BIOLOGY OF REPRODUCTION **74,** 1073–1082 (2006)
Published online before print 25 January 2006.
DOI 10.1095/biolreprod.105.047969

Mutual Regulation of Follicle-Stimulating Hormone Signaling and Bone Morphogenetic Protein System in Human Granulosa Cells[1]

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ABSTRACT

Bone morphogenetic proteins (BMPs) play critical roles in folliculogenesis by modulating the actions of follicle-stimulating hormone (FSH) in the ovary. However, the effects of FSH on the BMP system remain unknown. Here, we have investigated the effects of FSH on BMP signaling using the human granulosa-like tumor cell line KGN. KGN cells express BMP type I and type II receptors and the BMP signaling molecules SMADs. FSH administration upregulated BMP type IA (*BMPR1A*) and IB (*BMPR1B*) receptors, activin type II receptor (*ACVR2*), and BMP type II receptor (*BMPR2*). FSH also augmented *SMAD1* and *SMAD5* expression, and conversely, FSH suppressed the expression of the inhibitory SMADs, *SMAD6* and *SMAD7*. Bioassays revealed that FSH enhances BMP-induced SMAD1/5/8 phosphorylation and cellular DNA synthesis induced by BMP6 and BMP7. Since overexpression of BMPR1A and BMPR1B, but not SMADs, significantly enhanced the BMP responses, these type I receptors were revealed to be limiting factors for BMP signaling in KGN cells. BMPs significantly suppressed progesterone synthesis induced by forskolin and dibutyryl-cAMP (BtcAMP) but had no effect on estradiol induced by the same factors. KGN cAMP levels induced by forskolin were not altered by BMPs, suggesting that BMPs regulate steroidogenesis at a level downstream of cAMP synthesis in KGN cells. In this regard, BMPs specifically reduced the *STAR* transcription, whereas the levels of *CYP11A*, *HSD3B2*, and *CYP19* stimulated by forskolin as well as BtcAMP were not altered. Collectively, the two major factors, FSH-cAMP pathway and BMP system, are reciprocally and functionally linked. Given that BMPs downregulate FSH receptors in KGN cells, this interaction may contribute to fine-tuning of the mutual sensitivity toward BMP ligands and FSH.

follicle-stimulating hormone, granulosa cells, growth factors, ovary, signal transduction, steroiodogenesis

INTRODUCTION

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β (TGFβ) superfamily and are key factors for normal folliculogenesis in the ovary. The spatiotemporal expression patterns of the ovarian BMP system, including BMP ligands, receptors, and binding proteins, have been demonstrated throughout the normal estrous cycle, in which each transcript of the BMP system components exhibited cell-specific pattern in ovarian cells [1]. Recent studies have established the concept that the BMP system plays a crucial role in female fertility in mammals by regulating steroidogenesis as well as mitogenesis in granulosa cells [2, 3].

In granulosa cells, a major regulatory process governed by BMPs involves the control of follicle-stimulating hormone (FSH) signaling. For instance, theca-derived factors BMP4 and BMP7 enhance estradiol and suppress progesterone production induced by FSH in rat primary granulosa cells [4, 5]. BMP2 action in sheep primary granulosa cells mimics the steroidogenic properties of BMP4 and BMP7 in rat granulosa cells [6]. Oocyte/granulosa-derived BMP6 inhibits FSH-induced progesterone synthesis through suppression of cellular cAMP synthesis [7]. Another key factor, BMP15, which is specifically expressed by oocytes in the ovary, also exhibits potent suppression of FSH actions by inhibiting FSH receptor (FSHR) expression [8]. In addition, oocyte-derived growth and differentiation factor-9 (GDF9) that shares the highest homology with BMP15 also inhibits FSH-induced steroidogenesis and luteinizing hormone (LH) receptor expression in rat granulosa cells [9].

Thus, all these BMPs are potent regulators of FSHR signaling at various sites of the signaling pathway. Despite the established importance of the BMP system in the regulation of FSH action in granulosa cells, the effects of FSH in the regulation of the ovarian BMP system remain poorly understood. In mammals, the ability of the ovary to produce dominant follicles capable of ovulating fertilizable oocytes in response to FSH stimulation is a fundamental requirement for female fertility. The actions of FSH during the folliculogenesis process are tightly regulated by dynamic systems of paracrine and autocrine factors as the follicles grow and differentiate [10].

In the present study, we investigated FSH effects on BMP signaling using the human granulosa-like tumor cell line KGN. As reported previously, this cell line expresses functional FSHR [11, 12], steroidogenetic properties [11, 12], and aromatase activity [13, 14], reminiscent of primary granulosa cells. By utilizing the KGN cell line, we performed molecular analysis regarding the functional roles of BMPs and FSH effects on the BMP system.

[1]Supported in part by Grants-in-Aid for Scientific Research (No. 16790525), Uehara Memorial Foundation, Kanzawa Medical Research Foundation, Yamaguchi Endocrine Research Association, Life Science Foundation of Japan, Takeda Science Foundation, and The Tokyo Biochemical Research Foundation.
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Received: 27 September 2005.
First decision: 3 November 2005.
Accepted: 24 January 2006.

ISSN: 0006-3363. http://www.biolreprod.org

MATERIALS AND METHODS

Reagents and Supplies

A 1:1 mixture of Dulbecco Modified Eagle Medium/Ham F-12 medium (DMEM/F12), human pituitary FSH, forskolin (FSK), N^6,$O^{2'}$-Dibutyryl adenosine 3′,5′-cyclic monophosphate monosodium salt (BtcAMP), 4-androstene-3,7-dion (androstenedione) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human TGFB1 was from PeproTech EC Ltd. (London, UK), and human BMP2, 4, 6, 7, and activin A were from R&D Systems (Minneapolis, MN). Human ovary total RNA was purchased from Stratagene Inc. (La Jolla, CA). Plasmids of pGL2-StAR (−235 bp) were kindly provided from Dr. Jerome F. Strauss III; Tlx2-Luc from Dr. Jeff Wrana; and 3TP-Luc, Id1-Luc and expression plasmids for BMP type I receptors (pcDNA3-HASL-wtALK2, wtALK3 and wtALK6) and SMADs (pcDEF3-Flag(N)-SMAD1, 2, 3 and 5) were from Drs. Tetsuro Watabe and Kohei Miyazono, Tokyo University, Japan.

Cell culture and RNA extraction

The human ovarian granulosa-like tumor cell line, KGN, was from Drs. Masatoshi Nomura and Hajime Nawata, Kyushu University, Japan [11]. KGN cells were cultured in DMEM/F12 supplemented with 10% FCS and antibiotics in a 5% CO_2 atmosphere at 37°C. To prepare total cellular RNA for cDNA array analysis, KGN cells (3×10^5 viable cells) were cultured on 6-well plate and treated with or without FSH (10 ng/ml) in DMEM/F12 containing 1% FCS for 24 h. For the other RNA preparations, the cells were treated with or without BMP2, 4, 6, and 7 (100 ng/ml) in the presence or absence of FSK (10 μM) or BtcAMP (1 mM) with DMEM/F12 containing 1% FCS for 24 h. The culture medium was then removed and total cellular RNA was extracted using TRIzol (Invitrogen Corp.) and quantified by measuring absorbance at 260 nm and stored at −80°C until assay.

cDNA array analysis

A GEArray system (SuperArray Bioscience Corp., Frederick, MD) that includes 96 genes of human TGFβ and BMP signaling pathway was used for analyzing the expression pattern of the BMP signaling system in KGN cells. Extracted total RNAs (2.5 μg) were used as templates to generate Biotin-16-dUTP-labeled cDNA probes according to the manufacturer's instructions. The cDNA probes were denatured and hybridized at 60°C with the cDNA array membranes, which were washed and exposed to x-ray films with use of chemiluminescent substrates. To analyze the array results, we scanned the x-ray film, and the image was inverted as grayscale TIFF files. The spots were digitized and analyzed using GEArray analyzer software (SuperArray Bioscience Corp.) and the data were normalized by subtraction of the background as the average intensity levels of three spots containing plasmid DNA of pUC18. The averages of two spots of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and four spots of cyclophilin A (PPIA) were used as positive controls to compare the membranes. Using these standardized data, we compared the signal intensity of the membranes using the GEArray analyzer program (SuperArray Bioscience Corp.).

Western immunoblotting analysis

KGN cells (3×10^5 viable cells) were seeded in 12-well plates in DMEM/F12 containing 1% FCS. After 24-h preculture, BMP2, 4, 6, 7, activin A, and TGFB1 (100 ng/ml) were added to the culture media in the presence or absence of FSH (10 ng/ml). Following 1-h stimulation with growth factors, cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS, and 4% β-mercaptoethanol. The cell lysates were then subjected to SDS-PAGE immunoblotting analysis as we previously reported [15], using anti-phospho-SMAD1/5/8 antibody (Cell Signaling Technology, Inc., Beverly, MA).

