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Differential Regulation of Steroidogenesis by Bone Morphogenetic Proteins in Granulosa Cells: Involvement of Extracellularly Regulated Kinase Signaling and Oocyte Actions in Follicle-Stimulating Hormone-Induced Estrogen Production

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In the present study, we investigated the cellular mechanism by which oocytes and bone morphogenetic proteins (BMPs) govern FSH-induced steroidogenesis using rat primary granulosa cells. BMP-6 and BMP-7 both inhibited FSH- and forskolin (FSK)-induced progesterone synthesis and reduced cAMP synthesis independent of the presence or absence of oocytes. BMP-7 also increased FSH-induced estradiol production, and the response was further augmented in the presence of oocytes. In contrast, BMP-6 had no impact on estradiol synthesis regardless of the presence of oocytes. Because BMP-7 changed neither FSK- nor cAMP-induced estradiol production, the BMP-7 action was mediated through a FSH receptor signaling mechanism that was independent of cAMP-protein kinase A pathway. Treatment with FSH but not cAMP activated ERK1/2 phosphorylation in granulosa cells, which was further accelerated by oocytes. A specific ERK inhibitor, U0126, increased estradiol production and decreased FSH- and FSK-induced progesterone production and cAMP synthesis. This suggests that ERK activation is directly linked to inhibition of estradiol synthesis and amplification of cAMP. Moreover, FSH-induced ERK1/2 phosphorylation was inhibited by BMP-7 but not influenced by BMP-6. In contrast, BMP signaling including Smad1/5/8 phosphorylation and Id-1 transcription was up-regulated by FSH and oocytes in granulosa cells through inhibition of Smad6/7 expression. Collectively, oocytes enhance FSH-induced MAPK activation and BMP signaling in granulosa cells, which leads to differential regulation of steroidogenesis elicited by BMPs in the presence of FSH in developing follicles. (*Endocrinology* 148: 337–345, 2007)

NORMAL FOLLICULAR DEVELOPMENT is controlled by gonadotropins and by ovarian autocrine/paracrine factors. Of these autocrine/paracrine factors, recent studies have established the concept that the bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, play a key role in female fertility in mammals by regulating steroidogenesis and mitogenesis in granulosa cells (1, 2).

The major regulatory process by BMPs in steroidogenesis is the control of FSH receptor signaling. Oocyte/granulosa-derived BMP-6 inhibits FSH-induced progesterone synthesis through suppression of cellular cAMP synthesis (3). Another key factor, BMP-15, which is specifically expressed by oocytes in the ovary, also suppresses FSH action by inhibiting FSH receptor expression (4). Likewise, growth differentiation factor-9 (GDF-9), which is oocyte-derived and shares the highest homology with BMP-15, also inhibits FSH-induced steroidogenesis and LH receptor expression in rat granulosa cells (5). In contrast to the *in vitro* actions of BMP-6 and BMP-15 on steroidogenesis, theca-derived BMP-4 and BMP-7 elicit stimulating effects on FSH-induced estradiol production in addition to a common BMP effect: luteinizing inhibition that suppresses FSH-induced progesterone production in rat primary granulosa cells (6, 7). Despite the recognition of the physiological importance of the BMP system in regulation of FSH action in the ovary, the mechanism of how the BMP effects are linked to oocyte function and FSH receptor signaling remains poorly understood. Recent studies have introduced the concept that communication networks between oocytes and somatic follicular cells are crucial not only for the oocyte maturation but also for the growth and differentiation of surrounding granulosa cells (8, 9). Vanderhyden and colleagues (10–12) demonstrated a key role for oocytes in enhancing estradiol and suppressing progesterone production induced by FSH using mouse granulosa cells. We have also discovered that oocytes are obligatory for estrogen-dependent augmentation of FSH action in rat granulosa cells (13). However, the detailed mechanism of oocyte-somatic cell interaction for steroidogenesis remains uncertain.

The experiments of the present study were designed to

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Abbreviations: ALK, Activin receptor-like kinase; ActRII, activin type II receptor; BMP, bone morphogenetic protein; BMPRII, BMP type II receptor; BtcAMP, N^6,O^2-dibutyryl adenosine-3′,5′-cyclic monophosphate monosodium salt; DES, diethylstilbestrol; FSK, forskolin; βgal, β-galactosidase; GDF-9, growth differentiation factor-9; IBMX, 3-isobutyl-1-methylxanthine; P450arom, P450 aromatase; RPL19, ribosomal protein L19; StAR, steroidogenic acute regulatory protein.

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elucidate the molecular mechanism of the integration of steroidogenesis performed through "oocyte-granulosa cell communication" using rat primary granulosa cells focusing on effects of BMP-6 and BMP-7.

Materials and Methods

Reagents and supplies

Female Sprague Dawley rats were purchased from Charles-River (Wilmington, MA). Medium 199, McCoy's 5A medium, and HEPES buffer solution were purchased from Invitrogen Corp. (Carlsbad, CA). Diethylstilbestrol (DES), ovine pituitary FSH, forskolin (FSK), N^6,O^2-dibutyryl adenosine-3′,5′-cyclic monophosphate monosodium salt (BtcAMP), 4-androstene-3,17-dione, 3-isobutyl-1-methylxanthine (IBMX), BSA, and penicillin-streptomycin solution were from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human BMP-6 and BMP-7 were purchased from R&D Systems (Minneapolis, MN), and U0126 was from Promega Co. (Madison, WI). Human ovary total RNA was purchased from Stratagene Inc. (La Jolla, CA). Plasmids of Id-1-Luc were kindly provided from Drs. Tetsuro Watabe and Kohei Miyazono (Tokyo University, Tokyo, Japan).

Rat primary granulosa cell culture and coculture with oocytes

Female 22-d-old Sprague Dawley rats were implanted with silastic capsules containing 10 mg of DES to increase granulosa cell number. After 4 d of DES exposure, the ovarian follicles were punctured with a 28-gauge needle, and the isolated mixture of granulosa cells and oocytes was cultured for 48 h in serum-free McCoy's 5A medium supplemented with penicillin-streptomycin at 37 C in an atmosphere of 5% CO_2. Granulosa cell and oocyte numbers were counted in the oocyte/granulosa cell suspension which was filtered by cell strainers (100-μm nylon mesh; BD Falcon, Bedford, MA) to eliminate cell aggregation. For indicated experiments, granulosa cells were separated from oocytes by filtering the oocyte/granulosa cell suspension through additional 40-μm nylon mesh (BD Falcon) that allowed granulosa cells but not oocytes to pass through (14). The purified granulosa cells were cultured in serum-free McCoy's 5A medium as described above. The animal protocols were approved by Okayama University Institutional Animal Care and Use Committee.

Steroid and cAMP measurements

Rat granulosa cells (1×10^5 viable cells in 200 μl) with or without oocytes (100 oocytes/ml) were cultured in 96-well plates with serum-free McCoy's 5A medium containing 100 nM of androstenedione [a substrate for P450 aromatase (P450arom)]. FSH (10–100 ng/ml), FSK (10 μM), and BtcAMP (1 mM) were added to the culture medium either alone or with a combination of BMPs (10–100 ng/ml) and U0126 (1–3 μM). After a 48-h culture, the culture media was collected and stored at −80 C until assay. The levels of progesterone and estradiol in the media were determined by RIA (Schering Co., Osaka, Japan). Steroid contents were undetectable (progesterone $<$ 0.1 ng/ml and estradiol $<$ 8 pg/ml) in cell-free medium. To assess cellular cAMP synthesis, rat granulosa cells (1×10^5 viable cells in 200 μl) with or without oocytes (100 oocytes/ml) were cultured in 96-well plates with serum-free McCoy's 5A medium containing 0.1 mM of IBMX (specific inhibitor of phosphodiesterase activity). After 48-h culture with indicated treatments, the conditioned medium was collected and stored at −80 C until assay. The extracellular contents of cAMP were determined by enzyme immunoassay (Sigma-Aldrich Co. Ltd.) after the acetylation of each sample with assay sensitivity of 0.039 nM.

