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Activin stimulates *CYP19A* gene expression in human ovarian granulosa cell-like KGN cells via the Smad2 signaling pathway

Masatoshi Nomura *, Ryuichi Sakamoto, Hidetaka Morinaga, Lixiang Wang, Chizu Mukasa, Ryoichi Takayanagi

Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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ABSTRACT

Activin, a transforming growth factor β family member, has a wide range of physiological roles during embryonic development and organogenesis. In the ovary, activin, secreted from ovarian granulosa cells, not only acts on the pituitary gland to regulate the gonadotropin secretion from the pituitary gland in an endocrine manner but also acts on granulosa cells in a paracrine/autocrine manner to regulate folliculogenesis. Previously, we showed that activin signals through activin type IB receptor (ActRIB) and up-regulates follicle-stimulating hormone receptor expression and P450 aromatase activity in human ovarian granulose cell-like KGN cells. In the current study, we demonstrate the direct involvement of Smad2 as a downstream signal mediator of ActRIB in the transcriptional regulation of the P450 aromatase gene (*CYP19A*) in KGN cells. Upon activin stimulation, Smad2 activation and an increase in P450 aromatase messenger RNA (mRNA) were observed in KGN cells. Interestingly, Smad2 phosphorylation correlated well with the increase in P450 aromatase mRNA. Reciprocally, knockdown of *Smad2* mRNA in KGN cells led to a decrease in the P450 aromatase mRNA expression, suggesting that Smad2 regulates *CYP19A* gene expression. Further analysis of *CYP19A* promoter activity revealed that the 5′ upstream region between −2069 and −1271 bp is required for the activation by Smad2. Finally, we provide compelling evidence that Smad2 shows follicular stage-specific expression, which is high in granulosa cells of preantral or early antral follicles in mice. Our results suggest that activin signaling through the ActRIB-Smad2 pathway plays a pivotal role in *CYP19A* expression and thus in follicular development.

1. Introduction

Activin, a TGF-β family member, was initially identified as a secreted protein from ovarian follicular fluid, stimulating FSH production by the pituitary gland [1,2]. A growing number of studies have demonstrated its involvement in a wide variety of physiological processes, including embryogenesis and carcinogenesis, as well as reproduction [3]. In the ovary, activin is expressed predominantly in the granulosa cell layer of follicles [4]. Activin A has been demonstrated to potentiate FSH-stimulated aromatase activity as well as progesterone and inhibin production in rat immature granulosa cells [5]. These results strongly suggest its important roles in processes such as folliculogenesis, steroid hormone production, and oocyte maturation as a paracrine or autocrine factor. However, the signaling mechanism of activin in ovarian granulosa cells is still not well understood, mainly because of the limitations in molecular biological analyses that use primary culture systems. To overcome this problem, we have established the human ovarian granulose cell-like KGN cells [6]. As originally reported, this cell line expresses functional FSH receptors and has aromatase activity, reminiscent of ovarian granulosa cells *in vivo*. Thus, the KGN cell line could be a very useful model for studying the role of activin in the regulation of the expression of P450 aromatase during folliculogenesis in the ovary.

P450 aromatase, encoded by the *CYP19A* gene, is a key enzyme involved in estrogen biosynthesis [7]. *CYP19A* has nine coding exons (exon II–X) and the 5′ untranslated region is encoded by exon I, which is alternatively used by different tissues. The gene uses multiple promoters in a tissue-specific manner, resulting in a tissue-specific regulation of aromatase activity [8]. In the ovary, the 5′ flanking region of exon II has been shown to direct the tissue-specific expression. The first 300-bp region of the proximal promoter II contains the elements necessary for the expression of aromatase in mural granulosa cells [9].

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; TGF-β, transforming growth factor β; ActRIB, activin type IB receptor; ActRIA, activin type IA receptor; *CYP19*, cytochrome P450 aromatase gene; FSH, follicular-stimulating hormone; CRE, cyclic AMP responsive element; CLS, CRE-like sequence; SF-1, steroidogenic factor 1.