Transient transfection and luciferase assay

KGN cells (2×10^5 viable cells) were cultured in 12-well plates in DMEM/F12 with 10% FCS. The cells were then transiently transfected with 500 ng of each luciferase reporter plasmid (3TP-Luc, Id1-Luc, Tlx2-Luc or pGL2-StAR-235) and 50 ng of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using SuperFect Transfect Reagent (Quiagen Inc., Valencia, CA). In the indicated experiments, 500 ng of expression plasmids encoding wild-type DNA of either *ACVR1, BMPR1A,* and *BMPR1B* or *SMAD1, 2, 3* and *5* were co-transfected. The cells were then treated with BMP2, 4, 6, 7, activin A and TGFB1 (100 ng/ml), in the presence or absence of FSH (10 ng/ml) for 24 h in DMEM/F12 containing 1% FCS. The cells were washed with PBS and lysed with Cell Culture Lysis Reagent (TOYOBO, Osaka, Japan). Luciferase activity and β-galactosidase (βgal) activity of the cell lysate were measured by luminescencer-PSN (ATTO, Tokyo, Japan). The data were shown as the ratio of luciferase to βgal activity.

RT-PCR and quantitative real-time PCR analysis

The extracted RNA (1 μg) was subjected to RT reaction using First-Strand cDNA Synthesis System (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U) and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42°C for 50 min, 70°C for 10 min. Subsequently, hot-start PCR was performed using $MgCl_2$ (1.5 mM), dNTP (0.2 mM), and 2.5 U of Taq DNA polymerase (Invitrogen Corp.). Oligonucleotides used for PCR were custom-ordered from Kurabo Biomedical Co. (Osaka, Japan). PCR primer pairs were selected from different exons of the corresponding genes as follows: *ACVR1* (also known as *ALK2*), 599–621 and 1060–1081 (from GenBank accession #Z22534); *SMAD1*, 722–743 and 1037–1058 (from U59912); *SMAD2*, 304–324 and 651–673 (from NM_005901); *SMAD3*, 348–370 and 624–646 (from NM_005902); *SMAD4*, 526–546 and 853–874 (from NM_005359); *SMAD5*, 806–828 and 1232–1253 (from NM_005903); *SMAD6*, 1450–1470 and 1861–1881 (from NM_005585); *SMAD7*, 763–783 and 1179–1199 (from NM_005904); FSH receptor (*FSHR*), 229–249 and 415–435 (from NM_000145); steroidogenic acute regulatory protein (*STAR*), 449–469 and 757–775 (from U17280); P450 cholesterol side-chain cleavage enzyme (P450scc, *CYP11A*), 188–207 and 660–680 (from M14565); 3 beta-hydroxysteroid dehydrogenase (3βHSD, *HSD3B2*), 307–327 and 441–461 (from NM_000198); P450 aromatase (P450arom, *CYP19*), 703–724 and 1235–1256 (from M22246); and house-keeping gene, ribosomal protein L19 (*RPL19*), 401–420 and 571–590 (from NM_000981); *BMPR1A* (also known as *ALK3*), *ACVR1B* (also known as *ALK4*), *BMPR1B* (also known as *ALK6*), activin type II receptor, *ACVR2A* (also known as *ActRII*) and BMP type II receptor, *BMPR2* (also known as *BMPRII*) as we reported previously [16]. For the quantification of *ACVR1, BMPR1A, BMPR1B, ACVR2A, BMPR2, SMAD1, SMAD5, SMAD6, SMAD7*, *FSHR*, *STAR, CYP11A*, *HSD3B2*, *CYP19* and *RPL19* mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic Co., Tokyo, Japan) under the optimized conditions of annealing at 60 to 62°C with 4 mM $MgCl_2$ following the manufacturer's protocol. Accumulated levels of fluorescence during amplification were analyzed by the second-derivative method after the melting-curve analysis (Roche Diagnostic), and then the expression levels of target genes were standardized by *RPL19* level in each sample.

Thymidine incorporation assay

KGN cells (1.5×10^5 viable cells) were cultured in 12-well plates with 1 ml of DMEM/F12 culture medium containing 1% FCS. After 24-h preculture, BMP2, 4, 6, 7, activin A, and TGFB1 (100 ng/ml) were added in the presence or absence of FSH (10 ng/ml). After 24-h culture, 0.5 μCi/well of [methyl-^{3}H] thymidine (Amersham Pharmacia, Piscataway, NJ) was added and incubated for 3 h at 37°C. The incorporated thymidine was detected as we previously reported [7]. Cells were then washed with PBS, incubated with 10% ice-cold trichloroacetic acid for 30 min at 4°C, solubilized in 0.5 M NaOH, and its radioactivity determined by liquid scintillation counter (TRI-CARB 2300TR, Packard Co., Meriden, CT).

Measurement of progesterone and estradiol

KGN cells (6×10^4 viable cells) were precultured in 96-well plates with DMEM/F12 containing 10% FCS for 48 h. The medium was changed to DMEM/F12 containing 1% FCS and 100 nM of androstenedione (a substrate for P450arom) and then FSH (10 to 100 ng/ml), FSK (10 μM), or BtcAMP (1 mM) was added to the culture medium either alone or in combination with BMP2, 4, 6, and 7 (30 to 100 ng/ml). After 48 h culture, the supernatant of culture media was collected and stored at −80°C until assay. The levels of progesterone and estradiol in the media were measured by radioimmunoassay using Progesterone-Coatria and Estradiol-Coatria kits (Schering Co., Osaka, Japan), respectively. Steroid contents were negligible (progesterone < 0.1 ng/ml and estradiol < 8 pg/ml) in cell-free medium containing 1% FCS.

Measurement of cAMP level

To assess the BMP effects on cAMP synthesis, KGN cells (6×10^4 viable cells) were cultured in 96-well plates with DMEM/F12 containing 1% FCS and then indicated concentrations (ng/ml) of BMP2, 4, 6, and 7 in a combination

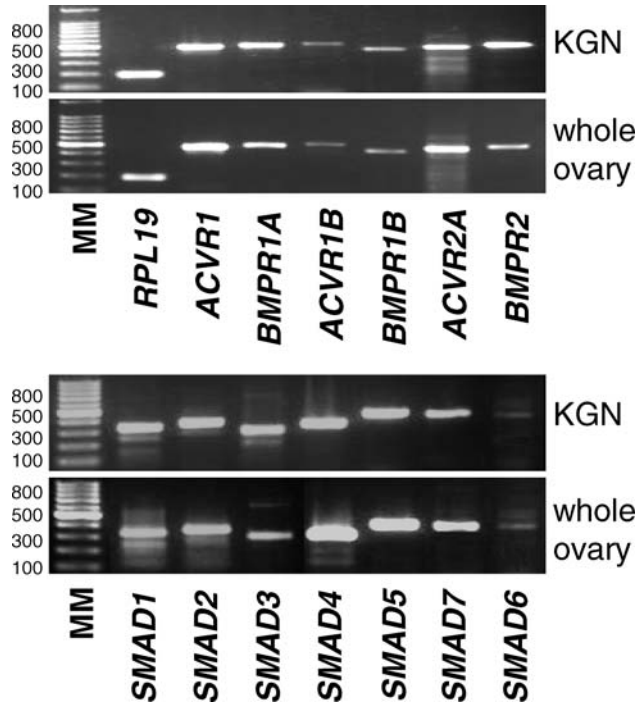

FIG. 1. Expression of BMP system in KGN cells. The expression of mRNAs encoding BMP type I and II receptors, SMADs and house-keeping gene L19 (*RPL19*) were examined in total cellular RNAs extracted from KGN cells and human whole ovary tissue by RT-PCR analysis. Aliquots of PCR products were electrophoresed on 1.5% agarose gel, visualized by ethidium bromide staining and shown as representative of those obtained from three independent experiments. MM indicates molecular weight marker.

with FSH (10 to 100 ng/ml) or FSK (10 μM) were added in the presence of 0.1 mM IBMX (specific inhibitor of phosphodiesterase activity). After 12-, 24- and 48-h culture the conditioned medium was collected and the extracellular content of cAMP was determined by cAMP enzyme immunoassay kit (Sigma-Aldrich Co. Ltd.) after acetylation of each sample to increase assay sensitivity (sensitivity = 0.039 nM).

Statistical analysis

All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance using ANOVA with Fisher protected least significant difference (PLSD) test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). P values < 0.05 were accepted as statistically significant.

RESULTS

We first attempted to characterize the presence of endogenous BMP system in KGN cells compared with human whole ovary by RT-PCR. As shown in Fig. 1, KGN cells expressed key molecules for the BMP signaling, including BMP type I receptors, type II receptors, and SMAD signaling molecules. As for the type I receptors, the expression of *ACVR1* (also known as *ALK2*), *BMPR1A* (*ALK3*), *ACVR1B* (*ALK4*), and *BMPR1B* (*ALK6*) was clearly detected. The expression of BMP type II receptors including *ACVR2A* (also known as *ActRII*) and *BMPR2* (*BMPRII*) was also shown. Regarding SMAD signaling molecules, the expression of *SMAD1, 2, 3, 4* and *5* was demonstrated by RT-PCR. As for inhibitory SMADs, *SMAD7* expression was evident while the expression of *SMAD6* mRNA was marginally detected.

