stimulating hormone treatment of granulosa cells from WT ovaries upregulated Fshr, Cyp19a1, and Ccn2 expression. However, FSH did not increase these mRNAs in Smad3-deficient granulosa cells. When Smad3 was introduced into Smad3-deficient granulosa cells with adenosirus vectors, FSH responsiveness was restored, and FSH was able to upregulate Fshr expression. Furthermore, SMAD3 interacts with a palindromic SMAD binding element in the Fshr promoter, and TGF can activate promoter constructs containing this element. Collectively, these observations establish an essential role for Smad3 in regulating the response of ovarian follicles to FSH.

fertility, follicle-stimulating hormone receptor, folliculogenesis, Fshr, mechanisms of hormone action, ovary, Smad3

INTRODUCTION

Fertility in mammalian females is dependent on the growth of follicles, structural units consisting of an oocyte, granulosa cells, and theca cells with an extracellular matrix separating the cell layers. Follicles develop through stages beginning with primordial follicles, containing a small oocyte surrounded by a layer of fibroblast-like granulosa cells, through primary, preantral, antral, to the preovulatory or Graafian stage, containing a fully grown oocyte and multiple layers of granulosa cells. After ovulation and release of the oocyte, the remaining cells participate in the formation of a corpus luteum, which sustains pregnancy in a fertile cycle. Significant disruption occurring at any of the stages of follicle development impairs fertility and alters ovarian hormone production [1].

The regulation of follicle growth has been studied for many years; however, regulation of the early stages of folliculogenesis has lagged behind the study of antral and preovulatory follicles. Early follicles express follicle-stimulating hormone (FSH) receptors and can respond to FSH treatment with increased follicle growth and development [1, 2]. Early follicles also respond to other growth factors that augment or modify the effects of FSH on granulosa cells [3]. Many members of the transforming growth factor (TGF) beta superfamily are thought to have important roles in several stages of folliculogenesis [1, 4]. Activin and TGF beta family members have both been reported to be involved in FSH receptor upregulation in granulosa cells [5, 6]. Various effects of TGF beta family members have been also demonstrated in whole-follicle studies [7–10].

A family of closely related proteins known as SMADs mediates signal transduction of the TGF beta family of growth factors [11]. They are divided into three functional groups with mediating, receptor-activated, and inhibitory roles. The bone morphogenic protein subgroup of the TGF beta family largely signals via SMAD1, SMAD5, and SMAD8. TGF beta and activin subgroups signal via SMAD2 and SMAD3.

SMAD2 and SMAD3 are both highly expressed in follicles from the primordial stage to the late preantral and preovulatory stages. Expression decreases in large antral and preovulatory follicles. Although a low level of SMAD2 expression returns to the corpus luteum, SMAD3 is not expressed in the corpus luteum [12]. Female mice deficient in Smad3 have impaired fertility [13, 14], but the cause of the infertility has not been specifically determined. These mice have a normal follicle endowment of primordial follicles but have reduced numbers of small growing follicles [13]. There are no litters from females, and at age 90 days they do not ovulate in response to exogenous gonadotropins [14]. Markers for cell division are normal at age 18 days but are reduced by age 30 days [13, 14]. However, these studies did not explore the dynamics of gonadotropin treatment on follicle growth and development.

In this study, we evaluated the role of Smad3 in FSH-stimulated follicle growth, granulosa cell division, and FSH receptor expression. Preantral follicles have a markedly reduced growth rate in response to FSH stimulation in the absence of Smad3. Granulosa cells also have reduced cell division and Cyp19a1 expression. There is impaired expression of Fshr in the absence of Smad3 that can be restored with Smad3 expression. Smad3 associates with a SMAD binding element (SBE) in the Fshr promoter, and TGF treatment activates a promoter construct containing this site. Thus, Smad3 is required for the optimal regulation of FSH receptor expression during folliculogenesis in the mouse.
Materials and Methods

Animals

All animal experiments were performed in accordance with National Institutes of Health guidelines and with institutional approval. Smad3-deficient mice (Smad3<sup>−/−</sup>) were generated by disrupting exon 8 of the Smad3 gene by homologous recombination, which is described in detail elsewhere [15]. These animals were created on a C57BL6/SEV129 hybrid background and do not express SMAD3 protein [16, 17]. Animals were housed under standard conditions for breeding colonies under 12L:12D cycles and with standard rodent chow continuously available. Offspring were genotyped as previously described [13, 15]. Ovulation induction was carried out in the standard fashion [18]. Equine chorionic gonadotropin (10 IU; Sigma-Aldrich, St. Louis, MO) was administered s.c., followed 48 h later by human chorionic gonadotropin (10 IU; Sigma-Aldrich). Approximately 2 h later, animals were euthanized, and fallopian tubes and uteri were dissected and searched for ovulated oocytes as previously described [18].