* Corresponding author. Fax: +81 92 642 5287.
E-mail address: nomura@med.kyushu-u.ac.jp (M. Nomura).

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The basal components of the TGF-β family signaling pathways are the type I and II transmembrane serine and threonine kinase receptors and the cytoplasmic Smad proteins [10]. Upon ligand binding, the type II receptor recruits and transphosphorylates the type I receptor. The type I receptor acts downstream of the type II receptor, and has been shown to determine signaling specificity within the heteromeric receptor complex [11,12]. The type I receptor, activated by phosphorylation, subsequently propagates signals to the Smad pathway. Once phosphorylated, the receptor-regulated Smads (R-Smads) dissociate from the receptor complex, bind to a Co-Smad (Smad4), and enter the nucleus as a complex where they participate in the transcriptional regulation of the target genes. Activin, like most of the TGF-β family members, mediates its action by binding to a complex of type I and II receptors. The type I receptors comprise type IA (ActRIA) and type IB (ActRIB). The type II receptors comprise type IIA (ActRIIA) and type IIB (ActRIIB). Activated type I receptors propagate the signal to Smad2 and Smad3. The precise roles of the different activin receptors and Smads are still not clear. The different usage of the type I and II receptors and Smads may be responsible for the different actions of activin on different tissues and target genes, explaining their functional diversity. Thus, delineation of the cellular components of the system may be necessary for understanding the regulation of numerous biological systems.

To clarify the signaling pathway and the role of activin in ovarian granulosa cells, we performed a detailed analysis in KGN cells. We have previously reported that activin signaling through ActRIB up-regulates FSH receptor expression and aromatase activity in KGN cells [13]. In addition, blockage of the protein kinase A (PKA) pathway resulted only in partial cancelation of the increase in aromatase activity, suggesting that other than the PKA pathway reinforcement there is an additional mechanism by which activin stimulates the aromatase activity synergistically with the FSH signal in granulosa cells. In this study, we showed the direct involvement of Smad2 as a downstream signal mediator of ActRIB in the activin signaling pathway to regulate *CYP19A* expression in KGN cells. In addition, we provide compelling evidence that Smad2 showed a follicular stage-specific expression in granulosa cells of preantral or early antral follicles in mice, suggesting an important role in ovarian follicular development. The activin signaling through the ActRIB-Smad2 pathway may thus be essential for *CYP19A* gene expression and hence follicular development.

2. Materials and methods

2.1. Cell culture

The human ovarian granulosa-like tumor cell line, KGN, was cultured as described [6].

2.2. Plasmids

The expression vectors for human ActRIBCA were kindly provided by Dr. Lawrence S. Mathews (University of Michigan, Ann Arbor, MI). A constitutively active form of ActRIB, designated ActRIBCA, was generated by amino acid substitution of the threonine at position 206 with glutamine in human ActRIB complementary DNA [14]. The expression vectors for Smad2 and CBP/p300 were constructed by inserting their entire coding sequence into the pCDNA3 vector. The reporter plasmids Arom-Luc 0.8, 1.2, 2.0, and 4.0 K were generated by subcloning the 5′ flanking proximal region of the exon II of *CYP19A* with the length of 0.8, 1.2, 2.0 and 4.0 kb, respectively, into the luciferase reporter construct, pGL3 (Promega, Madison, WI, USA).

2.3. Luciferase assays

1×10^5 cells/well were transfected using 3 μl Superfect Transfection Reagents (Qiagen, Hilden, Germany) with 1 μg Arom-Luc reporter construct, 0.5 μg Smad2 and/or CBP, and 1 ng pRL-CMV (Promega) as an internal control. After overnight incubation in optimum medium, the medium was changed to a serum-containing culture medium. After another 24 h, luciferase activity was measured using the dual luciferase assay system (Promega) in a microLumat LB9507 luminometer. The luciferase activity was normalized against transfection efficiency using the *Renilla* luciferase activity from pRL-CMV.