Since the KGN cell line possesses functional FSHR on the cell surface as earlier reported [11], we examined the effects of

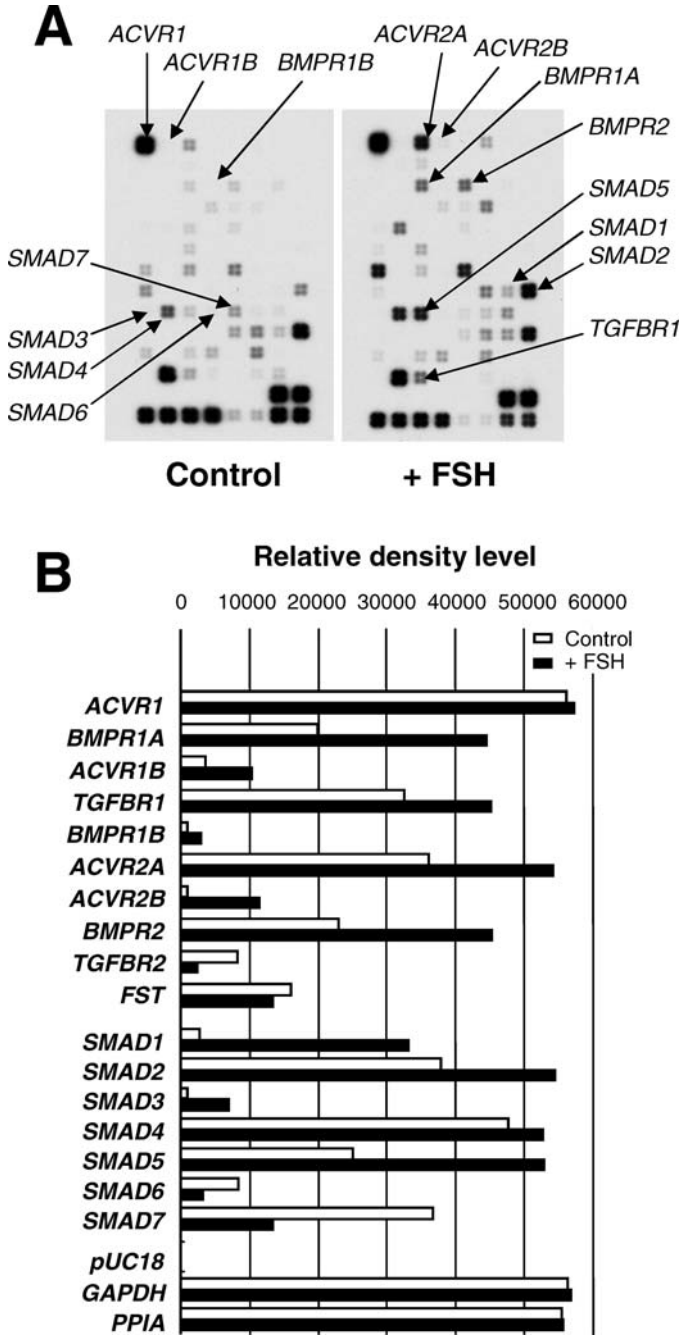

FIG. 2. FSH effects on the expression of BMP system in KGN cells. **A**) Total cellular RNAs (2.5 μg) were extracted from KGN cells cultured for 24 h in the absence (Control) or presence of FSH (10 ng/ml; + FSH). The RNAs were used as templates to generate Biotin-16-dUTP-labeled cDNA probes for GEArray membranes (SuperArray Bioscience Corp.) that include human TGFβ and BMP signaling molecules. The cDNA probes were denatured and hybridized with the cDNA array membranes and then the membranes were washed and exposed to X-ray films using chemiluminescent substrate. **B**) The spots on the X-ray films were scanned, digitized and the signal intensities were numerically converted. The signal intensities of the spots on the membranes obtained from two separate experiments were analyzed using the GEArray analyzer program (SuperArray Bioscience Corp.) after subtraction of the background levels of pUC18 DNA. The averages of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and cyclophilin A (*PPIA*) were used as positive controls to compare the membranes.

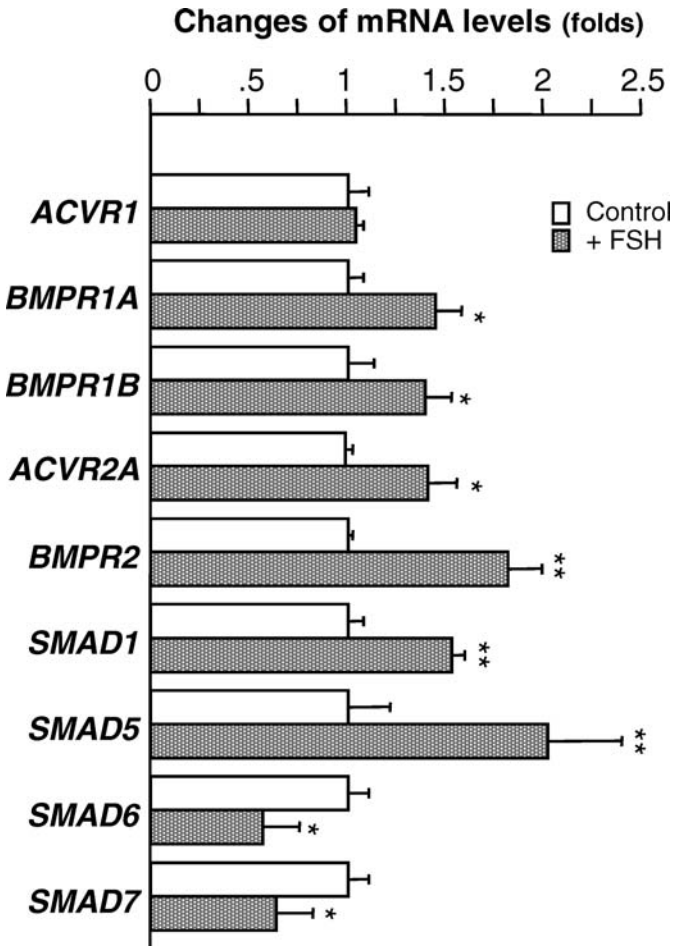

FIG. 3. Changes in mRNA levels of key BMP molecules by FSH. KGN cells (3×10^5 viable cells) were cultured in DMEM/F12 containing 1% FCS in 12-well plates. The cells were cultured for 24 h in the absence (Control) or presence of FSH (10 ng/ml; + FSH). Total cellular RNAs were extracted and subjected to RT reaction. Levels of steady-state mRNAs of key BMP molecules were analyzed by quantitative real-time PCR and standardized by level of *RPL19* in each sample. Changes of the mRNA levels by FSH treatment were graphed. Results show the mean ± SEM of data performed with triplicate treatments; $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. control.

FSH on the expression of various components of the BMP system using a cDNA array which includes the genes for human TGFβ and BMP signaling pathway. As seen in Fig. 2A, FSH (10 ng/ml) had pronounced effects on the modification of the expression pattern of BMP-related genes. Based on quantitative analysis (Fig. 2B), FSH upregulated *BMPR1A* in addition to the increase in type II receptors of *ACVR2A* and *BMPR2*. FSH also increased expression levels of *SMAD1* and *SMAD5* while inhibitory SMADs expression including *SMAD6* and *SMAD7* was instead reduced. Changes in the expression levels of key BMP molecules were further quantified by real-time PCR analysis, showing that FSH significantly increased mRNA levels of *BMPR1A*, *BMPR1B*, *ACVR2A*, *BMPR2*, *SMAD1*, and *SMAD5* while it decreased expression of *SMAD6* and *SMAD7* (Fig. 3).