RNA extraction, reverse transcription, and quantitative real-time PCR

Rat granulosa cells (5×10^5 viable cells in 1 ml) with or without oocytes (100 oocytes/ml) were cultured in 12-well plate with serum-free McCoy's 5A medium. FSH (30 ng/ml), FSK (10 μM), and BtcAMP (1 mM) were added to the culture medium either alone or with a combination of BMP-6 and BMP-7 (100 ng/ml). After a 48-h culture, the medium was removed and total cellular RNA was extracted using TRIzol (Invitrogen Corp.). Total RNA was quantified by measuring the absorbance of the sample at 260 nm, and stored at −80 C until assay. Oligonucleotides used for RT-PCR were custom-ordered from Kurabo Biomedical Co. (Osaka, Japan). PCR primer pairs were selected from different exons of the corresponding genes as follows: P450arom, 1180–1200 and 1461–1481 (from GenBank accession #M33986); steroidogenic acute regulatory protein (StAR), 395–415 and 703–723 (AB001349); activin receptor-like kinase (ALK)-2, 599–621 and 893–915 (Z22534); ALK-3, 426–445 and 916–935 (NM_009758); ALK-6, 792–813 and 1034–1053 (NM_007560); activin type II receptor (ActRII), 611–630 and 1083–1102 (NM_007396); BMP type II receptor (BMPRII), 13–32 and 515–534 (NM_007561); Smad1, 722–743 and 1037–1058 (U59912); Smad4, 526–546 and 853–874 (NM_005359); Smad5, 806–828 and 1232–1253 (NM_005903); Smad6, 1717–1737 and 1861–1881 (NM_005585); Smad7, 763–783 and 1179–1199 (NM_005904); Smad8, 420–440 and 687–707 (AF012347); and ribosomal protein L19 (RPL19), 401–421 and 575–595 (J02650). The extracted RNA (1 μg) was subjected to a reverse transcription reaction using First-Strand cDNA Synthesis System (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (0.5 mM) at 42 C for 50 min, 70 C for 10 min. For the quantification of each mRNA expression, real-time PCR was performed using LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic Co., Tokyo, Japan) under the condition of annealing at 60–62 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 Co.), and then the expression levels of target genes were standardized by RPL19 level in each sample.

Immunoblot analysis of phosphorylated proteins

Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured with or without oocytes (100 oocytes/ml) in 12-well plates in serum-free McCoy's 5A medium. After a 3-h preculture, cells were treated with FSH (30–100 ng/ml) or BtcAMP (1 mM) for 1 h in the presence or absence of U0126 (3 μM). In indicated experiments, cells were treated with FSH (30–100 ng/ml) either alone or combination of BMP-6 and BMP-7 (100 ng/ml) for 1 h. Cells were then 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 using anti-phospho- and anti-total-ERK1/2 MAPK antibodies (Cell Signaling Technology, Inc., Beverly, MA), and anti-phospho-Smad1/5/8 antibody (Cell Signaling Technology, Inc.). The relative integrated density of each protein band was digitized by NIH image J 1.34s.

Transient transfection and luciferase assay

Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured with or without oocytes (100 oocytes/ml) in 12-well plates in McCoy's 5A medium. After a 3-h preculture, the cells were transiently transfected with 500 ng of luciferase reporter plasmid Id-1-Luc and 50 ng of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). After transfection, cells were treated with or without FSH (100 ng/ml) in combination with BMP-6 and BMP-7 (100 ng/ml) for 24 h. The cells were then 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.

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's protected least significant difference test (StatView 5.0 software; Abacus Concepts, Inc., Berkeley, CA). *P* values less than 0.05 were accepted as statistically significant.

Results

We first examined oocyte effects on steroidogenesis regulated by BMP-6 and BMP-7 using rat primary granulosa cells. In preliminary studies, ovine FSH (30 ng/ml) elicited equivalent effects on steroid production as those induced by FSK (10 μM) and BtcAMP (1 mM). As shown in Fig. 1A, BMP-7 increased FSH-induced estradiol production in a concentration-dependent manner and BMP-6 had no significant effect on FSH-induced estradiol. Coculture with oocytes significantly up-regulated the effects of BMP-7 on FSH-induced estradiol production (Fig. 1A). However, in contrast to the effects induced by FSH, neither of BMP-6 and BMP-7 had any impact on estradiol levels induced by FSK and BtcAMP (Fig. 1B). Thus, BMP-7 specifically activated estradiol production induced by FSH in cooperation with oocytes.

BMP-6 and BMP-7 inhibited FSH- and FSK-induced progesterone production in a dose-dependent manner. The presence or absence of oocytes in the cultures had no effect on the actions by BMP-6 and BMP-7 (Fig. 2, A and B). In contrast to their effects on FSH and FSK induction of progesterone synthesis, neither BMP-6 nor BMP-7 had any effect on BtcAMP-induced progesterone levels (Fig. 2B). To confirm whether the effects of BMP-6 and -7 were involved in the expression changes of steroidogenetic factors, levels of mRNA encoding P450arom and StAR, a key molecule for regulating progesterone production in granulosa cells (7), were examined by quantitative real-time PCR. Consistent with its effects on estrogen production, BMP-7 up-regulated P450arom mRNA

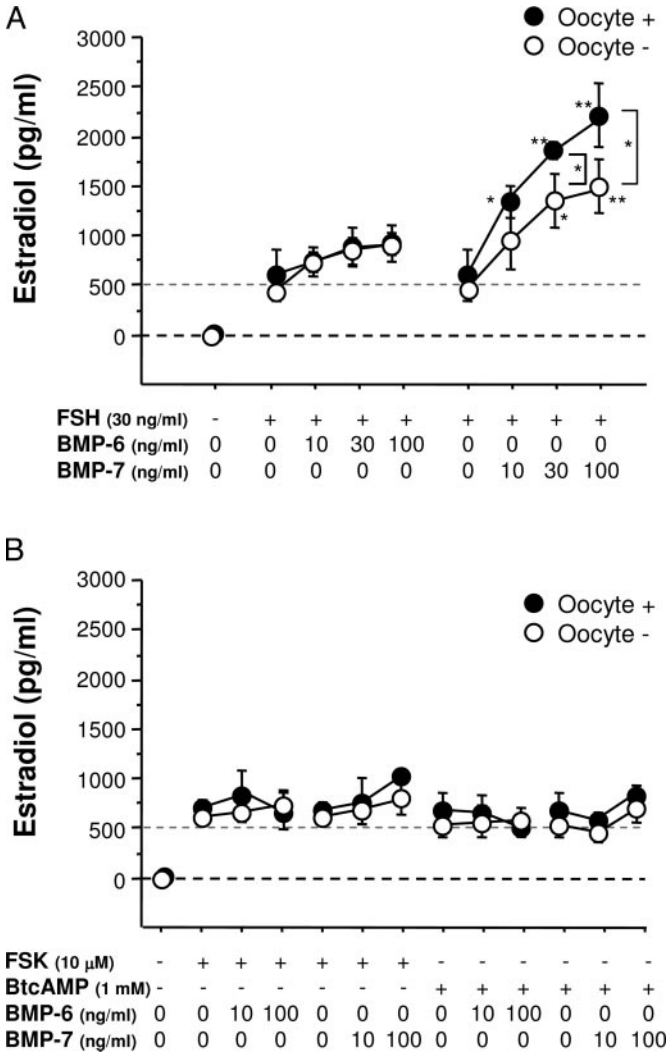

FIG. 1. Effects of oocytes and BMPs on FSH-induced estradiol production by rat granulosa cells. Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured with or without oocytes (100 oocytes/ml). The cells were treated with FSH (30 ng/ml) (A) or FSK (FSK; 10 μM) or BtcAMP (1 mM) (B) in serum-free medium containing 100 nM androstenedione (a substrate for P450arom) either alone or with a combination of BMP-6 and BMP-7 (10–100 ng/ml). After a 48-h culture, the culture media was collected and estradiol levels were determined by RIA. Results show the mean ± SEM of data performed with triplicate treatments; **, $P < 0.01$ and *, $P < 0.05$ *vs.* basal levels treated with FSH or between the indicated groups.