Follicle Culture

Ovaries were dissected from 18-day-old wild-type (WT) and knockout (KO) (Smad3<sup>−/−</sup>) mice and placed into embryo culture dishes in warmed Leibovitz L-15 medium as previously described [8, 19, 20]. Preantral follicles of 130–140 μm in diameter were dissected microscopically using fine needles. Follicles were cultured individually in 96-well dishes in α-modified Eagle medium (Invitrogen, Carlsbad, CA), supplemented with ITS + Culture Supplement (contains 1.0 mg/ml insulin, 0.55 mg/ml human transferrin, 0.5 μg/ml selenium, 50 μg/ml bovine serum albumin (BSA), and 470 μg/ml linoleic acid; Sigma-Aldrich), 100 mM β-mercaptoethanol, and Pen/Strep (100 U/ml penicillin and 100 μg/ml streptomycin; Invitrogen) at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>. Follicles were obtained from two different WT mice and two different KO mice for each experiment. At least 20 follicles were included in each treatment group.

Granulosa Cell Culture

For primary granulosa cell culture, ovaries from 25- to 30-day-old female mice were removed and dissected free of connective tissue. Ovaries were incubated in McCoy 5A (Sigma-Aldrich) medium containing 1.8 mM ethyleneglycoltetraacetic acid and 26 mM sodium bicarbonate at 37°C for 10 min, and then in McCoy 5A with 0.5 M sucrose at 37°C for 5 min as previously described [21]. Puncture of antral follicles was performed under microscopic visualization. The cells were pelleted and resuspended and were counted using a hemocytometer. Viability was determined by trypan blue staining. Cells were cultured in McCoy 5A medium containing 10% fetal bovine serum (FBS) and Pen/Strep at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>. After 24 h, the medium was changed to serum-free McCoy 5A medium containing 0.1% BSA and 10 μMitis + Culture Supplement for 24 h before performing the experiments.

Histology and Immunofluorescence Staining

For histological examination of tissue sections, ovaries were fixed in Bouin reagent or 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C and then embedded in paraffin. Six-micrometer sections were cut and stained with hematoxylin-eosin.

Granulosa cells were grown in glass chamber slides. After treatments, cells were washed with PBS three times, fixed in 4% paraformaldehyde for 10 min, and then preincubated with 10% goat serum/PBS for 20 min. Cells were incubated with rabbit polyclonal antibody against SMAD3 (1:100 dilution in PBS; Zymed Laboratories, South San Francisco, CA) for 2 h at room temperature. Slides were washed with cold PBS and incubated with biotinylated anti-rabbit IgG antibody (1:200 in PBS; Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Slides were washed and incubated with Texas Red Avidin D (6 μg/ml in PBS; Vector Laboratories) for 10 min and were mounted with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories).

For proliferation studies, cells were labeled with bromodeoxyuridine (BrdU) at 37°C for 2 h, fixed with 70% ethanol, and stained according to the manufacturer’s instructions using the BrdU staining kit from Invitrogen. One hundred cells from each well were counted and scored as stained or not stained by a counter unaware of the treatment group or source of cells.

For apoptosis studies, cells were fixed in 4% paraformaldehyde and permeabilized by 0.2% Triton X-100 for 10 min. Cells were then analyzed using a DeadEnd colorimetric TUNEL system (Promega, Madison, WI) according to the manufacturer’s protocol. The percentage of dark-staining apoptotic nuclei was determined by counting 1000 cells on four different coverslips per group and scoring each cell as stained or not stained. Groups were unknown to the person counting the cells.

RNA Isolation and Real-Time PCR Analysis

RNA was isolated from ovaries or granulosa cells using TRIzol reagent (Invitrogen) following the manufacturer’s protocol. The cDNA synthesis reactions were performed using oligo (dT) M-MLV RT (Ambion, Austin, TX) with 0.5–1.5 μl of total RNA under the conditions described by the manufacturer.