We next investigated the activation of BMP signaling in KGN cells (Fig. 4). Western immunoblotting analysis demonstrated that 100 ng/ml of BMP2, 4, 6, and 7 induces SMAD1/5/8 phosphorylation, although the effects of BMP6 and BMP7 were less potent than those of BMP2 and BMP4 (Fig. 4A). Notably, FSH treatment (10 ng/ml) significantly enhanced

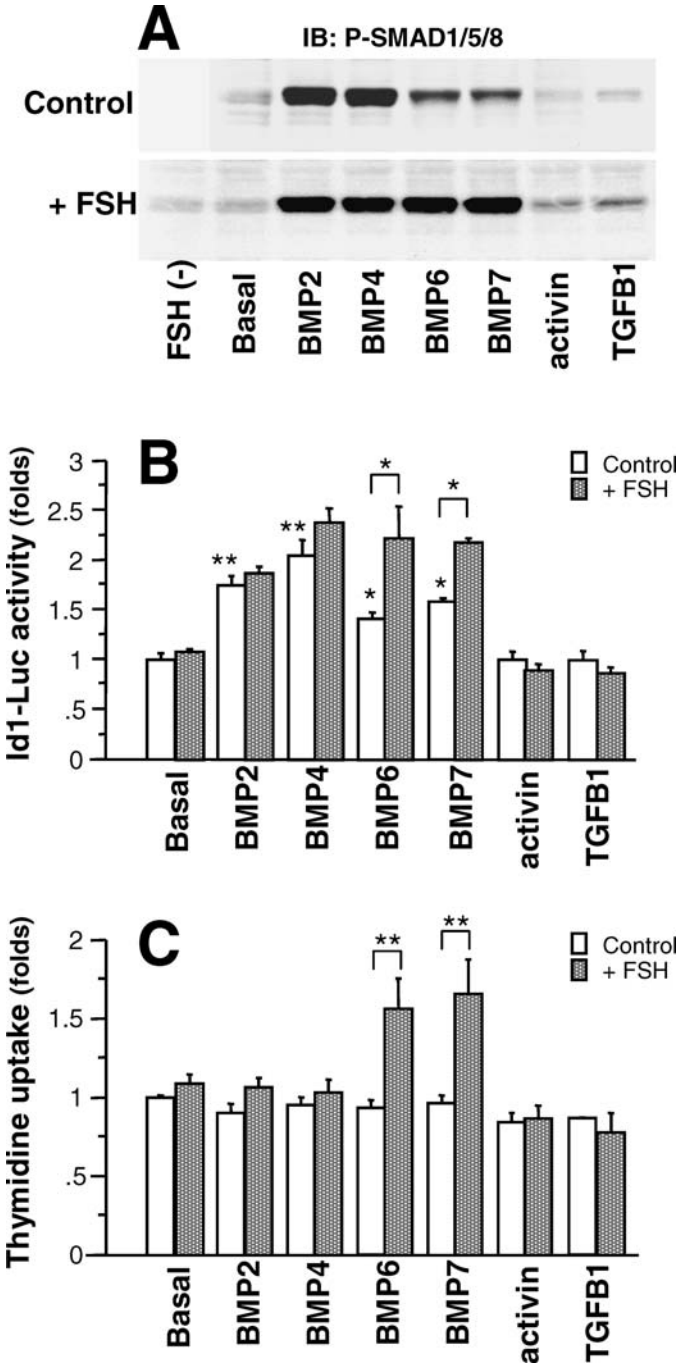

FIG. 4. FSH effects on BMP signaling in KGN cells. **A**) SMAD1/5/8 phosphorylation: KGN cells (3×10^5 viable cells) were cultured in 12-well plates in DMEM/F12 containing 1% FCS. After 24-h preculture, BMP2, 4, 6, 7, activin A, and TGFB1 (100 ng/ml) were added to the culture media in the absence (Control) or presence (+ FSH) of FSH (10 ng/ml). Following 1-h stimulation with growth factors, cells lysates were obtained and then subjected to SDS-PAGE immunoblotting (IB) analysis using anti-phospho-SMAD1/5/8 anitibody (P-SMAD1/5/8). **B**) FSH effects on Id1-Luc activities: Cells were transiently transfected with Id1-Luc reporter plasmid and cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal). The cells were then treated with BMP2, 4, 6, 7, activin A, and TGFB1 (100 ng/ml) in the absence (Control) or presence of FSH (10 ng/ml; + FSH) for 24 h. The cells were washed with PBS, lysed, and the luciferase activity and β-galactosidase (βgal) activity were measured by luminometer. Results were shown as the ratio of luciferase to βgal activity and graphed as mean ± SEM of data performed with triplicate treatments; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. basal levels or between the indicated groups. **C**) BMP effects on DNA synthesis by KGN cells: Cells were precultured in 12-well plate (1.5×10^5 viable cells) and then BMP2, 4, 6, 7, activin A and TGFB1 (100 ng/

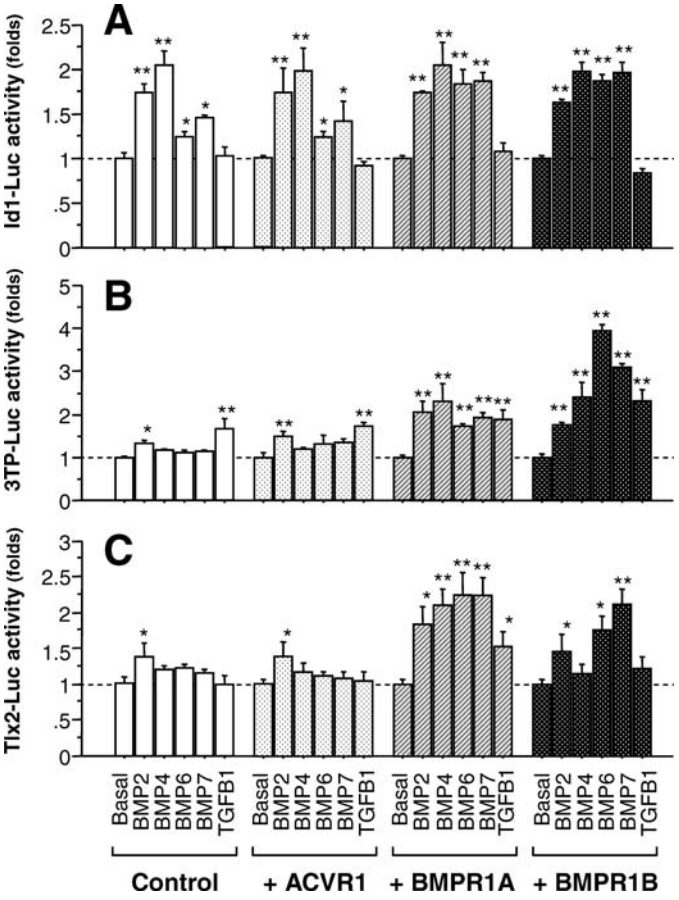

FIG. 5. BMP signaling in KGN cells overexpressing BMP type I receptors. KGN cells (2×10^5 viable cells) were precultured in 12-well plates and then transiently transfected with 500 ng of Id1-Luc (**A**), 3TP-Luc (**B**), or Tlx2-Luc (**C**) and 50 ng of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) with co-transfection of plasmids encoding wild-type constructs of ACVR1, BMPR1A or BMPR1B (500 ng). The cells were treated with BMP2, 4, 6, 7 and TGFB1 (100 ng/ml) for 24 h. The cells were washed with PBS, lysed and the luciferase activity and β-galactosidase (βgal) activity were measured by luminometer. Results were shown as the ratio of luciferase to βgal activity and graphed as mean ± SEM of data performed with triplicate treatments; $^*P < 0.05$ and $^{**}P < 0.01$ vs. basal levels of each group.

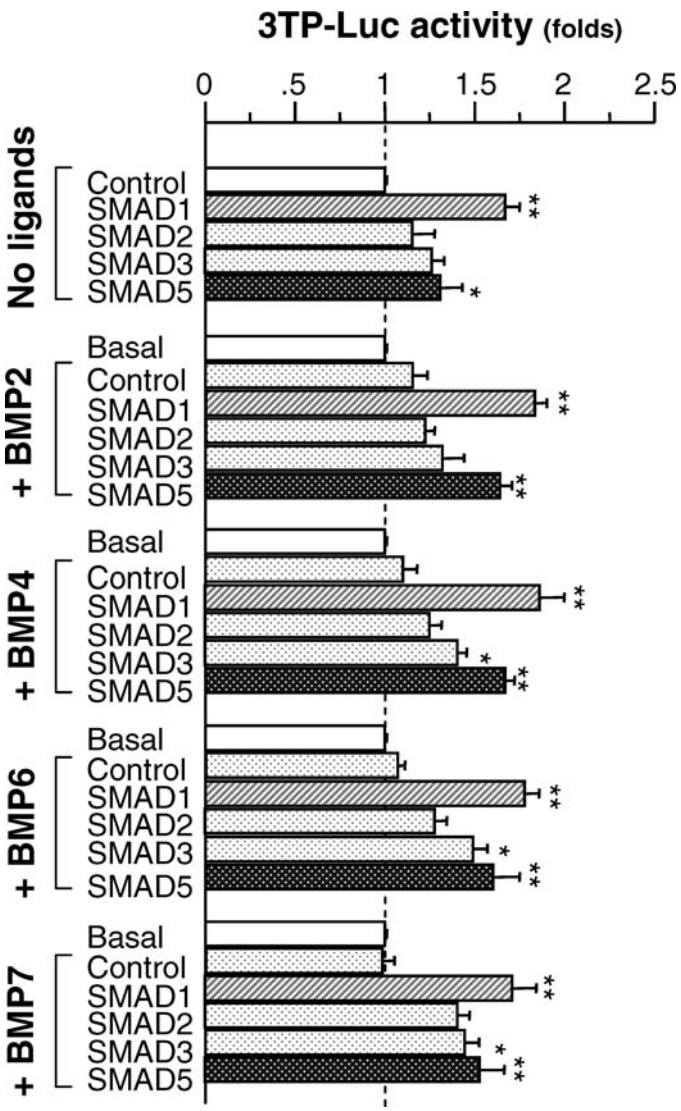

FIG. 6. BMP signaling in KGN cells overexpressing SMADs. KGN cells (2×10^5 viable cells) were precultured in 12-well plates and then transiently transfected with 500 ng of 3TP-Luc and 50 ng of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) with co-transfection of plasmids encoding wild-type constructs of SMAD1, SMAD2, SMAD3 or SMAD5 (500 ng). The cells were treated with BMP2, 4, 6, and 7 (100 ng/ml) for 24 h. The cells were washed with PBS, lysed and the luciferase activity and β-galactosidase (βgal) activity were measured by luminometer. Results were shown as the ratio of luciferase to βgal activity and graphed as mean ± SEM of data performed with triplicate treatments; $^*P < 0.05$ and $^{**}P < 0.01$ vs. control or basal levels.