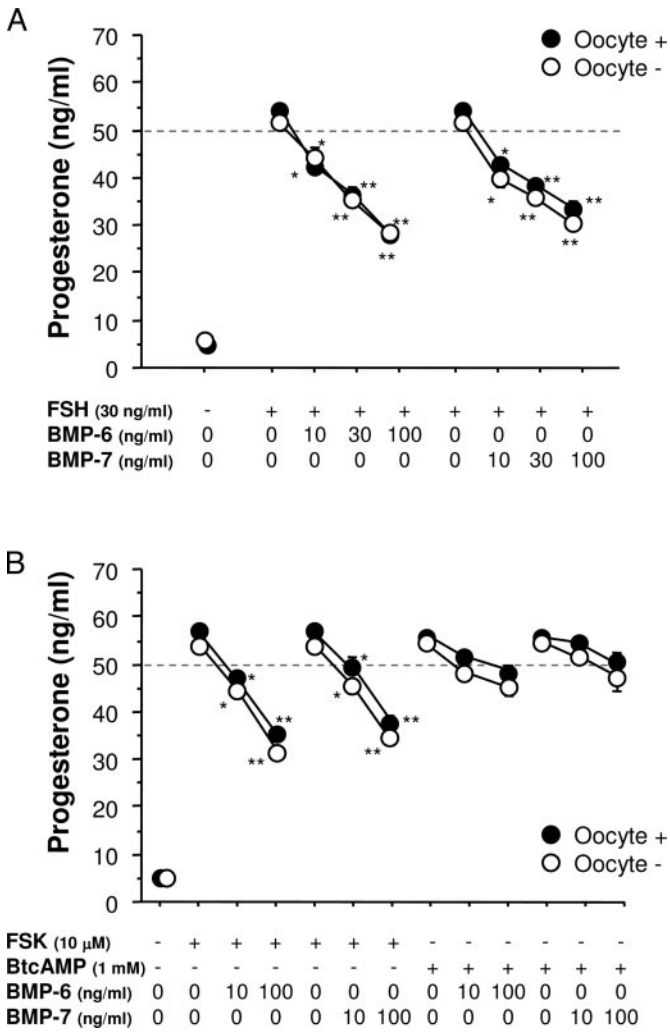

FIG. 2. Effects of oocytes and BMPs on FSH-induced progesterone production. A, Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured with or without oocytes (100 oocytes/ml). The cells were treated with FSH (30 ng/ml) (A) or FSK (10 μM) or BtcAMP (1 mM) (B) in the culture medium either alone or with a combination of BMP-6 and BMP-7 (10–100 ng/ml). After a 48-h culture, the culture media was collected and progesterone levels were determined by RIA. Results show the mean ± SEM of data performed with triplicate treatments; **, $P < 0.01$ and *, $P < 0.05$ *vs.* basal levels treated with FSH or FSK.

expression induced by FSH, which was further increased ($P = 0.0323$) in the presence with oocytes (Fig. 3A). In contrast, BMP-6 had no effect on FSH-induced P450arom expression regardless of the presence of oocytes (Fig. 3A). StAR mRNA levels induced by FSH were significantly suppressed by both BMP-6 and BMP-7 regardless of oocyte actions (Fig. 3B). Thus, the BMP effects in regulating steroidogenesis were accompanied by transcriptional changes of P450arom and StAR in granulosa cells.

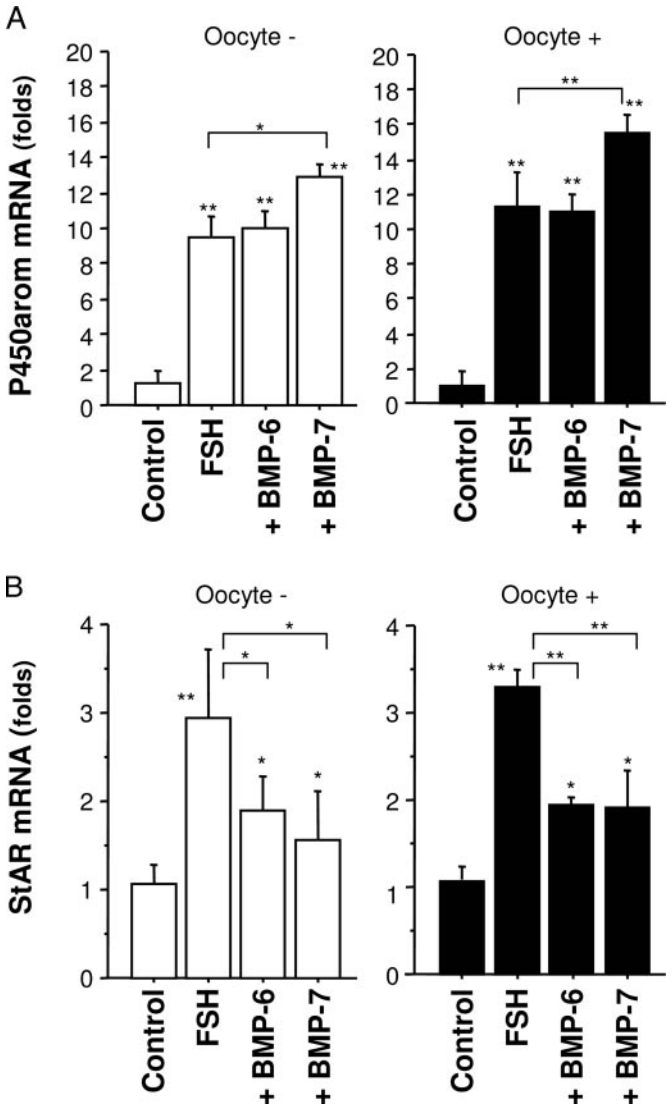

FIG. 3. Effects of oocytes and BMPs on FSH-induced P450arom and StAR mRNA. Rat primary granulosa cells (5×10^5 viable cells in 1 ml) were cultured with or without oocytes (100 oocytes/ml) in serum-free medium containing 100 nM of androstenedione (a substrate for P450arom). The cells were treated with FSH (30 ng/ml) either alone or with a combination of BMP-6 and BMP-7 (100 ng/ml). After a 48-h culture, total cellular RNA was extracted. For the quantification of P450arom (A) and StAR (B) mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic Co.). Accumulated levels of fluorescence were analyzed by the second-derivative method after the melting-curve analysis (Roche Diagnostic Co.) and 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; **, $P < 0.01$ and *, $P < 0.05$ *vs.* control or between the indicated groups.