2.4. mRNA analysis

KGN cells were cultured in a 6-well dish containing 3×10^5 viable cells in 3 ml of medium in the presence or absence of activin A (0, 12.5, 25, 50, or 100 ng) for 24 h, followed by total RNA preparation using Isogen solution (Molecular Research Center Inc., Tokyo, Japan). The final RNA pellet was dissolved in Tris–EDTA buffer. Total RNA was quantified by measuring the absorbance of the samples at 260 nm, and then stored at −80 °C until the assay. First-strand cDNA was synthesized with 1 μg total RNA using an RT-PCR kit (Stratagene, La Jolla, CA). Quantitative analysis of the expressions of *Smad2* and P450 aromatase was performed using a Roche LightCycler (Roche, Mannheim, Germany) as previously described [13]. Primer sets used in this study were as follows: *Smad2*, 5′-AGAAGTCAGCTGGTGGGTCTG-3′/5′-TCATGATGACTGTGAAGATCAGG-3′; P450 aromatase, 5′-AAGGCTCTGAGAAGACCTCAACGATG-3′/5′-CATCGTTGAGGTCTTCTCAGAGCCTT-3′. The primer set for human *β-actin* was supplied in the LightCyclerTM-Primer set (Roche). The relative abundance of the mRNAs, expressed as fold change, was extrapolated from crossing point data. A difference of one PCR cycle in crossing point number translates into a two-fold change in mRNA expression.

2.5. RNA interference

Stealth RNAi™ siRNAs targeting Smad2, siRNA-1 (ID: VHS41106) and siRNA-2 (ID: VHS41107), were purchased from Life technologies (Carlsbad, CA, USA). RNA oligomers were reconstituted and annealed following a previously described protocol [15]. KGN cells were plated 24 h prior to transfection and siRNAs were transfected with Lipofectamine RNAiMAX (Life technologies,) following the manufacturer's instructions. For the Smad2 silencing experiments, total RNAs were prepared 48 h after transfection.

2.6. Western blot analysis

Proteins were extracted by cell lysis in a buffer containing 50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, and 1 tablet/10 ml of protease inhibitor mix (Roche). Extracts were subjected to 10% SDS–polyacrylamide gel electrophoresis, and blotted onto a PVDF membrane (Millipore, Billerica, MA, USA). The PVDF membrane was blocked with 4% Block Ace in TBST and incubated with specific antibodies. The primary antibodies used were rabbit anti-Smad2 and rabbit anti-phospho-Smad2 (both 1:250; Cell Signaling Technology, Danvers, MA, USA). Chemiluminescent signals were generated by incubation with an ECL reagent (GE Healthcare, Buckinghamshire, UK).

2.7. X-gal staining of ovaries

Whole ovaries were excised from $Smad2^{mh2\text{-}lacZ/+}$ mice [16] and X-gal staining was performed as described [17]. After X-gal staining, the ovaries were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated in 100% ethanol, and embedded in paraffin. The sections (6-μm thick) were counterstained with Mayer's hematoxylin (5 min), dehydrated, and mounted. X-gal staining was viewed under microscopy BioZero (Keyence, Osaka, Japan).

2.8. Statistics

All experiments were carried out at least three times. In the luciferase and the aromatase assays, each independent experiment was run in triplicate or duplicate plates and these were used to generate a single mean value, which was then used to generate the mean ± standard deviation (SD) shown in the figures. All values represent the mean ± SD. Statistical significance was determined by one-factor analysis of variance followed by a posthoc test (Fisher's PSLD).