SMAD1/5/8 phosphorylation stimulated by BMP6 and BMP7. FSH alone had no effect on SMAD1/5/8 phosphorylation (Fig. 4A). The FSH effects on the BMP signaling induced by BMP6 and BMP7 were confirmed by the BMP-specific reporter gene analysis using Id1-Luc, which contains the promoter region of a direct BMP target gene *ID1*, a dominant negative inhibitor of basic helix-loop-helix proteins [17]. As shown in Fig 4B, Id1-Luc activities induced by BMP6 and BMP7 were specifically augmented in the presence of FSH. Furthermore, thymidine uptake analysis showed that the FSH augmentation in the BMP signaling induced by BMP6 and BMP7 is functionally linked to the mitotic activity of KGN cells (Fig. 4C). Namely, the cellular DNA synthesis induced by BMP6 and BMP7 was selectively amplified in the presence of FSH through enhanced BMP-SMAD signal activation.

To investigate the molecular mechanism involved in the ligand-dependent control shown in Fig. 4, each wild-type BMP type I receptor (ACVR1, BMPR1A, and BMPR1B) was transfected to KGN cells (Fig. 5). The BMP signaling was evaluated by reporter gene analysis using Id1-Luc, 3TP-Luc, and Tlx2-Luc. BMP/TGFβ reporter gene analysis using 3TP-Luc, which includes three repeated TGFβ-responsive elements (TRE) and the plasminogen activator inhibitor-1 (PAI-1) promoter sequence [18], was also performed. 3TP-Luc can work for the responses to activin/TGFβ-SMAD2/3 and weakly to BMP-SMAD1/5/8 as well [19, 20]. *TLX2* is a homeobox gene expressed in the primitive streak of mouse embryos and

ml) were added in the absence (Control) or presence of FSH (10 ng/ml; + FSH). After 24-h cell culture, 0.5 μCi/well of [methyl-^{3}H] thymidine was added and incubated for 3 h at 37°C. The cells were then washed, precipitated by trichloroacetic acid and solubulized in NaOH and its radioactivity was counted. Results show the mean ± SEM of data performed with triplicate incubations; $^{**}P < 0.01$ between the indicated groups.

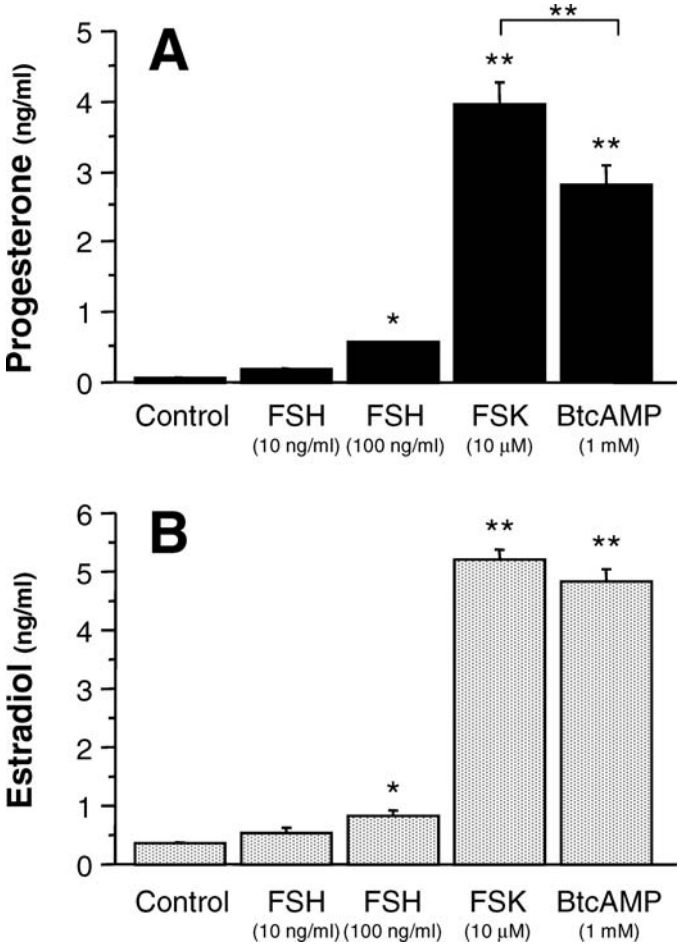

FIG. 7. Hormone-induced steroidogenesis in KGN cells. KGN cells (6×10^4 viable cells) were precultured in 96-well plates and then changed to fresh DMEM/F12 medium containing 100 nM of androstenedione, a substrate for P450arom and then FSH (10 to 100 ng/ml), FSK (10 μM) or BtcAMP (1 mM) was added. After 48-h culture, the supernatant of culture media was collected and the levels of progesterone (**A**) and estradiol (**B**) in the media were measured by radioimmunoassay. Results show the mean ± SEM of data performed with triplicate treatments; $*P < 0.05$ and $**P < 0.01$ vs. control or between the indicated groups.

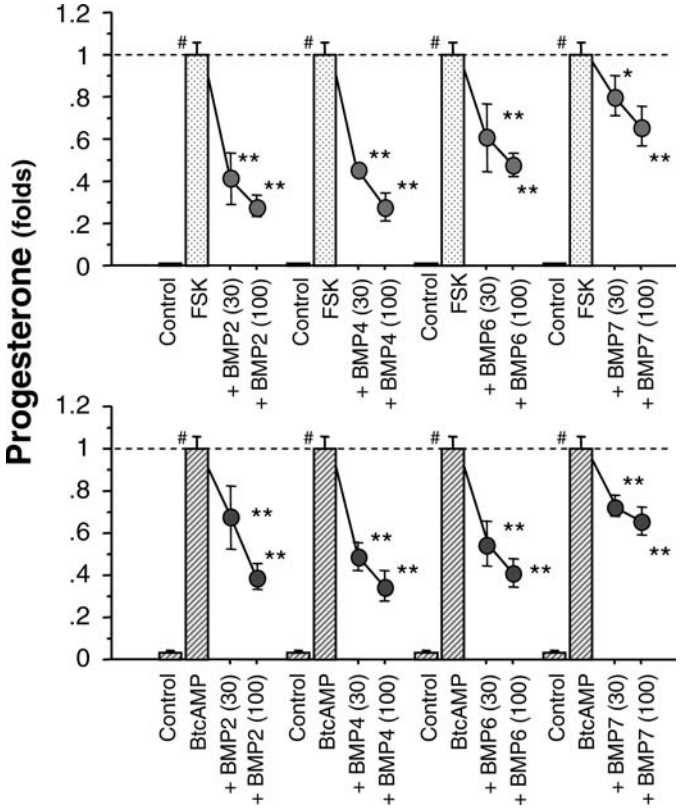

FIG. 8. BMP effects on progesterone production in KGN cells. Cells were cultured as mentioned in the legend of Fig. 7. BMP2, 4, 6, and 7 (30 to 100 ng/ml) were added to the culture medium in combination with FSK (10 μM) or BtcAMP (1 mM). After 48-h culture, the supernatant of culture media was collected and the levels of progesterone in the media were measured by radioimmunoassay. Results show the mean ± SEM of data performed with triplicate treatments; $\#P < 0.01$ vs. control; and $*P < 0.05$ and $**P < 0.01$ vs. FSK or BtcAMP groups.

found to be a downstream target gene for BMP signaling, which is specifically responsive to the BMP-SMAD1/5/8 pathway but unresponsive to TGFβ and activin signaling [21]. BMP2 and BMP4 stimulated Id1-Luc activation more potently than BMP6 and BMP7. Overexpression of wild-type BMPR1A or BMPR1B significantly enhanced Id1-Luc responses to BMP6 and BMP7, although ACVR1 was not effective in inducing this effect (Fig. 5A). BMP2 and TGFB1 showed marginal activation of 3TP-Luc (Fig. 5B) and BMP2 activated Tlx2-Luc (Fig. 5C) in control KGN cells, whereas BMP6 and BMP7 failed to activate these responses. Notably, BMP6 and BMP7 increased 3TP-Luc activity in the KGN cells overexpressing BMPR1A and in particular BMPR1B, but not ACVR1. In addition, BMP6 and BMP7 also potently enhanced Tlx2-Luc activity in the KGN cells overexpressing BMPR1A and BMPR1B (Fig. 5C). Thus, ligand-specific activation of the BMP signaling can be caused by the upregulation of BMPR1A or BMPR1B. Given that BMPR1B is much less abundant compared to BMPR1A in KGN cells regardless of the presence of FSH (Fig. 1 and 2), BMPR1A could be a crucial type I receptor for activation of BMP signaling stimulated by BMP6 and BMP7 in FSH-induced KGN cells.