To investigate the effects of cAMP-protein kinase A signal activation, cellular production of cAMP by granulosa cells was examined in conditioned medium from granulosa cells cultured with FSH, FSK, and BMPs in the presence or absence of oocytes. As shown in Fig. 4, cAMP production induced by FSH (Fig. 4A) and FSK (Fig. 4B) was inhibited by BMP-6 and BMP-7 regardless of the presence or absence of oocytes. Thus, the inhibitory effects of BMP-6 and BMP-7 on cAMP synthesis were in parallel with the reduction in progesterone production.

Recent studies have highlighted the involvement of MAPK activation in the regulation of steroidogenesis by FSH

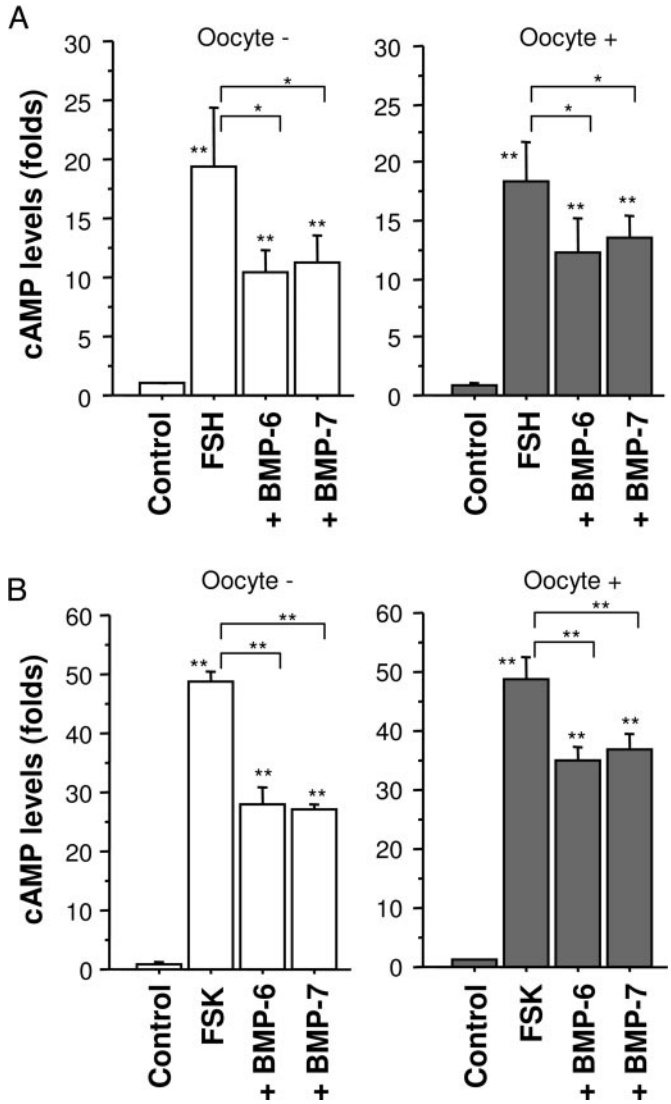

FIG. 4. Effects of oocytes and BMPs on FSH-induced cAMP production. A, Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured with or without oocytes (100 oocytes/ml) in serum-free medium containing 0.1 mM of IBMX. Cells were treated with FSH (30 ng/ml) (A) or FSK (10 μM) (B) either alone or with a combination of BMP-6 and BMP-7 (100 ng/ml). After a 48-h culture, the conditioned medium was collected and the extracellular contents of cAMP were determined by enzyme immunoassay after the acetylation of each sample. Results show the mean ± SEM of data performed with triplicate treatments; **, $P < 0.01$ and *, $P < 0.05$ *vs.* control or between the indicated groups.

in granulosa cells (15–22). As shown in Fig. 5A, FSH activated ERK1/2 phosphorylation, whereas BtcAMP treatment did not activate ERK signaling. A specific inhibitor of ERK1/2 activation, U0126 by itself had no effect on ERK activation but potently abolished FSH-induced ERK phosphorylation in granulosa cells. U0126 also potently increased FSH-induced estradiol production concentration dependently without having any significant effects on FSK- and BtcAMP-induced estradiol (Fig. 5B). These effects were not affected by coculture conditions with oocytes. U0126, in contrast, decreased FSH- and FSK-induced progesterone production (Fig. 5C) and suppressed cAMP production induced by FSH (Fig. 6A) and FSK (Fig. 6B) regardless of the presence or absence of oocytes. Taken together, ERK1/2 activation induced by FSH was functionally linked to increases in cAMP and progesterone production as well as suppression of estradiol production in granulosa cells.

Next, the impact of BMPs on FSH-induced MAPK activation in granulosa cells was examined in the presence or absence of oocytes. BMP-6 and BMP-7 had no effects on ERK1/2 phosphorylation when added to the cultures alone; however, when added with FSH, BMP-7 abolished FSH-induced ERK1/2 phosphorylation. BMP-6 had no effect on

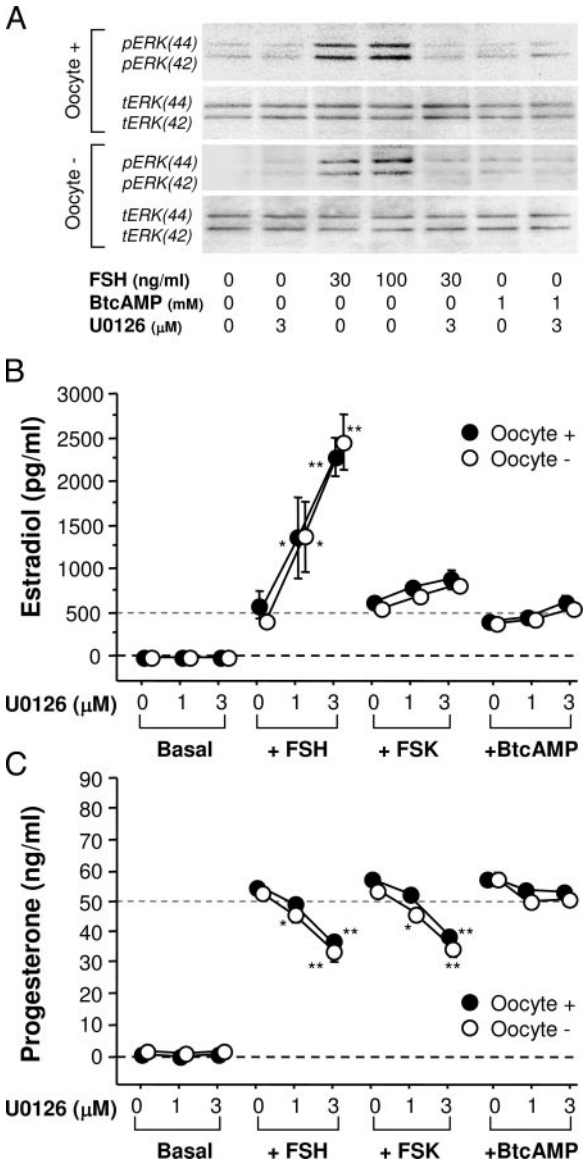

FIG. 5. Effects of FSH on ERK signaling in rat granulosa cells and inhibitory effects of ERK activation on steroidogenesis. A, Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured with or without oocytes (100 oocytes/ml). After preculture, cells were treated with U0126 (3 μM) and then exposed to either FSH (30 and 100 ng/ml) or BtcAMP (1 mM) for 1 h. The cell lysates were then subjected to SDS-PAGE immunoblotting analysis using anti-phospho ERK1/2, anti-total ERK1/2 antibodies. B, Rat granulosa cells (1×10^5 viable cells in 200 μl) cultured with or without oocytes (100 oocytes/ml) in serum-free medium containing 100 nM of androstenedione (a substrate for P450arom). Cells were treated with FSH (30 ng/ml), FSK (10 mM), or BtcAMP (1 mM) in the presence or absence of U0126 (1 and 3 μM) for 48 h. The levels of estradiol (B) and progesterone (C) in the media were determined by RIA. Results show the mean ± SEM of data performed with triplicate treatments; **, $P < 0.01$ and *, $P < 0.05$ *vs.* basal levels treated with FSH or FSK.