For real-time PCR, the TaqMan assay system was used (Applied Biosystems, Foster City, CA) with specific probes for mouse FSH receptor (Fshr [catalog number Mm00442819]), TATA (Tbp [Mm00446973]), aromatase (Cyp19a1 [Mm00484049]), and cyclin D2 (Ccn2 [Mm00438071]). Probes were labeled at the 5’ end with FAM (6-carboxy-fluorescein) reporter dye. The real-time PCR reaction was completed on an ABI 7500 Thermocycler (Applied Biosystems) with 2 min at 50°C (AmpliTag Gold DNA polymerase activation), 10 min at 95°C (AmpliTaq Gold DNA polymerase activation), and 40 cycles each with 15 sec at 95°C (melting) and 1 min at 60°C (annealing/extension). The C<sub>T</sub> value was determined for each reaction (run in duplicate) using Sequence Detection Software version 1.7a (Applied Biosystems), and quantification was completed using the ΔΔC<sub>T</sub> method [22]. Tbp is a well-characterized housekeeping gene [23] that is expressed at low levels, similar in magnitude to Fshr.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using an EZ ChIP kit (Upstate, Temecula, CA). Wild-type mouse granulosa cells were cultured for 48 h with FSH (1 IU/ml). Cells were treated with 1% formaldehyde (Sigma-Aldrich) at 37°C for 10 min to cross-link proteins to DNA. The reaction was stopped with glycine, and the cells were lysed. Samples were then sonicated on ice for 30 min in 30-sec bursts each at a maximal input using Bioruptor UCD-200TM-EX (Diagenode, Sparta, NJ) and then centrifuged for 10 min at 12,000 g at 4°C. Chromatin was precleared with salmon sperm DNA/protein G agarose for 1 h at 4°C, and 1% of this solution was saved for use as input chromatin. Samples were incubated overnight with a ChIP-grade SMAD3 antibody (Abcam, Cambridge, MA) or with normal rabbit IgG (negative control; Santa Cruz Biotechnology, Santa Cruz, CA). Samples were then incubated, pelleted, and washed as per the manufacturer’s instructions. Immunocomplexes were eluted from the agarose beads. Input and immunoprecipitated chromatin was incubated with 200 ml NaCl at 65°C overnight to reverse DNA–protein cross-links. DNA was purified as per the manufacturer’s instructions. PCR was performed using the 1.55-kilobase PCR primer pair for the BSE site (forward primer 5′-CAGGTGTAACCATTTTCAGGAGA-3′ and reverse primer 5′-TCCACCCAGAGTCTAGAAACTCA-3′), the PCR product is 135 base pair [bp] in length, and the non-BSE site (forward primer 5′-AGGTCTGAAGGATAACAGGTT-3′ and reverse primer 5′-ACA CAGTCCGGATAAGAGAT-3′; the PCR product is 198 bp in length).

FSH Receptor Promoter-Luciferase Reporter Constructs

Mouse Fshr promoter-luciferase reporter constructs were created using the pGL-3 basic vector system (Promega). A genomic DNA fragment containing the 1.55-kilobase Fshr 5′-flanking region was amplified by PCR using WT female mouse genomic DNA with forward primer 5′-GGTACCTTTGCAACTCTCCACCATG-3′ (Kpn I) and reverse primer 5′-CTCCAGCTATTITATCTCCAC-3′ (XhoI). The PCR product was cloned into pCR2.1-TOPO TA vector (Invitrogen). The -1035/+566 promoter fragment was then subcloned into the KpnI/XhoI sites of the pGL-3 basic vector. A truncated Fshr promoter construct (FRP truncated) was also generated. The fragment was amplified with forward primer 5′-GGTACCGATCTATTAGTATGTTATAAC-3′ (KpnI) and reverse primer 5′-CTCCAGCTATTITATCCATCCAC-3′ (XhoI). An Fshr promoter construct with all BSE deletion was generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The sequence “TAGAC” was deleted from the BSE “CTAGAC” sequence. All Fshr promoter constructs were verified by sequencing.

Cell Culture, Transient Transfection, and Luciferase Assays

The luciferase reporter constructs were transfected into a well-characterized granulosa cell line [24, 25] that was kindly provided by Dr. Robert Burglard, Texas A&M University, College Station, TX [26]. Cells were cultured in Dulbecco modified Eagle medium (DMEM)/F-12 with 5% FBS, 100 U/ml
penicillin, and 100 μg/ml streptomycin (all from Invitrogen) and were seeded at a density of $8 \times 10^3$ cells/well in 12-well plates 1 day before transfection, which was performed with FuGene 6 (Roche Diagnostics, Indianapolis, IN) following the manufacturer’s protocol. Cells were transfected with 0.5 μg of indicated plasmid DNA and 10 ng of internal control Renilla DNA. Cells were serum starved for 8 h before incubating with or without 1 ng/ml of TGF-β1 for 16 h. The Dual-Luciferase Reporter Assay System (Promega, Minneapolis, MN) was used following the manufacturer’s protocol. Luciferase activity was normalized relative to the activity of pGIII-3 basic vector.

**Adenoviral Infection of Primary Granulosa Cells**

Adenoviruses containing Smad3 (AdSmad3) and Gfp (AdGFP) were constructed and prepared using the AdMax system (MobiBix, Toronto, ON, Canada) according to the manufacturer’s directions with assistance from Dr. Anthony Zeleznik (Pittsburgh, PA). This construct is described in detail elsewhere [27]. Initially, 293 cells were transfected with the adenoviral vector DNA and maintained in DMEM containing 3% FBS at 37°C in 5% CO₂ for 10 days or until plaques exhibiting viral cytopathic effects were identified. A second large-scale amplification was carried out, and purification was performed using CsCl density gradient ultracentrifugation. The optical density of the samples at 260 nm was used to calculate virus content using the relationship of $1.1 \times 10^{12}$ virus particles/ml per A₂₆₀ unit. Expression of SMAD3 protein after adenovirus infection of granulosa cells was confirmed by both Western analysis and immunohistochemistry. Transfection efficiency routinely exceeded 90% [28].