3. Results

3.1. Smad2 is activated downstream of activin type IB receptor in KGN-ActRIBCA cells

The human ovarian granuulosa tumor cell line, KGN, is known to express functional FSH receptors and P450 aromatase. Previously, we have established the KGN-ActRIBCA cell line, which stably expresses the constitutively active form of ActRIB [13]. The aromatase activity of KGN-ActRIBCA at basal level was dramatically increased by about 20-fold and reached a level much higher than that of the KGN cells treated with FSH. These results suggested that activation of activin signaling stimulates aromatase activity not only by increasing FSH receptor expression but also by a mechanism independent of FSH signaling. To clarify the signaling pathway of activin in KGN cells, we first analyzed the expression of Smad2 in KGN-ActRIBCA cells. Analysis of mRNA expression by real-time RT-PCR showed that there was no difference in mRNA expression between KGN-ActRIBCA and KGN cells (Fig. 1A). This was also confirmed at the protein level by western blotting with a Smad2 antibody. As shown in Fig. 1B, the total amount of Smad2 protein was similar, while the level of the phosphorylated Smad2 protein was higher in KGN-ActRIBCA cells than in KGN cells. To ensure that Smad2 was activated by phosphorylation, immunostaining of Smad2 was performed. As shown in Fig. 1D, Smad2 was found to be predominantly located in the nucleus of KGN-ActRIBCA cells. However, in KGN cells, only a few cells showed nuclear staining of Smad2 (Fig. 1C, arrowheads). These results indicated that Smad2 acts downstream of ActRIB and is activated in KGN-ActRIBCA cells.

3.2. Smad2 is required for transcription of the aromatase gene in the KGN cell

To confirm that activin signaling employs Smad2 as a downstream effector to regulate aromatase gene expression in KGN cells, first we analyzed the phosphorylation of Smad2 in KGN cells after treatment with various ligands, including activin. As shown in Fig. 2A, Smad2 was strongly phosphorylated after activin A treatment, while it was weakly phosphorylated after TGF-β1 treatment in KGN cells. No phosphorylation of Smad2 was observed after treatment with FSH. Next, we examined the *CYP19A* expression in KGN cells after activin stimulation. As shown in Fig. 2B, aromatase mRNA was induced by activin stimulation at any concentration up to 100 ng/mL, with the highest value at 25 ng/mL. Interestingly, the phosphorylation levels of Smad2 correlated well with aromatase mRNA expression levels, suggesting a direct involvement of Smad2 in the transcriptional regulation of *CYP19A*. To confirm this hypothesis, we employed siRNA-mediated silencing of Smad2. We constructed two siRNAs, siRNA1 and siRNA2, specific to Smad2. Transfection of KGN cells with either siRNA1 or siRNA2 reduced Smad2 transcription levels by approximately 80% and 90%, respectively (Fig. 2C). As shown in Fig. 2D, the transcription levels of *CYP19A* dramatically reduced concomitantly with the silencing of Smad2, indicating that Smad2 is required for the transcription of *CYP19A* in KGN cells.

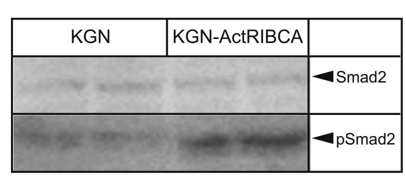
3.3. Smad2 upregulates aromatase gene transcription

To confirm that activin acts through Smad2 to regulate aromatase gene transcription, promoter assays with *CYP19A* were performed. It has already been shown that the promoter region that directs ovary-specific [18], as well as KGN cell-specific [19], expression of the aromatase gene resides in the 5′ flanking promoter region of exon II. Examination of this promoter region revealed the presence of a CRE (TGAAGTCA, located at −409 to −402), CLS (TGCACGTCA, located at −328 to −320) [20], SF-1

A

Smad2 mRNA expression	
Cells	crossing point
KGN	21.85 ± 0.15
KGN-ActRIBCA	21.55 ± 0.10