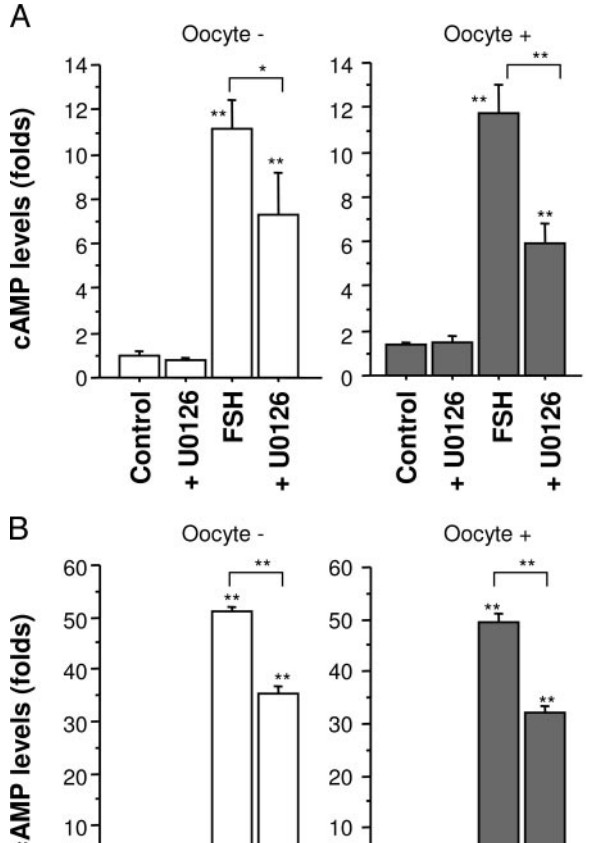

FIG. 6. Effects of ERK inhibition on FSH-induced cAMP production. A, Rat granulosa cells (1×10^5 viable cells in 200 μl) cultured with or without oocytes (100 oocytes/ml) in serum-free medium containing 0.1 mM of IBMX. Cells were treated with FSH (30 ng/ml) (A) or FSK (10 μM) (B) in the presence or absence of U0126 (3 μM). After a 48-h culture, the extracellular contents of cAMP was determined by enzyme immunoassay after the acetylation of each sample. Results show the mean ± SEM of data performed with triplicate treatments; **, $P < 0.01$ and *, $P < 0.05$ *vs.* control or between the indicated groups.

ERK1/2 phosphorylation induced by FSH. In addition, the FSH induction of ERK phosphorylation was more potent in the presence of oocytes (Fig. 7). Thus, BMP-7, but not BMP-6, plays an inhibitory role in ERK1/2 activation elicited by FSH in cooperation with oocytes.

To characterize the effect of FSH and oocytes on BMP signaling in granulosa cells, detection of Smad1/5/8 phosphorylation and promoter assays for BMP signaling were performed. As seen in Fig. 8A, Smad1/5/8 phosphorylation was induced by BMP-6 and BMP-7. Interestingly, Smad1/5/8 phosphorylation was further enhanced in the presence of FSH and oocytes (Fig. 8A). Using the BMP target gene reporter, Id-1-Luc, it was found that oocytes augment BMP signaling in combination with FSH (Fig. 8B). Thus, FSH and oocytes synergistically enhance BMP signaling in granulosa cells.

To elucidate the mechanism by which FSH and oocytes up-regulate BMP signaling in granulosa cells, expression levels of key BMP molecules, including Smads (Smad1, 5, 8, 4, 6, and 7), and BMP type I (ALK-2, -3, and -6) and type II (ActRII and BMPRII) receptors were examined in the pres-

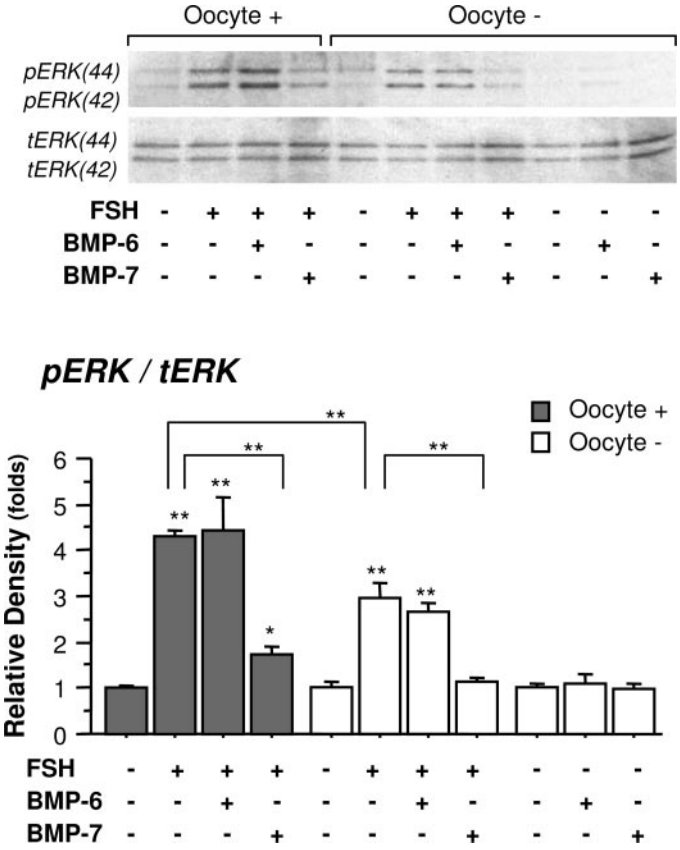

FIG. 7. Effects of BMPs and oocytes on FSH-induced ERK activation by rat granulosa cells. Rat primary granulosa cells (5×10^5 viable cells in 1 ml) were cultured with or without oocytes (100 oocytes/ml). Cells were treated with FSH (30 ng/ml) either alone or with a combination of BMP-6 and BMP-7 (100 ng/ml) for 1 h. The cell lysates were then subjected to SDS-PAGE immunoblotting analysis using anti-phospho ERK1/2 and anti-total ERK1/2 antibodies. The bands on the x-ray film were scanned and digitized, and the signal intensities were numerically converted. Results show the mean ± SEM of data performed with triplicate treatments; **, $P < 0.01$ and *, $P < 0.05$ *vs.* control or between the indicated groups.