Smad3-deficient granulosa cells were seeded into 48-well tissue culture plates or eight-well glass chamber slides and were allowed to attach for 24–48 h. Before infection, medium and unattached cells were removed, and granulosa cell monolayers were exposed to recombinant adenovirus vector AdSmad3 (multiplicity of infection [MOI] = 200) in McCoy 5A medium for 2 h. Medium was then replaced with fresh serum-free medium (McCoy 5A medium containing 0.1% BSA and 10 μl/ml ITS + Culture supplement) for 24 h before experiments.

**Data Analysis**

Experiments were repeated at least three times, and results were reported as the mean ± SEM. Data were subjected to ANOVA and post hoc testing with Tukey honestly significant difference test when more than two groups were compared. Student t-test was used for single comparisons. Statistical significance was accepted at $P < 0.05$.

**RESULTS**

The Ovaries of Smad3-Deficient Mice Did Not Respond Normally to Endogenous or Exogenous Gonadotropins

Observations of female mice (WT and heterozygous and homozygous for the mutant Smad3 allele) were made on a daily basis. Vaginal opening occurred at a similar age in all three genotypes. However, following vaginal opening, Smad3-deficient females seldom had normal estrous cycles. In a group of six Smad3 KOs and six WT sister pairs, daily vaginal smears revealed that over a 4-wk period the six WT females had 40 estrous cycles, while there was only one normal estrous cycle in the KO group over the same observation time.

The capacity of Smad3-deficient mice to ovulate in response to exogenously administered gonadotropins was evaluated at age 56–60 days. Wild-type females (n = 6) ovulated 12–16 eggs when treated with exogenous gonadotropins. Most homozygous mutant mice (n = 6) did not ovulate, except for one animal that ovulated one egg. We maintained histological or morphological observations of all ovaries harvested from the colony, and ovaries from untreated Smad3-deficient mice only rarely contained corpora lutea and never past age 60 days. This is similar to a previous study [14] in which no ovulations were observed in Smad3-deficient animals at age 90 days. Figure 1 shows representative ovary sections from both a Smad3-deficient and a WT sister pair at age 48 days.

Because of diminished cyclicity and poor ovulatory response to exogenous gonadotropins in the Smad3-deficient ovaries, we next determined the ability of early preantral follicles to respond to FSH treatment. Table 1 summarizes the growth of individually isolated preantral follicles in four experimental groups that included 20–30 follicles in each group. Follicles of 130–140 μm in diameter were mechanically dissected from whole ovaries from WT and Smad3-deficient animals and were individually cultured in 96-well plates. In the absence of FSH, neither WT nor Smad3-deficient follicles increased in diameter. As expected, follicles from WT ovaries grew in response to FSH treatment. A 60-μm increase over 3 days of culture with FSH is consistent with what we and others have shown previously [8, 29]. However, the follicles from Smad3-deficient ovaries did not increase in diameter in response to treatment with FSH. Thus, Smad3-deficient preantral follicles exhibited a limited response to FSH stimulation in vitro.

We next determined whether preantral follicles from Smad3-deficient mice respond to FSH treatment in vivo. In these studies, FSH was administered every 12 h from age 15 days to age 19 days. The average weight of untreated ovaries at age 19 days is $0.42 \pm 0.04$ mg (n = 6). The weight of the FSH-stimulated WT ovaries at age 19 days increased three-fold to $1.40 \pm 0.25$ mg (n = 8) ($P < 0.05$ compared with untreated control). In contrast, ovarian weight did not increase in the Smad3-deficient animals treated with FSH ($0.37 \pm 0.10$ mg for treated ovaries [n = 4] versus $0.25 \pm 0.05$ mg for untreated ovaries [n = 4]).

To determine the effect that FSH treatment had on cell division within follicles, we also treated the animals with the marker BrdU 1 h before termination of the experiment [30]. Cells that are in the synthesis phase of cell division stain brown. Figure 2, A and B, shows BrdU-stained sections of ovaries from these experiments, whereas Figure 2, D and D,
shows higher-magnification representative staining patterns of follicles from each group. The ovaries from the FSH-treated WT animals showed robust follicle growth and had large preantral follicles containing multiple layers of granulosa cells with readily apparent BrdU staining (Fig. 2C). In contrast, the Smad3-deficient ovaries had minimal follicle growth response to FSH treatment. The follicles contained few layers of granulosa cells and only sporadically stained for BrdU, consistent with reduced granulosa growth response (Fig. 2D).

The proliferation index was determined for small preantral follicles with 3–5 layers of granulosa cells (and follicle diameters between 130 and 150 μm) from the ovaries from these experiments. Twenty-five to 30 follicles that were sectioned through the oocyte were identified in sections from four ovaries from each group. More than 100 total follicles were assessed each in WT and KO ovaries. All of the granulosa cells in the follicle cross-section were counted, and the number of cells that stained for BrdU was noted. The percentage of stained cells reflects the proliferation index of the follicle. The WT preantral follicles had a proliferation index of 23.3% ± 2.9%, whereas the Smad3-deficient follicles had a proliferation index of only 3.5% ± 0.6% (P < 0.05). Thus, the number of dividing cells in similarly sized follicles was reduced in Smad3-deficient ovaries treated with FSH compared with WT ovaries.