B



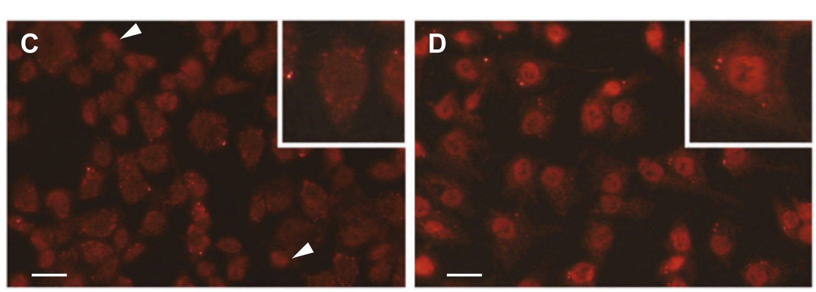


Fig. 1. Smad2 is activated downstream of activin type IB receptor in KGN-ActRIBCA cells. (A) Smad2 expression analyzed by real-time RT-PCR. (B) Increased phosphorylation of Smad2 in KGN-ActRIBCA cells. (C and D) Immunohistochemical staining of Smad2 in KGN (C) and KGN-ActRIBCA cells (D). The immunoreactivity of Smad2 in the nucleus was observed only in a few KGN cells (C, arrowheads), whereas in KGN-ActRIBCA cells all the cells showed nuclear staining of Smad2 (D). High magnification of each staining is shown in the right upper corner. Bars, 10 μm.

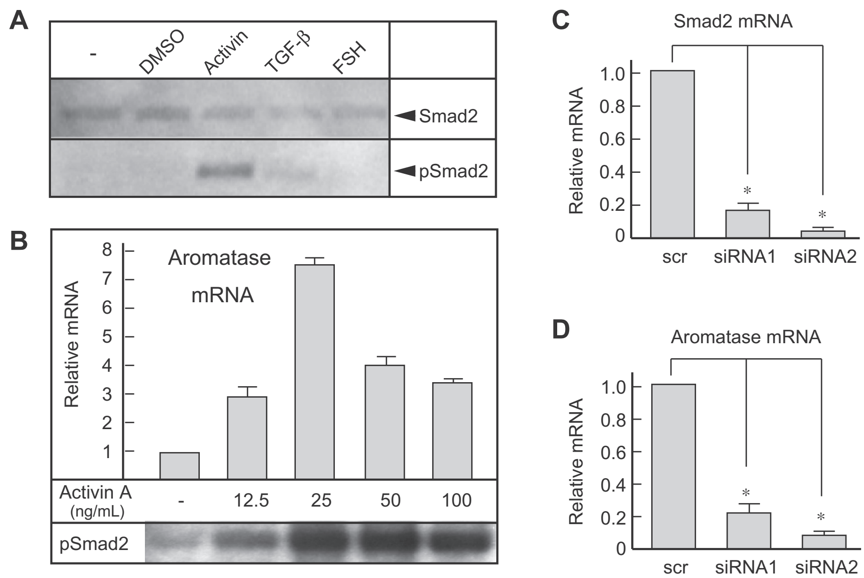


Fig. 2. Smad2 is involved in the transcriptional regulation of *CYP19A* in KGN cells. (A) Immunoblot analysis of Smad2 phosphorylation in KGN cells. Protein lysates were prepared from the cells treated for 1 h with activin A (100 ng/mL), TGF-β1 (50 ng/mL), or FSH (50 ng/mL). (B) Smad2 phosphorylation by activin correlates well with aromatase mRNA. (C and D) Smad2 silencing experiments with siRNA. (C) Both of the siRNAs, siRNA1 and siRNA2, efficiently knocked down Smad2 transcript in KGN cells. scr, scramble RNA. (D) Aromatase mRNA was reduced in parallel with the knockdown of Smad2 in KGN cells in the presence of 25 ng/mL activin A.