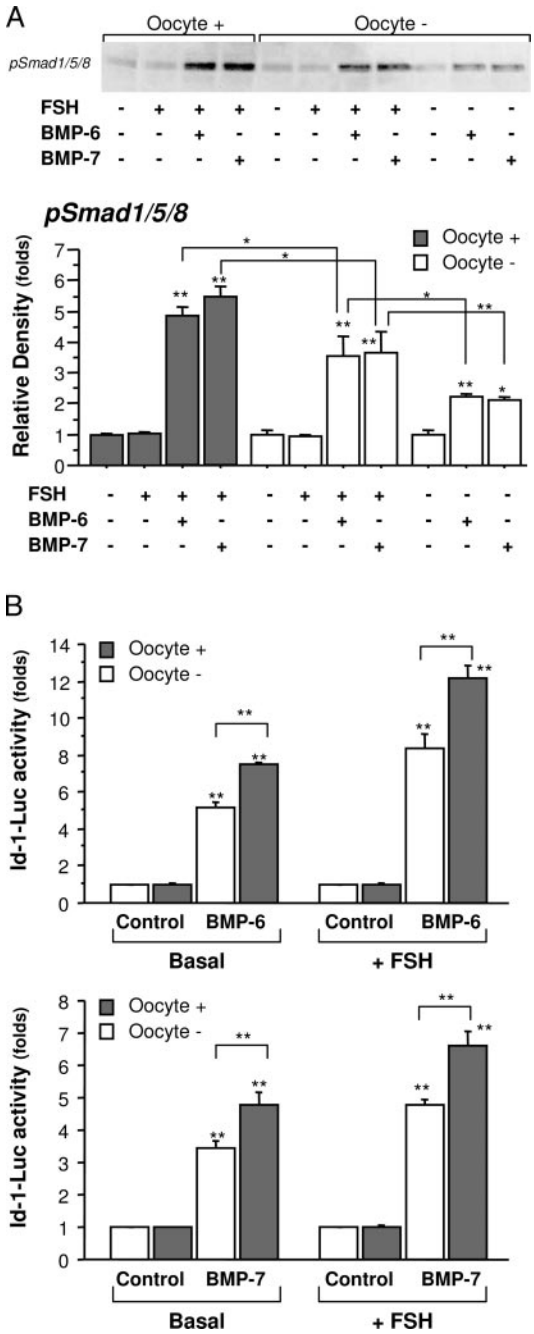

FIG. 8. Effects of FSH and oocytes on BMP-Smad signaling by rat granulosa cells. A, Rat primary granulosa cells (5×10^5 viable cells in 1 ml) were cultured with or without oocytes (100 oocytes/ml). Cells were treated with FSH (30 ng/ml) in combination with BMP-6 or BMP-7 (100 ng/ml) for 1 h. The cell lysates were then subjected to SDS-PAGE immunoblotting analysis using anti-phospho Smad1/5/8 antibody. The bands on the x-ray film were scanned and digitized, and the signal intensities were numerically converted. B, Rat primary granulosa cells (5×10^5 viable cells in 1 ml) were cultured with or without oocytes (100 oocytes/ml). After a 3-h preculture, the cells were transiently transfected with 500 ng Id-1-Luc reporter plasmid and 50 ng cytomegalovirus-βgal plasmid (pCMV-β-gal). After transfection, cells were treated with FSH (100 ng/ml) in combination with BMP-6 and BMP-7 (100 ng/ml) for 24 h, and then luciferase activity and βgal activity of the cell lysate were measured. The data were shown as the ratio of luciferase to βgal activity. Results show the mean ± SEM of data performed with triplicate treatments; **, $P < 0.01$ and *, $P < 0.05$ *vs.* control or between the indicated groups.

ence or absence of oocytes and FSH. As shown in Fig. 9A, all the BMP molecules examined except Smad5 were clearly detected in our culture system by RT-PCR analysis. Furthermore, the changes of mRNA levels of Smads and BMP type I and type II receptors were confirmed by quantitative real-time PCR. As shown in Fig. 9B, the expression levels of inhibitory Smads, *i.e.* Smad6 and Smad7, were impaired in the presence of FSH while Smad8 expression was up-regulated in turn by FSH, in which oocytes had no specific effects on the expression of Smads. Levels of BMP type I and type II receptor expression were not influenced by FSH and oocyte conditions. It appears that FSH modulates BMP signaling activity by altering the expression levels of Smad6, 7, and 8 in granulosa cells.

Discussion

The regulation of FSH responsiveness in granulosa cells is critical for the establishment of dominant follicles and subsequent ovulation in mammals. In the present study, we have uncovered a novel cellular mechanism of regulation of granulosa cell steroidogenesis involving interactions between oocytes, FSH, and BMPs. BMP-7 up-regulated FSH-induced estradiol production, which was further enhanced in the presence of oocytes. The BMP-7 action on estrogen production is likely to occur via an independent pathway of cAMP-protein kinase A cascade because cAMP levels were rather decreased by BMPs. The involvement of ERK1/2 molecules was highlighted here in regulating FSH-induced estrogen production by granulosa cells.

Although the importance of BMPs in the ovary has become well established (23), specific BMP signaling pathways and their particular regulatory roles in granulosa cells have yet to be completely elucidated. In granulosa cells, BMP-2, -6, -7, and -15 have been found to stimulate the phosphorylation of Smad1/5/8 in human and rat granulosa cells (24, 25). In addition to the Smad pathway induced by BMP ligands, involvement of MAPK signaling has also been implicated in BMP signaling in granulosa cells.

As for the functional link between MAPK and BMP system in the ovary, it has been reported that inhibitors of ERK1/2 phosphorylation suppress granulosa cell mitosis induced by BMP-15 (25) and cumulus cell expansion induced by GDF-9 (26). Yet, the inhibition of ERK1/2 phosphorylation does not reverse the suppressive effects of BMP-15 on FSH-induced progesterone production by rat primary granulosa cells (25). Considering that ERK inhibition increased estradiol yet decreased progesterone and cAMP production induced by FSH, ERK activation leads to inhibition of estradiol synthesis and amplification of cAMP. It is notable that FSH-induced ERK1/2 phosphorylation is suppressed by BMP-7 but not affected by BMP-6 in our study. Thus ERK signaling seems likely to be involved in the control of estradiol synthesis in granulosa cells.

BMP-6 and BMP-7 both inhibited FSH- and FSK-induced progesterone and reduced cAMP synthesis in our study. Thus, a signaling action that is common between BMP-6 and BMP-7 is the inhibition of cAMP synthesis, which is functionally linked to progesterone production. Selective inhibitors of progesterone synthesis, *i.e.* a luteinization inhibitor,