To further investigate the molecular mechanism by which FSH augments BMP-specific activities in KGN cells, the roles of SMADs (SMAD1, 2, 3 and 5) in BMP signal activation were studied using 3TP-Luc, which can respond to all of SMAD1, 2, 3 and 5. Among the cells transfected with wild-type SMADs, the cells overexpressing SMAD1 and SMAD5 significantly increased 3TP-Luc activities (Fig. 6), suggesting that FSH-induced increases in SMAD1/5 expression lead to enhancement of the BMP signaling by KGN cells. Furthermore, the SMAD-induced effects were not significantly influenced by exogenously added BMP ligands. Taken together with the results on overexpression of BMP type I receptors, BMPR1A and BMPR1B are limiting factors in KGN cells and critical for the signaling of BMP6 and BMP7.

To research the effects of BMPs on the FSHR-cAMP signaling pathway, BMP actions on steroidogenesis by KGN cells were investigated. FSH effects on progesterone (Fig. 7A) and estradiol (Fig. 7B) synthesis were negligible compared to the effects induced by FSK or BtcAMP. Progesterone synthesis induced by FSK or BtcAMP was dose-dependently suppressed by BMP2, 4, 6, and 7 (Fig. 8), whereas BMPs had no effects on estradiol production induced by either FSK or BtcAMP (Fig. 9). The second messenger cAMP levels were moderately increased by a high concentration (100 ng/ml) of FSH and markedly stimulated by FSK (10 μM) during 48-h culture with a saturated effect at 24-h incubation (Fig. 10A). Notably, the

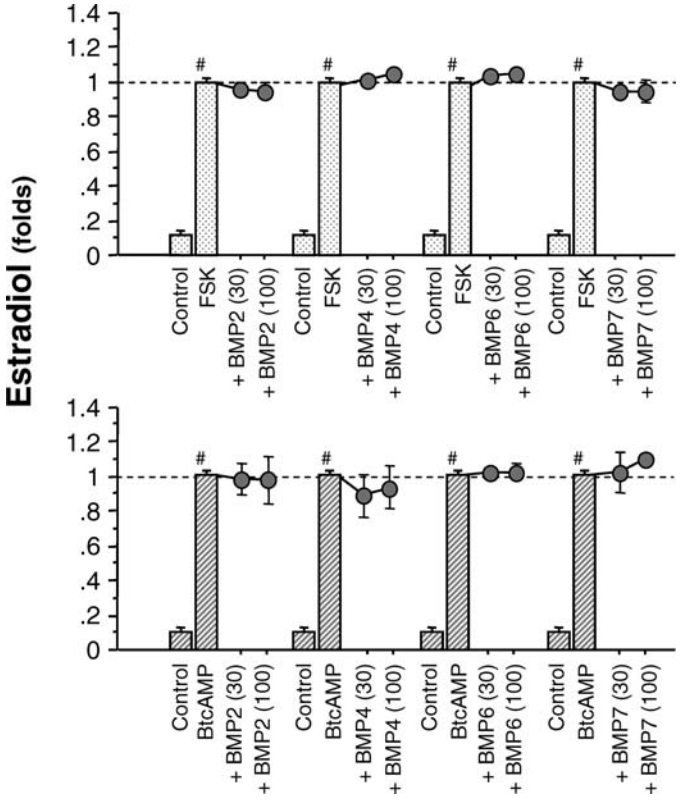

FIG. 9. BMP effects on estradiol production in KGN cells. Cells were cultured as mentioned in the legend of Fig. 7. BMP2, 4, 6, and 7 (30 to 100 ng/ml) were added to the culture medium in combination with FSK (10 μM) or BtcAMP (1 mM). After 48-h culture, the supernatant of culture media was collected and the levels of estradiol in the media were measured by radioimmunoassay. Results show the mean ± SEM of data performed with triplicate treatments; #$P < 0.01$ vs. control; and *$P < 0.05$ and **$P < 0.01$ vs. FSK or BtcAMP groups.

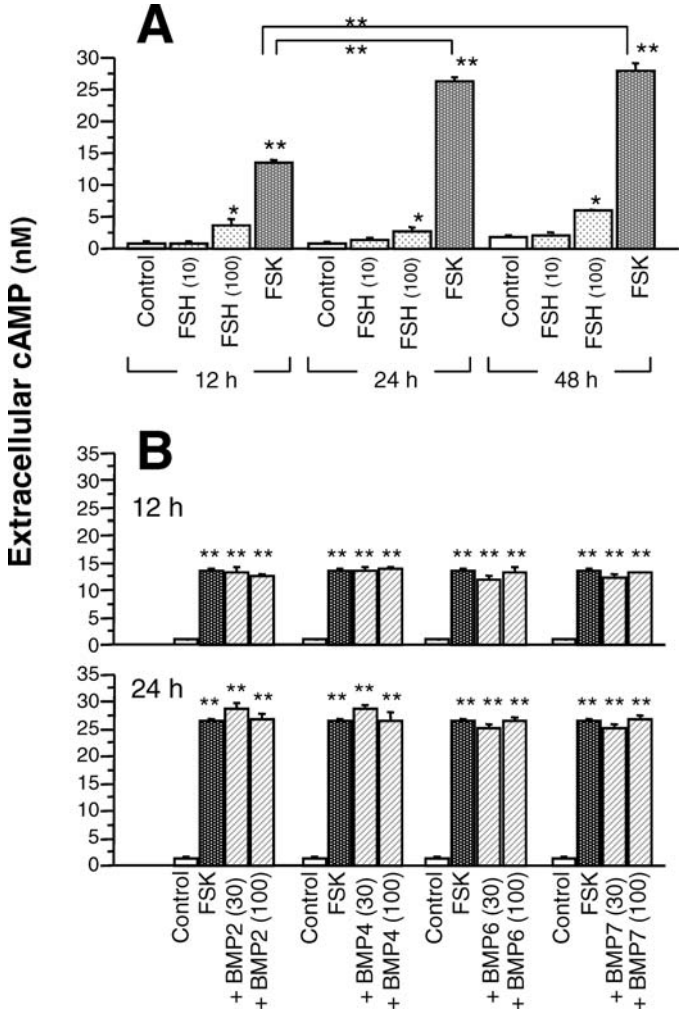

FIG. 10. BMP effects on cAMP production by KGN cells. KGN cells (6×10^4 viable cells) were cultured in 96-well plates with DMEM/F12 containing 1% FCS and 0.1 mM of IBMX (specific inhibitor of phosphodiesterase activity). **A**) Low (10 ng/ml) and high concentrations (100 ng/ml) of FSH and FSK (10 μM) were added to culture medium and the conditioned medium was collected after 12-, 24- and 48-h culture. **B**) Indicated concentrations (ng/ml) of BMP2, 4, 6, and 7 in a combination with FSK (10 μM) were added to culture medium and the conditioned medium was collected after 12- and 24-h culture. The extracellular content of cAMP in the collected conditioned medium was determined by enzymeimmunoassay after acetylation of each sample. Results show the mean ± SEM of data performed with triplicate treatments; *$P < 0.05$ and **$P < 0.01$ vs. control or between the indicated groups.

cAMP levels for 12 to 24 h in the presence of FSK were not altered by BMP treatment (Fig. 10B). Moreover, changes of mRNA encoding *STAR* and steroidogenic enzymes including *CYP11A*, *HSD3B2*, and *CYP19* were examined by quantitative real-time PCR, showing that BMPs specifically reduced *STAR* mRNA levels stimulated by FSK as well as BtcAMP while the levels of *CYP11A*, *HSD3B2*, and *CYP19* were not altered by addition of BMP (Fig. 11).