**Granulosa Cells from Ovaries of Smad3-Deficient Mice Showed Diminished Mitotic Activity in Response to FSH Treatment In Vitro**

To analyze the FSH effect on granulosa cell division more closely, we performed granulosa cell culture with cells isolated from the ovaries of mice aged 21–25 days (Fig. 3A). Cells were grown in chamber slides as described in Materials and Methods. Cells from either WT or Smad3-deficient mice were pooled from 2 to 4 animals per experiment and grown in the presence or absence of FSH (1 IU/ml) for 24 h. At the end of the experiment, the cells were incubated with BrdU to mark the cells in the synthesis phase of cell division. Positively stained cells were counted from at least three wells per group, and the experiment was repeated three times. As shown in Figure 3A, there was a low baseline rate of cell division in both WT and Smad3-deficient cells. However, FSH stimulated cell division in WT cells to a much greater extent than that in Smad3-deficient cells (P < 0.05). Thus, cultured granulosa cells were less responsive to FSH treatment in the absence of Smad3.

Cells were also analyzed for apoptosis at the end of the experiments using a TUNEL assay to label free DNA ends. Figure 3B shows the low but consistent rate of apoptosis in all the experimental groups of cells. Both Smad3-deficient and WT cells survive well in this culture system.

**Expression of Key Genes Involved in Granulosa Cell Function Was Reduced in the Ovaries and Granulosa Cells of Smad3-Deficient Mice**

We next evaluated the expression of Fshr, Ccnd2, and Cyp19a1 mRNAs as indices of the response to FSH in ovaries and granulosa cells of WT and Smad3-deficient mice. Ccnd2 was used as a marker of cell division and Cyp19a1 as a marker of granulosa cell differentiation [1]. In whole ovaries (Fig. 4A), endogenous expression of all of these mRNAs was markedly reduced in the Smad3-deficient ovaries as determined by quantitative real-time PCR. In granulosa cells isolated from Smad3-deficient ovaries (Fig. 4B), the expression of Fshr, Cyp19a1, and Ccnd2 mRNAs was reduced in granulosa cells compared with WT matched controls.

To determine the effect of FSH treatment on gene expression in granulosa cells in vitro, we cultured granulosa cells from both WT and Smad3-deficient mice with or without FSH for 24 h. As expected, FSH doubled the percentage of BrdU-stained cells in the WT cells (P < 0.05), but the KO cells had no change in the percentage of BrdU-stained cells. Therefore, FSH treatment increased the number of BrdU-stained cells in WT mice, whereas in KO mice, FSH treatment had no effect on BrdU-stained cell numbers. These results are consistent with the in vivo findings, in which Smad3-deficient mice had reduced follicle growth and reduced gene expression in response to FSH treatment.
without FSH (1 IU/ml) for 24 h (Fig. 4C). The cells were then lysed, and mRNA was isolated for quantitative real-time PCR determination of expression of Cyp19a1, Fshr, and Ccnd2 mRNAs. In the WT cells, FSH treatment stimulated increased expression of Cyp19a1, Fshr, and Ccnd2 [1]. However, the expression of Cyp19a1, Fshr, and Ccnd2 mRNAs was not increased in the granulosa cells from the KO mice (P < 0.05). Thus, FSH was not able to upregulate the expression of its own receptor in the absence of Smad3. Furthermore, the expression of downstream markers of cell division and differentiation was also impaired in the cells in the absence of Smad3, consistent with diminished FSH receptor function.

Granulosa cells from WT and Smad3-deficient mice were also treated with forskolin, an activator of adenylate cyclase (Fig. 4D). Forskolin treatment had the same effect as FSH treatment on Fshr and Cyp19a1 mRNA expression but did not stimulate Ccnd2 expression. This is not unexpected given that Ccnd2 is not thought to be a cAMP-regulated response to FSH receptor activation [31]. Activin augmented the FSH-induced effects in the WT cells but not in the Smad3-deficient cells. In the absence of Smad3, FSH, forskolin, and FSH/activin were all unable to upregulate FSH receptor expression.

Restoration of Smad3 Signaling Corrected the Defects in FSH Responsiveness in Granulosa Cells of Smad3-Deficient Mice

To determine if the absence of Smad3 prevented the upregulation of FSH receptor in Smad3-deficient mice, we performed experiments to restore SMAD3 to the Smad3-deficient granulosa cells (Fig. 5). Granulosa cells from WT and Smad3-deficient mice were infected with adenovirus vectors containing a functional Smad3 construct or Gfp as a control. In the absence of FSH treatment, cells had low basal expression of Fshr. However, in the presence of FSH, the WT cells had increased expression of Fshr. The Smad3-deficient cells did not respond to FSH treatment. However, when functional Smad3 was added back to the KO cells with the adenovirus, the ability of the granulosa cells to respond to FSH treatment was restored (Fig. 5A). Although SMAD3 was not detected in the uninfected granulosa cells (Fig. 5B, a and b), SMAD3 protein...
was readily detected in cells infected with the Smad3 vector (Fig. 5B, c and d).