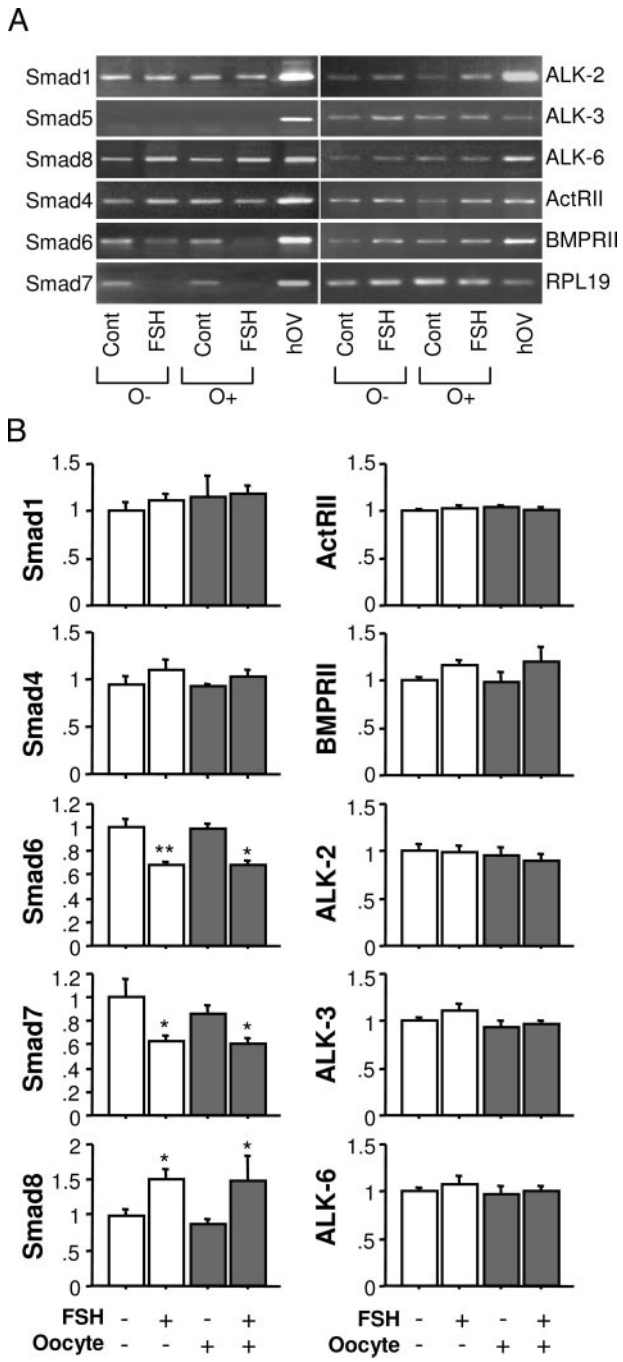

FIG. 9. Effects of FSH and oocytes on the expression of BMP system molecules by rat granulosa cells. A, Rat primary granulosa cells (5×10^5 viable cells in 1 ml) were cultured with (O+) or without (O−) oocytes (100 oocytes/ml) in the presence or absence of FSH (30 ng/ml) for 48 h. Total cellular RNA was then extracted, quantified by measuring absorbance at 260 nm, and stored at −80 C until assay. Expression of BMP receptors, Smads, and internal control RPL19 was detected by RT-PCR. hOV, Human control ovary total RNA. B, For the quantification of each molecule, real-time PCR analysis was performed using LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic Co.). Accumulated levels of fluorescence were analyzed by the second-derivative method after the melting-curve analysis (Roche Diagnostic Co.) and 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; **, $P < 0.01$ and *, $P < 0.05$ *vs.* control.

must act *in vivo* to modulate FSH bioactivity in a way that reflects normal follicle steroidogenesis during the estrous cycle (27, 28). Theca cell-derived BMP-7 is one of the candidates that are consistent with the long-sought luteinization inhibitor (6). As we reported previously, BMP-7 injection decreases the number of primordial follicles but increases the number of primary, secondary, and antral follicles *in vivo*. Thus, BMP-7 can promote the recruitment of primordial follicles into the growing follicle pool and inhibit ovulation and progesterone production (7).

The receptors for TGF-β superfamily members consist of type I and type II receptors, each of which exhibit serine/threonine kinase activity. ALK-2, -3, and -6 have been identified as type I receptors for BMPs, whereas ALK-4 is the type I receptor for activin. BMP ligands act together to form a high-affinity complex with BMPRII and the appropriate type I receptor (29). The combinations of BMP ligands and receptors are not completely definitive, in which BMP-6 and BMP-7 can readily bind to ALK-2 and/or ALK-6 (30–32). Once phosphorylated 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 (33).

Using a human granulosa KGN cell line, we recently discovered that FSH preferentially up-regulates the expression of type I receptors (ALK-3 and ALK-6) as well as type II receptors (ActRII and BMPRII) (34). In addition, FSH increased expression levels of Smad1/5 and decreased expression of inhibitory Smads, Smad6/7. Thus, it is most likely that FSH augments the bioavailability of BMP signaling in human granulosa cells (34). In the present study, the Smad1/5/8 phosphorylation induced by BMPs was further enhanced in the presence of FSH. Oocytes also augmented BMP signal transduction in combination with FSH, in which FSH up-regulated BMP signaling activity by reducing Smad6/7 expression while increasing Smad8 expression in granulosa cells.

FSH receptor signaling in the granulosa cells is required for follicular selection and dominant follicle formation, which is precisely modulated by autocrine/paracrine factors within the follicles (23) as well as estrogen (13). In this regard, we earlier discovered that the oocyte is required for estrogen to amplify FSH-dependent granulosa differentiation (13). In contrast, the expression of BMP system components undergoes dynamic changes during folliculogenesis with changes of spatial and temporal expression patterns of these BMP genes (35). Therefore, the present data support the presence of functional positive feedback system between the BMP system and FSH receptor pathway, which plays key roles in regulating development of growing follicles by the reciprocal enhancement of BMP and FSH actions. Moreover, this reciprocal positive feedback in granulosa cells was further influenced by the presence of oocytes.

Candidates for oocyte factors that are responsible for the activation of MAPK as well as BMP signaling have yet to be elucidated in this study. There has been recent interest in the roles of oocyte-derived paracrine factors including BMP-15 and GDF-9. Based on studies using rat granulosa cell culture, estradiol production is not affected by BMP-15, whereas GDF-9 exhibits suppressive effects on estradiol synthesis induced by FSH (5, 36). Because oocytes act to augment FSH-induced estradiol synthesis in combination with BMP-7, it is unlikely that GDF-9 and BMP-15 are solely responsible for the oocyte activity observed in the present study. However, oocyte-derived GDF-9 could be partially involved in amplification of MAPK signaling in granulosa cells based on findings shown in mouse cumulus cells (26, 37). Future profiling would be necessary to determine such oocyte-derived factors that orchestrate steroidogenesis in the developing follicles. Collectively, the present data demonstrate a novel oocyte-granulosa cell communication (Fig. 10). ERK activation is directly linked to inhibition of estradiol synthesis and amplification of cAMP. Impairment of FSH-induced ERK1/2 phosphorylation by theca-derived BMP-7 is likely to be a key process for up-regulating estradiol production *in vivo*. A common signaling between BMP-6 and BMP-7 is the inhibition of cAMP synthesis linked to progesterone production as a central role, luteinizing inhibitor. The presence of oocytes facilitates BMP-Smad signaling in granulosa cells regardless of FSH. Oocytes also activate FSH induction of ERK signaling pathway. Thus, oocytes modulate the signaling interaction between the BMP system and ERK signaling in granulosa cells, which may be crucial for differential regu-

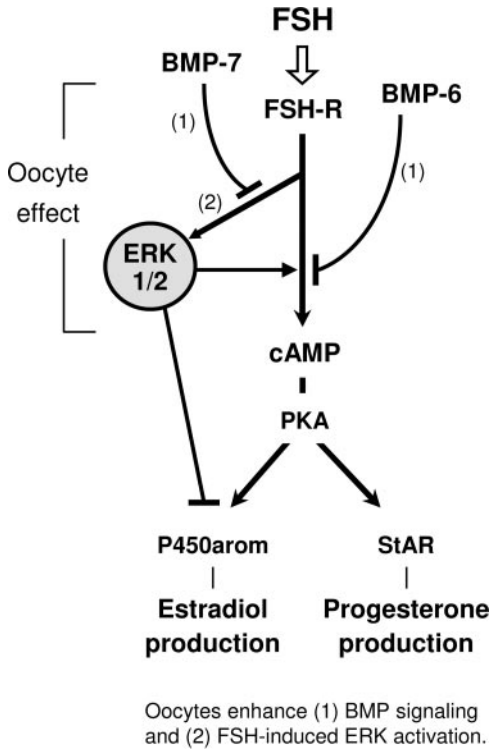

FIG. 10. A possible mechanism by which BMPs differentially regulate steroidogenesis in granulosa cells. ERK1/2 activation induced by FSH receptor (FSH-R) signaling is directly linked to inhibition of estradiol synthesis and amplification of cAMP. BMP-7 inhibits FSH-induced ERK1/2 phosphorylation, leading to up-regulation of estradiol production. A common signaling between BMP-6 and BMP-7 is the inhibition of cAMP synthesis linked to progesterone production. The presence of oocytes facilitates BMP-Smad signaling (1) and FSH induction of ERK signaling pathway in granulosa cells (2).

lation of FSH-induced steroidogenesis in developing follicles.