BMP regulation of *STAR* transcription was further evaluated by steady-state *STAR* mRNA levels and *STAR*-promoter activity using reporter gene analysis of the 5'-promoter region (−235 bp) of the *STAR* gene, showing that BMP2, 4, 6, and 7 directly inhibit *STAR* transcription in KGN cells (Fig. 12A). In addition, the changes in *FSHR* expression were examined by quantitative real-time PCR analysis. As shown in Fig. 12B, steady-state *FSHR* mRNA levels were significantly reduced by 24-h treatment with BMP2, 4, 6, and 7 (100 ng/ml) in contrast to the effects of FSK (10 μM). These results suggest that BMPs regulate steroidogensis at the levels downstream of cAMP synthesis as well as at the level of FSHR.

DISCUSSION

The receptors for TGFβ superfamily members consist of type I and type II receptors, both of which exhibit serine/threonine kinase activity [3]. ACVR1, BMPR1A, and BMPR1B have been identified as type I receptors for BMPs while ACVR1B is the type I receptor for activin. BMP ligands act together to form a high-affinity complex with BMP type II receptors and the appropriate type I receptor [22]. The combinations of BMP ligands and receptors are not completely definitive, in which BMP2 and BMP4 preferentially bind to BMPR1A and/or BMPR1B [23], BMP6 and BMP7 can readily bind to ACVR1 and/or BMPR1B [24–26], and BMP15 binds to BMPR1B with much lower affinity to BMPR1A [27]. In the signaling pathway of BMPs, the phosphorylated type I receptors interact with and phosphorylate SMAD1, 5 and 8 intracellular signaling molecules [28]. Once phosphorylated (activated) by type I receptors, SMAD1, 5 and 8 interact with a common-mediator SMAD4, and then translocate to the nucleus. SMAD6 and 7 are "inhibitory SMADs" and can prevent the phosphorylation and signaling activity of pathway-restricted SMADs (SMAD1, 5 and 8) by binding to the type I receptors [29].

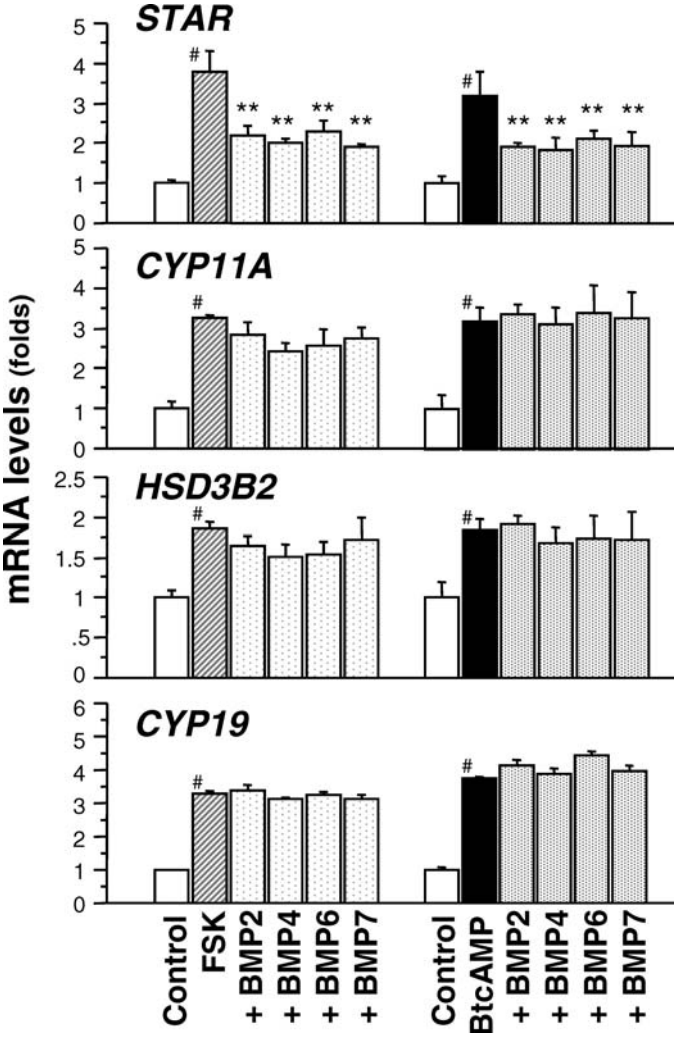

FIG. 11. BMP effects on key gene expression for steroidogenesis. KGN cells (3×10^5 viable cells) were cultured in DMEM/F12 containing 1% FCS in 12-well plates. The cells were treated with BMP2, 4, 6, and 7 (100 ng/ml) in the presence of FSK (10 μM) or BtcAMP (1 mM) for 24 h. Total cellular RNAs were extracted and subjected to RT reaction. Levels of steady-state mRNAs of *STAR, CYP11A, HSD3B2* and *CYP19* were analyzed by quantitative real-time PCR and standardized by level of *RPL19* in each sample. Results show the mean ± SEM of data performed with triplicate treatments; #$P < 0.01$ vs. control; and *$P < 0.05$ and **$P < 0.01$ vs. FSK or BtcAMP groups.

In the present study, the existence and functional role of the BMP system were investigated in the human granulosa-like tumor cell line KGN. Analysis using RT-PCR and cDNA arrays revealed that KGN cells express key components for the BMP system including BMP type I (ACVR1, BMPR1A, and BMPR1B) and type II (ACVR2A and BMPR2) receptors and its signaling molecules of SMAD1/5 and SMAD4. Functional experiments of the present study revealed a novel role of FSH action on BMP signaling in KGN cells. FSH preferentially upregulated the expression of type I receptors (BMPR1A and BMPR1B) as well as type II receptors (ACVR2A and BMPR2). In addition, FSH increased expression levels of SMAD1/5 while inhibitory SMADs expression such as SMAD6/7 was instead reduced. Thus, it is most likely that FSH augments the bioavailability of BMP signaling in KGN cells (Fig. 13).

Bioassays further revealed that FSH significantly enhanced SMAD1/5/8 phosphorylation, 3TP-Luc activity, and cellular