**Fshr Promoter Contains a SMAD Responsive-Element**

Examination of the 5' untranslated region of the mouse Fshr gene sequence revealed a palindromic SBE site [32, 33] –440 bp upstream of the transcription start site (Fig. 6); Fshr promoter constructs were generated to analyze the function of this site. Granulosa cells from a spontaneously immortalized cell line [26] were transfected with these constructs and treated with TGFβ. Cells transfected with the Fshr promoter constructs containing this SBE site (FRP) responded to TGFβ treatment but not when the SBE site was deleted (FRP-del-SBE) (Fig. 6A) or mutated (data not shown).

To investigate if SMAD3 binds to the SBE site in the Fshr promoter region at –440 bp, and the promoter activity was increased with the stimulation of TGFβ.

**DISCUSSION**

We have demonstrated that in the absence of Smad3 preantral follicles are unable to respond appropriately to FSH stimulation. It was known that there is a delay in the presence of multilayered follicles and antral follicles in the first wave of follicles in the ovaries in Smad3-deficient mice [13]. Although follicles can progress to the antral stage, they do not do so in a normal time frame or in conventional numbers. We have shown that there is a delay in the growth of isolated preantral follicles in response to FSH treatment, which may account for this disruption of the normal temporal pace of follicular development in vivo (Table 1). Follicle-stimulating hormone-stimulated cell division in preantral follicles in vivo is also reduced in the absence of Smad3 (Fig. 2). We have also shown reduced expression of Fshr and genes that are downstream of FSH receptor activation and involved in the replication and endocrine activity of granulosa cells in the absence of Smad3 (Fig. 4). Cyp19a1 (a marker of differentiation) and Ccnd2 (a marker of cell division) both have lower expression levels in Smad3-deficient granulosa cells than in WT cells, which is consistent with reduced responsiveness to gonadotropins.
However, these two genes may also be under direct regulation by Smad3 [31]. Of note, FSH serum levels are higher in Smad3-deficient mice than in WT mice [13], which is also consistent with reduced responsiveness to FSH at the level of the ovary.

Together, the results of the in vivo and in vitro studies lead to the conclusion that the delay in follicle progression in the Smad3-deficient mouse ovary results from reduced ability of the follicle to respond to FSH stimulation. This reduced ability correlates with reduced expression of Fshr in the granulosa cells of the Smad3-deficient ovaries (Fig. 4). The add-back experiments shown in Figure 5 further substantiate this conclusion. When Smad3 expression is restored in granulosa cells, they regain their ability to respond to FSH treatment with increased Fshr expression.

Our findings of reduced FSH receptor expression in Smad3-deficient ovaries (Fig. 4) are in contrast to those by Tomic et al. [13, 14] and by Looyenga and Hammer [36], who reported that there was none or only a modest decrease in Fshr expression as determined by real-time PCR. The apparent discrepancy in this important observation can be explained by differences in experimental protocol that obscured the discovery of reduced Fshr expression. In both of these studies [14, 36], whole-ovarian lysates from sexually mature WT ovaries were compared with similarly aged Smad3-deficient ovaries, which we have shown (Fig. 1) are smaller than WT ovaries and have few, if any, corpora lutea. Corpora lutea make up a large portion of ovarian volume and have a lower expression of FSH receptors than growing antral follicles [37]. Comparing cycling whole ovaries with noncycling ovaries introduces the possibility that Fshr expression as a percentage of all the mRNA in an ovary is less than the Fshr expression in the same ovary at diestrus or even before the first ovulation. Comparing relative RNA expression from a whole organ depends highly on the cellular composition and stages of maturation making up that organ. The difference in cycling WT versus noncycling Smad3-deficient ovaries is further emphasized and confirmed by examining the LH receptor expression in the two different ovary lysates from the study by Looyenga and Hammer [36].
In the WT ovaries, LH receptor is expressed at very high levels, yet LH receptor expression was almost nonexistent in the Smad3 KO lysates, consistent with the observation that there were fewer corpora lutea in the KO ovaries compared with the WT ovaries in that experiment. Our design bypasses the issue of comparing whole-ovary lysates of cycling animals with those of noncycling animals. We compared matched prepubertal sibling pair ovaries, as well as freshly isolated granulosa cells and cultured cells. Thus, in three different systems the basal expression of Fshr in granulosa cells is reduced in the absence of Smad3 expression. Our functional studies also confirm these conclusions because FSH-stimulated in vivo cell division, follicle growth, and in vitro cell division were all impaired in Smad3-deficient mice (Figs. 3 and 4). Furthermore, add-back of SMAD3 restored FSH responsiveness and FSH receptor expression (Fig. 5).