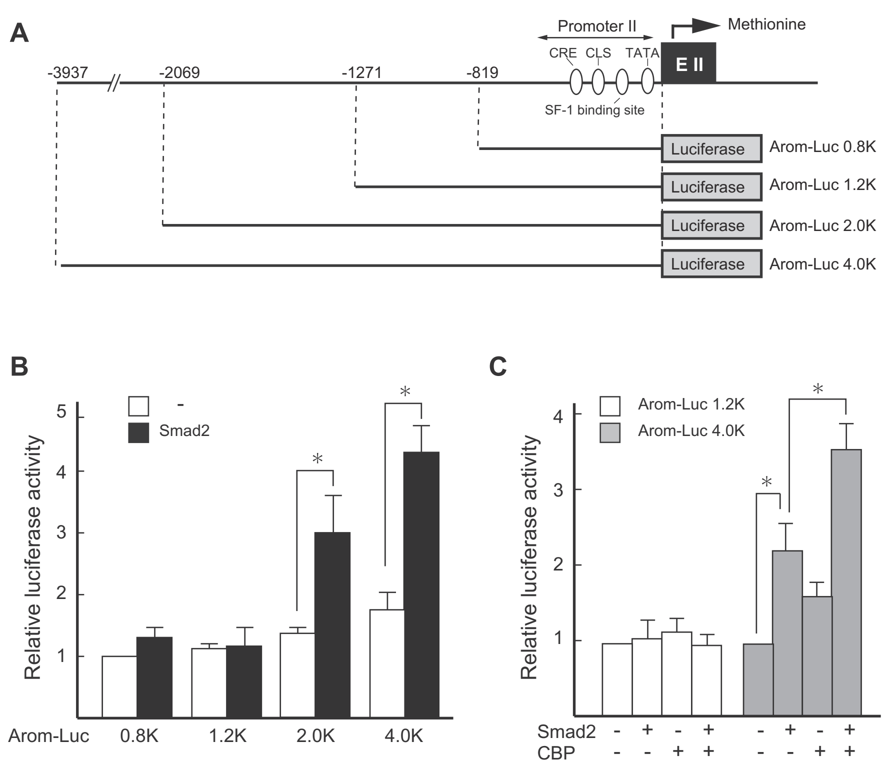


Fig. 3. Ovarian aromatase promoter constructs and the effect of Smad2 on promoter activity. (A) Various lengths (−3937 to −819) of the 5′ flanking region of exon II were cloned into the luciferase reporter construct, pGL3. The nucleotide sequences are numbered from the initiation methionine codon. The location of CRE, CLS, SF-1 binding site, and TATA box in promoter II region are indicated. (B) Smad2 up-regulates the promoter activity of the aromatase gene. Luciferase activities in cells transfected with the reporter constructs as listed in the figure with or without the Smad2 expression vector. $^{*}P < 0.01$. (C) CBP enhances the Smad2-induced promoter activity of the aromatase gene. Luciferase activities in cells transfected with either Arom-Luc 1.2 or 4.0 K together with combinations of Smad2 and CBP as listed in the figure. $^{*}P < 0.01$.

binding site (AGGTCAG, located at −247 to −241), and TATA box (TATAAAA, located at −148 to −142), suggesting the first 409-bp region of the proximal promoter contains the elements necessary for the expression of P450 aromatase in ovarian granulosa cells [9]. Four promoter constructs containing different lengths of the 5′ flanking sequence of exon II, designated as Arom-Luc 0.8, 1.2, 2.0 and 4.0 K, were made as shown in Fig. 3A. KGN cells were transiently transfected with each of the luciferase constructs along with the Smad2 expression vector. As shown in Fig. 3B, co-transfection with Smad2 up-regulated the promoter activity of