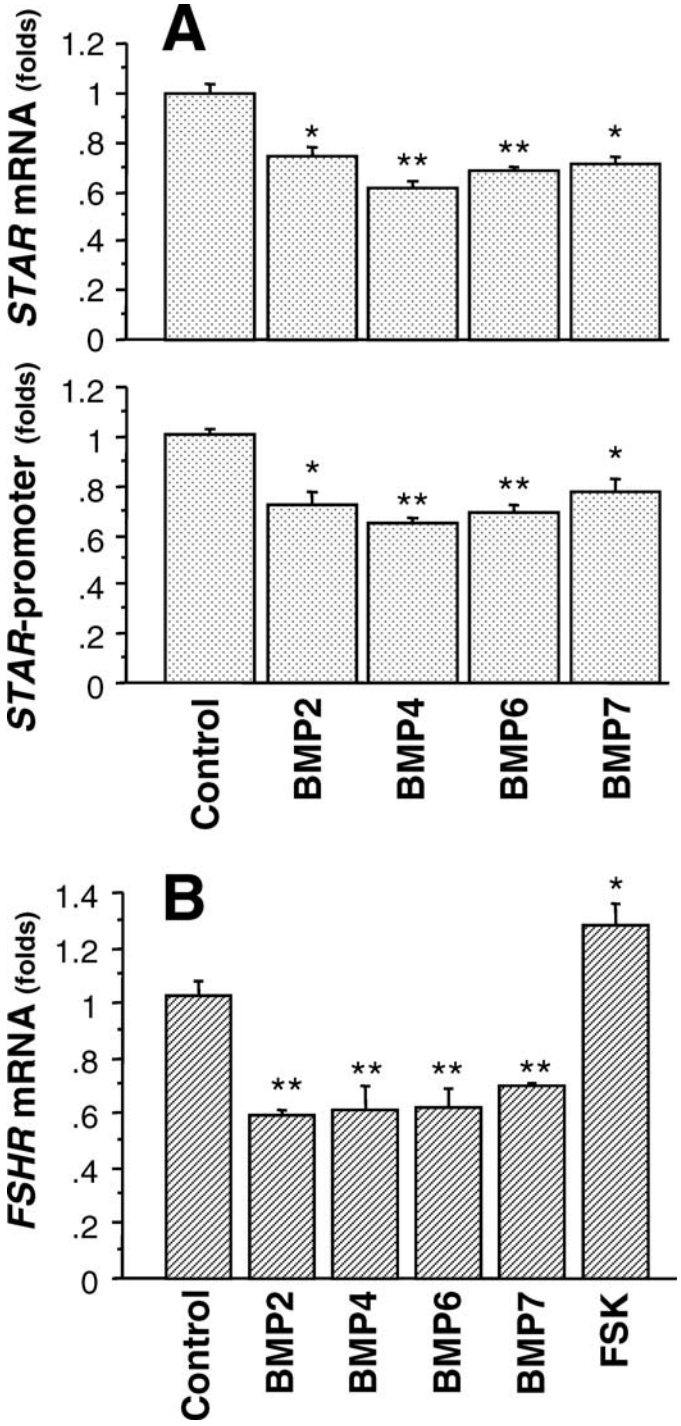

FIG. 12. BMP effects on *STAR* transcription and *FSHR* expression. KGN cells (3×10^5 viable cells) were cultured in DMEM/F12 containing 1% FCS in 12-well plates. After preculture, the cells were treated with or without BMP2, 4, 6, 7 (100 ng/ml) and FSK (10 μM) for 24 h. Total cellular RNA was extracted and subjected to RT reaction. For the quantification of *STAR* (**A**; upper panel) and *FSHR* (**B**) mRNA levels, real-time PCR analysis was performed. The expression levels of target genes were standardized by *RPL19* level in each sample. Results show the mean ± SEM of data performed with triplicate treatments; *$P < 0.05$ and **$P < 0.01$ vs. control. For the *STAR*-promoter assay (**A**; lower panel), KGN cells (2×10^5 viable cells) were cultured in 12-well plates in DMEM/F12 culture medium. The cells were then transiently transfected with 500 ng of each luciferase reporter plasmid (pGL2-*StAR-235*), 50 ng of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal). The cells were treated with BMP2, 4, 6, and 7 (100 ng/ml) for 24 h. The cells were washed with PBS, lysed and the luciferase activity and β-galactosidase (βgal) activity were measured by luminometer. Results were shown as the ratio of luciferase to βgal activity and graphed as mean ± SEM of data performed with triplicate treatments; *$P < 0.05$ and **$P < 0.01$ vs. control.

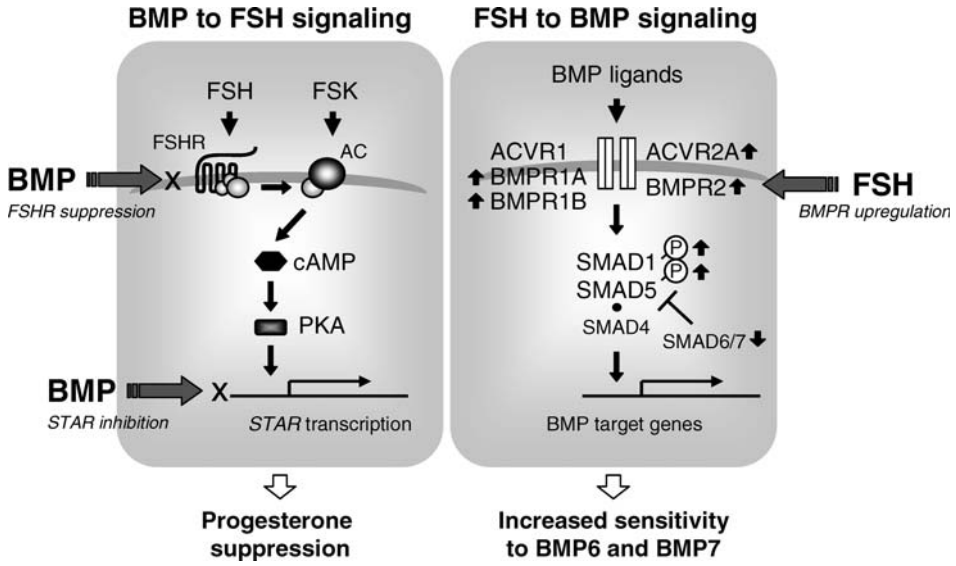

FIG. 13. Mutual regulation of the BMP system and FSH signaling in granulosa cells. FSH-cAMP pathway and BMP system are functionally linked, in which BMPs suppress progesterone production through inhibiting *STAR* transcription and *FSHR* expression: BMP to FSH signaling. On the other hand, FSH accelerates the endogenous BMP signaling by upregulating BMP receptors (BMPR): FSH to BMP signaling. AC, adenylate cyclase; PKA, protein kinase A; P in circles, phosphorylation.

DNA synthesis induced by BMP6 and BMP7 but not by BMP2 and BMP4. Based on the experiments using KGN cells overexpressing SMADs molecules, SMAD1 and SMAD5 were found to be ligand-nonspecific factors for BMP signal activation. Experiments using the KGN cells overexpressing BMP type I receptors support the idea that BMPR1A and BMPR1B play key roles in amplifying the BMP signaling induced particularly by BMP6 and BMP7 in KGN cells. Since BMPR1B expression in KGN cells is negligible, BMPR1A seems likely to be a critical type I receptor for ligand-dependent activation of BMP signaling in the presence of FSH.

In KGN cells, FSH effects on progesterone and estradiol synthesis were less potent than were the effects of cAMP donors such as FSK and BtcAMP. This could be due to much lower expression of functional FSHR and/or weaker FSHR signaling activation compared with primary granulosa cells. Activation of other pathways such as mitogen-activated protein kinase (MAPK) [30] via the cAMP-PKA system could also be involved in the potent induction of steroidogenesis by FSK and BtcAMP. It is of note that BMP2, 4, 6, and 7 selectively suppressed progesterone production stimulated by cAMP donors including FSK and BtcAMP, whereas BMPs had no effect on the corresponding estradiol production. In the actions of BMP6 and BMP7 on progesterone suppression induced by cAMP donors, upregulation of BMP receptors through cAMP-PKA signaling is possibly involved. As a major second messenger of FSHR signaling, cAMP levels activated by FSK were not affected by treatment of BMPs, suggesting that BMPs possibly regulate steroidogenesis at the sites downstream of cAMP synthesis in KGN cells. In this regard, the reduction of steady-state mRNA levels of steroidogenic enzymes and *STAR*-promoter assay revealed that the suppression of progesterone synthesis is most likely to occur through direct inhibition of *STAR* transcription by KGN cells (Fig. 13).

This regulatory mechanism of BMPs on KGN steroidogenesis is apparently different from the results demonstrated in primary granulosa cells. BMP4 and BMP7 differentially regulate FSH-induced progesterone and estradiol production [4, 5], while BMP6 and BMP15 suppress FSH-induced progesterone synthesis but do not affect FSH-induced estradiol levels in primary culture studies [7, 31]. Interestingly, that BMPs are able to disturb progesterone induction was found even in KGN cells as shown in primary cultured granulosa cells. This may imply an important consensus that all BMPs including BMP2, 4, 6, and 7 play roles as luteinizing inhibitors. These characteristics may reflect the specificity of the KGN cell line derived from an ovarian granulosa cell carcinoma. Yet, it is proven that the endogenous BMP system is bioactive in KGN cells and certainly involved in controlling the FSH-cAMP pathway.

The formation of a dominant follicle is a stepwise process that includes granulosa cell proliferation and differentiation [32]. During the early follicular phases, follicle growth and development are controlled by autocrine/paracrine mechanisms in the ovary and proceed independently of FSH [33]. In the later process, FSHR signaling in the granulosa cells is required for follicular selection and dominant follicle formation. FSHR signaling is precisely modulated by autocrine/paracrine factors residing in the follicles [34] as well as estrogen produced in the follicles [35]. On the other hand, the expression of BMP system components undergoes dynamic changes during folliculogenesis with change of spatial and temporal expression patterns of these BMP genes [1]. Therefore, it is very reasonable that the presence of a functional link between the BMP system and the FSH-cAMP pathway supports effective development of growing follicles by amplifying mutual effects of BMP and FSH. An interesting report regarding the TGFβ system in immature mouse ovary showed that in vivo treatment with FSH and LH increases the expression of TGFβ system molecules including TGFBR1 (also known as ALK5), TGFBR2, and SMAD2 and 4, while it decreases SMAD6 expression [36]. This strongly supports our hypothesis regarding the presence of crosstalk between the endogenous BMP/TGFβ system and gonadotropin signaling. Further investigation is necessary to determine the physiological significance of this functional cooperation between BMP and FSH signaling.

Collectively, the two major factors, the FSH-cAMP pathway and the BMP system, are reciprocally linked, in that BMPs suppress progesterone production through inhibiting STAR and FSHR, and on the other hand, FSH accelerates the endogenous BMP signaling (Fig. 13). A newly uncovered interrelationship between BMP and FSH-cAMP signaling could be physiologically important for maintenance and development of granulosa cell proliferation and differentiation. Given that BMPs downregulate FSHR in KGN cells, this interaction may contribute to maintenance of granulosa cell

functions during follicle development by modulating the receptor sensitivity to BMP ligands and FSH.

ACKNOWLEDGMENTS

We thank Dr. R. Kelly Moore and Dr. Shunichi Shimasaki for helpful discussion and critical reading of the manuscript. We also thank Drs. Masatoshi Nomura and Hajime Nawata, Dr. Jeff Wrana, Dr. Jerome F. Strauss III, and Drs. Tetsuro Watabe, and Kohei Miyazono for providing KGN cells, the plasmid of Tlx2-Luc, the STAR promoter plasmid, and the expression plasmids for SMADs and BMP type I receptors, respectively.

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