The Smad3-deficient mouse has an embryonic lethal phenotype, so its specific ovarian effects are unknown. SMAD2 and SMAD3 are very highly homologous molecules and share many functions [11, 38]. Thus, some of the functions of SMAD3 may be compensated for by SMAD2. However, there are differences in the two molecules. SMAD3 is able to bind DNA on its own, but SMAD2 must interact with other cofactors. SMAD2 and SMAD3 have different affinities for cofactors and can interact with different factors in different cell types [38]. SMAD3 and SMAD2 are both expressed in preantral follicles, and the expression of both declines in antral and larger follicles. However, only SMAD2 expression returns in the corpus luteum [12]. Because of this expression pattern, the effects of SMAD3 deletion must be predominantly in the smaller growing follicles. A recent study [39] has compared the roles of SMAD3 and SMAD2 in ovarian function by using conditional KO mice with a different Smad3 deletion construct from that in this colony. The study demonstrated redundancy in some roles of Smad2 and Smad3 in ovarian function, particularly in cumulus expansion. However, Fshr expression or function was not evaluated, so this effect cannot yet be compared in these models. Differences in the constructs used and the possibility of residual production of a small amount of SMAD3 protein with the exon 2 deletion construct also make direct comparison of preantral effects of Smad3 difficult. Further studies will be necessary to discern the causes of phenotypic differences in the two constructs used to create Smad3-deficient mice.

Our studies demonstrate that the ability of FSH to upregulate its own receptor is diminished in the absence of Smad3. This may be a critical process that is disrupted in the Smad3-deficient ovary and one that has been overlooked in previous studies. The restoration of functional SMAD3 to the granulosa cells allowed the expression of Fshr to recover to normal levels and restored the ability of FSH treatment to upregulate its receptor. Forskolin alone was not sufficient to increase Fshr in the Smad3-deficient cells (Fig. 4), although it did in the WT cells. Therefore, reduced cAMP levels do not appear to be solely responsible for the reduced expression of Fshr in the absence of SMAD3.

Both activin and TGFβ, which signal through both SMAD2 and SMAD3, increase mRNA expression for Fshr in cultured granulosa cells [5, 6]. In our studies, activin A did not increase FSH-stimulated Fshr expression in the absence of Smad3, although it did in the WT cells. Further studies are necessary to clarify the levels of regulation and the role of signaling cross talk in determining Fshr regulation and downstream function. However, SMAD3 can bind to and increase the activity of the Fshr promoter. An increase in Fshr expression stimulated by TGFβ (or a family member) in early follicles could be one mechanism by which Smad3 function enhances early follicle development.

The ability of a preantral follicle to respond to FSH treatment with growth and differentiation affects its ability to eventually become a preovulatory follicle. Factors that affect the responsiveness of follicles to FSH can affect fecundity and ultimate fertility. We have demonstrated that a signal transduction protein for a major growth factor family has a key role in Fshr expression in vitro and in vivo. Genes regulating FSH receptor activity may be of great functional importance. Infertility in women is not generally an all-or-nothing issue but is rather more appropriately described as subfertility. Smad3 deficiency does not turn off the FSH receptor, but it does reduce the ability of the granulosa cell to respond to gonadotropins. Thus, alterations in ovarian responsiveness and the dynamics of follicular development that are frequently found in the treatment of infertility may be caused, in part, by alterations in SMAD3 signaling. Normal dynamics of early follicle growth may be as important to fertility as the far more studied dynamics of later antral and preovulatory follicle growth and development. Although difficult to perform, studies of modulating influences on gamete production may produce rewards in our future understanding of the causes of human subfertility.