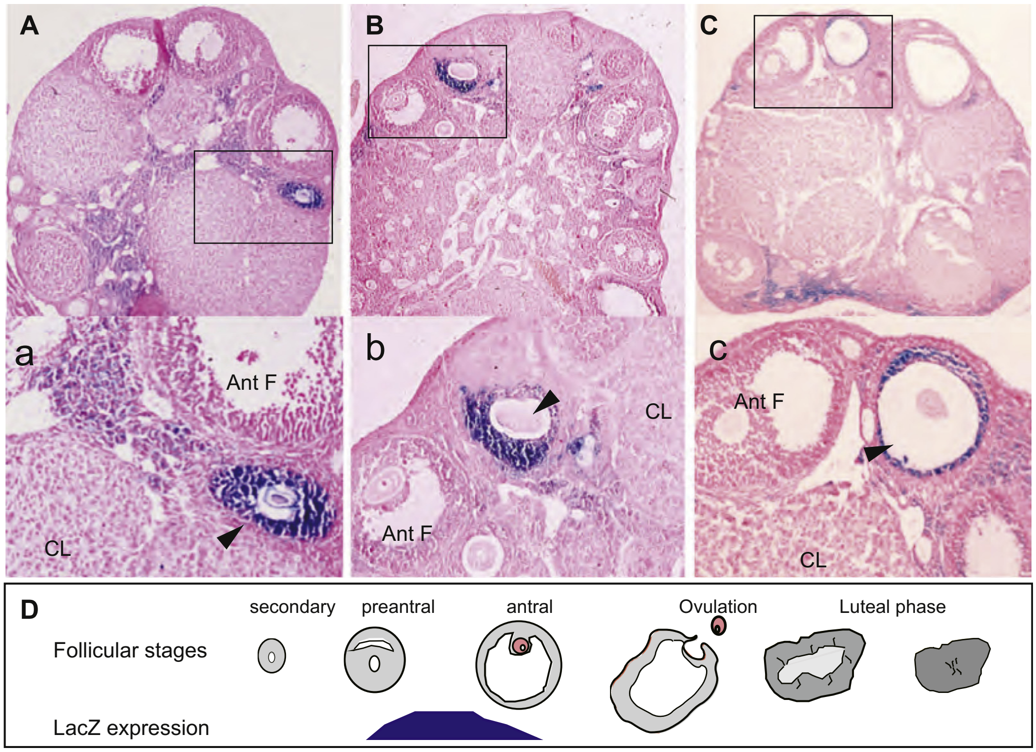


Fig. 4. Stage-specific expression of Smad2 during folliculogenesis in mice. (A–C) LacZ staining of ovaries from Smad2$^{mh2\text{-}lacZ/+}$ mice at 16 weeks of age are shown. The areas in the rectangle were magnified and are shown in the lower panels (a–c). The granulosa cells in preantral or early antral follicles are predominantly stained (arrowheads). CL, corpus luteum; Ant F, antral follicle. (D) Schematic representation of follicular development with a lacZ expression profile.

Armon-Luc 2.0 K and Arom-Luc 4.0 K constructs by approximately 2-fold. Deletion between −2069 and −1271 abolished the responsiveness to Smad2 in the promoter activity. These results strongly suggest that Smad2 is involved in activin-stimulated *CYP19A* transcription. Because Smad2 cannot directly bind to DNA, it requires formation of a DNA binding complex with co-factors. Smad proteins activate transcription by recruiting the CBP/p300 histone acetyltrasferase to the chromatin to remodel it into the active open confirmation [21]. CBP/p300 also acts as an adapter linking transcription factors to the general transcription machinery including RNA polymerase II [22]. To investigate whether CBP/p300 enhances the transcriptional activity of Smad2 on the aromatase promoter, co-transfection experiments were performed. As expected, co-transfection with the CBP expression vector further enhanced the Smad2-induced promoter activity of Arom-Luc 4.0 K, while it showed no effect on Arom-Luc 1.2 K (Fig. 3C). Taken together, Smad2 acts as a transcriptional activator of *CYP19A* gene expression.

3.4. Smad2 expression during folliculogenesis in mice

To address the physiological importance of Smad2 as a signaling molecule in ovarian folliculogenesis, its expression profile was investigated in Smad2$^{mh2\text{-}lacz/+}$ mice, which harbor the β-galactosidase gene in the *Smad2* locus, enabling visualization of Smad2 expression by lacZ staining *in vivo* [16]. LacZ staining of ovarian sections from 16-week-old mice revealed that Smad2 expression was strong in the granulosa cell layers of preantral or early antral follicles (Fig. 4A–C, arrowheads). In contrast, neither Graaf/preovulatory follicles nor corpus luteum showed lacZ expression, indicating Smad2 shows stage-specific expression during follicular development. The follicular development during folliculogenesis is shown as a schematic representation in Fig. 4D. LacZ expression is highest in preantral/early antral follicles and gradually decreases as follicles mature, and is hardly observed in large antral/preovulatory follicles, which is consistent with previous observations in rat ovaries [23].