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References

1. **Shimasaki S, Moore RK, Erickson GF, Otsuka F** 2003 The role of bone morphogenetic proteins in ovarian function. Reprod Suppl 61:323–337
2. **Shimasaki S, Moore RK, Otsuka F, Erickson GF** 2004 The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72–101
3. **Otsuka F, Moore RK, Shimasaki S** 2001 Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. J Biol Chem 276: 32889–32895
4. **Otsuka F, Yamamoto S, Erickson GF, Shimasaki S** 2001 Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. J Biol Chem 276:11387–11392
5. **Vitt UA, Hayashi M, Klein C, Hsueh AJ** 2000 Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. Biol Reprod 62:370–377
6. **Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S, Ueno N, Sampath K, Chang RJ, Erickson GF** 1999 A functional bone morphogenetic protein system in the ovary. Proc Natl Acad Sci USA 96:7282–7287
7. **Lee W, Otsuka F, Moore RK, Shimasaki S** 2001 The effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. Biol Reprod 65:994–999
8. **Eppig JJ** 2001 Oocyte control of ovarian follicular development and function in mammals. Reproduction 122:829–838
9. **Matzuk MM, Burns KH, Viveiros MM, Eppig JJ** 2002 Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296:2178–2180
10. **Vanderhyden BC, Cohen JN, Morley P** 1993 Mouse oocytes regulate granulosa cell steroidogenesis. Endocrinology 133:423–426
11. **Vanderhyden BC, Tonary AM** 1995 Differential regulation of progesterone and estradiol production by mouse cumulus and mural granulosa cells by a factor (s) secreted by the oocyte. Biol Reprod 53:1243–1250
12. **Vanderhyden BC** 1996 Oocyte-secreted factors regulate granulosa cell steroidogenesis. Zygote 4:317–321
13. **Otsuka F, Moore RK, Wang X, Sharma S, Miyoshi T, Shimasaki S** 2005 Essential role of the oocyte in estrogen amplification of follicle-stimulating hormone signaling in granulosa cells. Endocrinology 146:3362–3367
14. **Otsuka F, Shimasaki S** 2002 A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: its role in regulating granulosa cell mitosis. Proc Natl Acad Sci USA 99:8060–8065
15. **Kang SK, Tai CJ, Cheng KW, Leung PC** 2000 Gonadotropin-releasing hormone activates mitogen-activated protein kinase in human ovarian and placental cells. Mol Cell Endocrinol 170:143–151
16. **Moore RK, Otsuka F, Shimasaki S** 2001 Role of ERK1/2 in the differential synthesis of progesterone and estradiol by granulosa cells. Biochem Biophys Res Commun 289:796–800
17. **Tai CJ, Kang SK, Tzeng CR, Leung PC** 2001 Adenosine triphosphate activates mitogen-activated protein kinase in human granulosa-luteal cells. Endocrinology 142:1554–1560
18. **Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss 3rd JF, Amsterdam A** 2001 The ERK signaling cascade inhibits gonadotropin-stimulated steroidogenesis. J Biol Chem 276:13957–13964
19. **Dewi DA, Abayasekara DR, Wheeler-Jones CP** 2002 Requirement for ERK1/2 activation in the regulation of progesterone production in human granulosa-lutein cells is stimulus specific. Endocrinology 143:877–888
20. **Tajima K, Dantes A, Yao Z, Sorokina K, Kotsuji F, Seger R, Amsterdam A** 2003 Down-regulation of steroidogenic response to gonadotropins in human and rat preovulatory granulosa cells involves mitogen-activated protein kinase activation and modulation of DAX-1 and steroidogenic factor-1. J Clin Endocrinol Metab 88:2288–2299
21. **Amsterdam A, Tajima K, Frajese V, Seger R** 2003 Analysis of signal transduction stimulated by gonadotropins in granulosa cells. Mol Cell Endocrinol 202:77–80
22. **Yu FQ, Han CS, Yang W, Jin X, Hu ZY, Liu YX** 2005 Activation of the p38 MAPK pathway by follicle-stimulating hormone regulates steroidogenesis in granulosa cells differentially. J Endocrinol 186:85–96
23. **Findlay JK, Drummond AE, Dyson ML, Baillie AJ, Robertson DM, Ethier JF** 2002 Recruitment and development of the follicle; the roles of the transforming growth factor-β superfamily. Mol Cell Endocrinol 191:35–43
24. **Bondestam J, Kaivo-oja N, Kallio J, Groome N, Hyden-Granskog C, Fujii M, Moustakas A, Jalanko A, ten Dijke P, Ritvos O** 2002 Engagement of activin and bone morphogenetic protein signaling pathway Smad proteins in the induction of inhibin B production in ovarian granulosa cells. Mol Cell Endocrinol 195:79–88
25. **Moore RK, Otsuka F, Shimasaki S** 2003 Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. J Biol Chem 278:304–310
26. **Su Y-Q, Wigglesworth K, Pendola FL, O'Brien MJ, Eppig JJ** 2002 Mitogen-activated protein kinase activity in cumulus cells is essential for gonadotropin-induced oocyte meiotic resumption and cumulus expansion in the mouse. Endocrinology 143:2221–2232
27. **Falck B** 1959 Site of production of oestrogen in rat ovary as studied in microtransplants. Acta Physiol Scand 47:1–101
28. **Nalbandov AV,** Oogenesis. Symposium on Oogenesis, Baltimore, Maryland, 1970, pp 513–522
29. **Miyazono K, Kusanagi K, Inoue H** 2001 Divergence and convergence of TGF-β/BMP signaling. J Cell Physiol 187:265–276
30. **Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T** 1999 Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J Cell Sci 112:3519–3527
31. **Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, Heldin C-H, Miyazono K** 1995 Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. J Cell Biol 130:217–226
32. **Aoki H, Fujii M, Imamura T, Yagi K, Takehara K, Kato M, Miyazono K** 2001 Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction. J Cell Sci 114:1483–1489
33. **Kawabata M, Imamura T, Miyazono K** 1998 Signal transduction by bone morphogenetic proteins. Cytokine Growth Factor Rev 9:49–61
34. **Miyoshi T, Otsuka F, Suzuki J, Takeda M, Inagaki K, Kano Y, Otani H, Mimura Y, Ogura T, Makino H** 2006 Mutual regulation of follicle-stimulating hormone signaling and bone morphogenetic protein system in human granulosa cells. Biol Reprod 74:1073–1082
35. **Erickson GF, Shimasaki S** 2003 The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. Reprod Biol Endocrinol 1:9
36. **Otsuka F, Yao Z, Lee TH, Yamamoto S, Erickson GF, Shimasaki S** 2000 Bone morphogenetic protein-15: identification of target cells and biological functions. J Biol Chem 275:39523–39528
37. **Su YQ, Denegre JM, Wigglesworth K, Pendola FL, O'Brien MJ, Eppig JJ** 2003 Oocyte-dependent activation of mitogen-activated protein kinase (ERK1/2) in cumulus cells is required for the maturation of the mouse oocyte-cumulus cell complex. Dev Biol 263:126–138

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