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REFERENCES

1. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. Endocr Rev 2000; 21:200–214.
2. McGee EA, Perlas E, LaPolt PS, Tsafirri A, Hsueh AJ. Follicle-stimulating hormone enhances the development of preantral follicles in juvenile rats. Biol Reprod 1997; 57:990–998.
3. Adashi EY. With a little help from my friends: the evolving story of intraovarian regulation. Endocrinology 1995; 136:4161–4162.
4. Knight PG, Glister C, TGF-β superfamily members and ovarian follicle development. Reproduction 2006; 132:191–206.
5. Inoue K, Nakamura K, Abe K, Hirakawa T, Tsuchiya M, Oomori Y, Matsuda H, Miyamoto K, Minegishi T. Mechanisms of action of transforming growth factor beta on the expression of follicle-stimulating hormone receptor messenger ribonucleic acid levels in rat granulosa cells. Biol Reprod 2003; 69:1238–1244.
6. Minegishi T, Kishi H, Tano M, Kameda T, Hirakawa T, Miyamoto K. Control of FSH receptor mRNA expression in rat granulosa cells by 3'-5' cyclic adenosine monophosphate, activin, and follistatin. Mol Cell Endocrinol 1999; 149;71–77.
7. Hayashi M, McGee EA, Min G, Klein C, Rose UM, van Duin M, Hsueh AJ. Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. Endocrinology 1999; 140:1236–1242.
8. McGee EA, Smith R, Spears N, Nachtigal MW, Ingraham H, Hsueh AJ. Mullerian inhibitory substance induces growth of rat preovarial follicles. Biol Reprod 2001; 64:293–298.
9. Thomas FH, Armstrong DG, Telfer EE. Activin promotes oocyte development in ovine preovarial follicles in vitro. Reprod Biol Endocrinol 2003; 1:76–83.
10. Thomas FH, Ethier JF, Shimasaki S, Vanderhyden BC. Follicle-stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors. Endocrinology 2005; 146:941–949.
11. Wrana JL. Crossing Smads. Sci STKE 2000; 2000:eRE1.
12. Xu J, Oakley J, McGee EA. Stage-specific expression of Smad2 and Smad3 during folliculogenesis. Biol Reprod 2002; 66:1571–1578.
13. Tomic D, Brodie SG, Deng C, Hickey RJ, Babus JK, Mulkas LH, Flaws JA. Smad 3 may regulate follicular growth in the mouse ovary. Biol Reprod 2002; 66:917–923.
Ovarian follicle development requires Smad3. Mol Endocrinol 2004; 18: 2224–2240.

15. Yang X, Letterio JJ, Lechleider RJ, Chen L, Hayman R, Gu H, Roberts AB, Deng C. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. Embryo J 1999; 18:1280–1291.

16. Roberts AB, Russo A, Felici A, Flanders KC. Smad3: a key player in pathogenetic mechanisms dependent on TGF-β. Ann N Y Acad Sci 2003; 995:1–10.

17. Weinstein M, Yang X, Deng C. Functions of mammalian Smad genes as revealed by targeted gene disruption in mice. Cytokine Growth Factor Rev 2000; 11:49–58.

18. Nothnick WB, Solorway P, Curry TE Jr. Assessment of the role of tissue inhibitor of metalloproteinase-1 (TIMP-1) during the periovulatory period in female mice lacking a functional TIMP-1 gene. Biol Reprod 1997; 56: 1181–1188.

19. McGee EA, Chun SY, Lai S, He Y, Hsueh AJ. Keratinocyte growth factor promotes the survival, growth, and differentiation of preantral ovarian follicles. Fertil Steril 1999; 71:732–738.

20. McGee E, Spears N, Minami S, Hsu SY, Chun SY, Billig H, Hsueh AJ. Preantral ovarian follicles in serum-free culture: suppression of apoptosis after activation of the cyclic guanosine 3′,5′-monophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. Endocrinology 1997; 138:2417–2424.

21. Campbell KL. Ovarian granulosa cells isolated with EGTA and hypertonic sucrose: cellular integrity and function. Biol Reprod 1979; 21:773–786.

22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C (T)) method. Methods 2001; 25:402–408.

23. Jung M, Ramankulov A, Roigas J, Johannsen M, Ringsdorf M, Kristiansen G, Jung K. In search of suitable reference genes for gene expression studies of human renal cell carcinoma by real-time PCR. BMC Mol Biol 2007; 8:e47.

24. Peluso JJ, Pappalardo A. Progesterone regulates granulosa cell viability through a protein kinase G-dependent mechanism that may involve 14-3-3sigma. Mol Cell Biol 2008; 28:580:2811–2820.

25. Levallet J, Koskimies P, Rahman N, Huhtaniemi I. The promoter of vimentin gene expression during differentiation of the C2C12 skeletal myogenic cell line requires Smads, Ap-1 and Spl family members. Biochim Biophys Acta 2007; 1773:427–439.

26. Levallet J, Koskimies P, Rahman N, Huhtaniemi I. The promoter of murine follicle-stimulating hormone receptor: functional characterization and regulation by transcription factor steroidogenic factor 1. Mol Endocrinol 2001; 15:80–92.

27. Looyenga BD, Hammer GD. Genetic removal of Smad3 from inhibin-null mice attenuates tumor progression by uncoupling extracellular mitogenic signals from the cell cycle machinery. Mol Endocrinol 2007; 10:2440–2457.

28. Liu J, Aronow B, Witte DP, Pope WF, La Barbera A. Cyclic and maturation-dependent regulation of follicle-stimulating hormone receptor and luteinizing hormone receptor messenger ribonucleic acid expression in the porcine ovary. Biol Reprod 1998; 58:648–658.

29. Massague J, Gomis RR. The logic of TGFbeta signaling. FEBS Lett 2006; 580:2811–2820.

30. Li Q, Pangas SA, Jorgez CJ, Graff JM, Weinstein M, Matzuk MM. Redundant roles of SMAD2 and SMAD3 in ovarian granulosa cells in vivo. Mol Cell Biol 2008; 28:7001–7011.