4. Discussion

In this study, we demonstrated the direct involvement of Smad2 as a downstream signal mediator of ActRIB in the activin signaling pathway to regulate *CYP19A* expression in KGN cells. In KGN-ActRIBCA cells, endogenous Smad2 protein was phosphorylated and translocated into the nucleus, indicating Smad2 acts downstream of ActRIB in KGN cells. We also showed that the phosphorylation levels of Smad2 in response to various concentrations of activin correlated well with aromatase mRNA expression levels. Reciprocally, siRNA-mediated silencing of Smad2 led to a dramatic reduction in *CYP19A* transcription. These results strongly suggest that Smad2 is involved in the transcriptional regulation of the P450 aromatase gene, *CYP19A*.

Furthermore, using luciferase reporter assays, we found that Smad2 induced the promoter activity of *CYP19A*. Deletion analysis showed that removal of nucleotides between −2069 and −1271 resulted in a loss of response to Smad2, while the basal promoter activity was maintained. This finding suggests that an activin response element may reside in the region between −2069 and −1271. Because Smad2 cannot directly bind to DNA, it requires formation of a DNA-binding complex with other co-factors. Smads can positively regulate gene expression by recruiting coactivators, such as CBP/p300 [24,25], or negatively by direct recruitment of histone deacetylases or corepressors, such as c-Ski and Sno N [26]. CBP/p300 can act as an adapter linking transcription factors to the general transcription machinery including RNA polymerase II [22]. CBP/p300 also has histone acetyltransferase activity that can release DNA from the tight chromatin structure and increase its accessibility to transcription regulatory proteins [24]. Using luciferase reporter assays, we showed that CBP/p300 further enhances the transcriptional activity of Smad2 on the aromatase promoter. Although we could not identify the binding cis-element and the factors that link the transcription factor complex to DNA, our results clearly demonstrated that Smad2 acts as a transcription activator of the aromatase gene transcription in KGN cells through the ovarian-specific promoter. As no activin response element has been described in the aromatase proximal promoter, the effect of Smad2 is probably indirect. Further analyses will be required to unveil the entire complex regulating the transcription of the aromatase gene by activin stimulation.

Finally, we provide compelling evidence that Smad2 showed follicular stage-specific expression in granulosa cells of preantral or early antral follicles in mice, suggesting an important role in ovarian follicular development. Ovarian granulosa cells undergo a complete differentiation process during the growth and maturation of ovarian follicles that depends on pituitary gonadotropins. In this regard, the maintenance of the FSH receptor expression in the granulosa cells is important for the follicular maturation, and thus for fertility. FSH has been shown to increase the number of its own receptors on the cell surface, implying a positive regulatory loop in the FSH signaling pathway in granulosa cells [27]. This may explain, at least in part, the mechanism by which FSH receptor expression is maintained during follicular maturation. The response of granulosa cells to FSH changes dramatically during follicular growth *in vivo*. In small follicles, FSH regulates the proliferation of granulosa cells, while as follicles mature to a preovulatory stage FSH induces the expression of differentiation-specific genes such as *CYP19A*, encoding P450 aromatase. The precise molecular mechanism involved in this switch remains unknown. Based on the expression profile of Smad2 in granulosa cells, it is conceivable to speculate that activin through Smad2 may be one of the signals that modulates the FSH responsiveness and promotes the maturation of granulosa cells [5]. Studying the effects of granulosa cell-specific Smad2 disruption will be interesting.

In conclusion, the activin signaling through the ActRIB-Smad2 pathway plays a pivotal role in ovarian granulosa cell function by regulating *CYP19A* gene expression and thereby follicular development.

Acknowledgments

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