p38-Mitogen-Activated Protein Kinase Stimulated Steroidogenesis in Granulosa Cell-Oocyte Cocultures: Role of Bone Morphogenetic Proteins 2 and 4

Kenichi Inagaki, Fumio Otsuka, Tomoko Miyoshi, Misuzu Yamashita, Mina Takahashi, Junko Goto, Jiro Suzuki, and Hirofumi Makino

Departments of Medicine and Clinical Science (K.I., F.O., T.M., M.Y., J.G., J.S., H.M.) and Cancer and Thoracic Surgery (M.T.), Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama City 700-8558, Japan

Roles of the p38-MAPK pathway in steroidogenesis were investigated using coculture of rat granulosa cells with oocytes. Activin and FSH readily phosphorylated p38 in granulosa cells. Activin effect on p38 phosphorylation was abolished by a selective activin receptor-like kinase-4, -5, and -7 inhibitor, SB431542. SB431542 decreased FSH-induced estradiol but had no effect on progesterone production with a marginal cAMP reduction, suggesting that endogenous activin is primarily involved in estradiol synthesis. FSH-induced p38 activation was not affected either by SB431542 or follistatin, suggesting that FSH activates p38 not through the endogenous activin. Bone morphogenetic protein (BMP)-2 and BMP-4 also enhanced FSH-induced p38 phosphorylation, which was augmented by oocyte action. A specific p38 inhibitor, SB203580, decreased FSH-induced estradiol production. However, FSH-induced cAMP accumulation was not changed by SB203580, suggesting that p38 activation is linked to estradiol synthesis independently of cAMP. BMP-2 and BMP-4 inhibited FSH- and forskolin (FSK)-induced progesterone and cAMP synthesis regardless of oocyte action. BMP-2, BMP-4, and activin increased FSH-induced estradiol production, which was enhanced in the presence of oocytes. In contrast to activin that enhanced FSK-induced estradiol, BMP-2 and BMP-4 had no effects on FSK-induced estradiol production, suggesting that BMP-2 and BMP-4 directly activate FSH-receptor signaling. Given that activin increased, but BMP-2 and BMP-4 decreased, FSH-induced cAMP, the effects of BMP-2 and BMP-4 on estradiol enhancement appeared to be diverged from the cAMP-protein kinase A pathway. Thus, BMP-2 and BMP-4 differentially regulate steroidogenesis by stimulating FSH-induced p38 and suppressing cAMP. The former is involved in estradiol production and enhanced by oocyte action, whereas the latter leads to reduction of progesterone synthesis. (*Endocrinology* 150: 1921–1930, 2009)

Gonadotropins and various ovarian autocrine/paracrine factors orchestrate normal folliculogenesis. Among autocrine/paracrine molecules in the ovary, activins are well-known factors to stimulate FSH receptor expression in granulosa cells in the ovarian follicles and activate FSH-induced steroidogenesis in growing follicles (1). Recent studies have established the concept that bone morphogenetic proteins (BMPs), activins, and inhibins, all members of the TGF-β superfamily, play a key role in female fertility in mammals by regulating steroidogenesis and mitogenesis in granulosa cells (2, 3).

FSH influences the development of early stages of preantral follicles, and growth beyond the late-preantral stages is critically dependent on FSH. Accumulating evidence has indicated an autocrine/paracrine role for granulosa-derived activins and BMP-6, and a paracrine role of oocyte-derived growth differentiation factor-9 (GDF-9), BMP-15, and BMP-6 in regulating granulosa cell proliferation and FSH-

Abbreviations: ALK, Activin receptor-like kinase; βgal, β-galactosidase; BMP, bone morphogenetic protein; BMPRII, bone morphogenetic protein type II receptor; BtcAMP, N^6,O^2-dibutyryl adenosine-3′,5′-cyclic monophosphate monosodium salt; DES, diethylstilbestrol; FSK, forskolin; GDF-9, growth differentiation factor-9; IBMX, 4-androstene-3,17-dione, 3-isobutyl-1-methylxanthine; P450arom, P450 aromatase; pCMV-β-gal, cytomegalovirus-β-galactosidase plasmid; PKA, protein kinase A; RPL19, ribosomal protein L19; RT, reverse transcription, StAR, steroidogenic acute regulatory protein.

dependent follicle function (2, 3). For instance, in cultures of undifferentiated rat granulosa cells, activin promotes FSH receptor expression (1, 4), whereas mice overexpressing follistatin (5) or those with null mutations in activin type II receptor (6), exhibit arrested follicle development, suggesting a critical role for activin signaling in the maintenance of FSH receptors with cyclical recruitment of the growing follicles.

The major regulatory process by BMPs in steroidogenesis is control of the FSH receptor signaling. BMP-6 inhibits FSH-induced progesterone synthesis through suppression of cellular cAMP synthesis (7). BMP-15, which is specifically expressed by oocytes in the ovary, suppresses FSH action by inhibiting FSH receptor expression (8). Likewise, GDF-9, which shares the highest homology with BMP-15, also inhibits FSH-induced steroidogenesis and LH receptor expression in rat granulosa cells (9). BMP-6 and BMP-15 did not affect basal and FSH-induced estrogen production by granulosa cells, although these commonly suppress progesterone production induced by FSH (7). In contrast to the *in vitro* actions of BMP-6 and BMP-15 that had no effect on FSH-induced estrogen production, theca-derived BMP-7 elicits stimulating effects on FSH-induced estradiol production in addition to a common BMP effect: luteinizing inhibition that suppresses FSH-induced progesterone production (10–12). Moreover, the expression of BMP-2 and BMP-4 was also detected in granulosa cells and theca cells, respectively (13). However, the regulatory roles of BMP-2 and BMP-4 on ovarian steroidogenesis and the underlying mechanism have yet to be understood fully.

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. The differential exposure to BMPs/activin may be one of the ways in which certain follicles are sensitized to FSH and, thus, selected to become the dominant follicle(s) that continues growth to the preovulatory stage. Recent studies have identified specific oocyte-derived factors that play central roles in the communication network between oocytes and somatic follicular cells, which is crucial not only for the oocyte maturation but also for the growth and differentiation of surrounding granulosa and theca cells (14, 15).

Here, we investigated functional roles of the p38 MAPK pathway in regulating FSH-induced steroidogenesis by rat primary granulosa-oocyte coculture system. The experiments of the present study were designed to elucidate the molecular mechanism of the integration of steroidogenesis performed through "oocyte-granulosa cell communication," focusing the effects of BMP-2 and BMP-4 in comparison with activin actions.

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, pen-

icillin-streptomycin solution, recombinant human activin A, and SB431542 were from Sigma-Aldrich Corp. (St. Louis, MO). Recombinant human BMP-2, -4, -6, and BMP-7 were purchased from R&D Systems, Inc. (Minneapolis, MN), and SB203580 was from Promega Corp. (Madison, WI). Plasmid of (CAGA)⁹-Luc and recombinant human follistatin-288 (16) were kindly provided by Dr. Shunichi Shimasaki (University of California, San Diego, CA).

Primary culture of granulosa cells and coculture with oocytes

Female, 22-d-old Sprague Dawley rats were implanted with SILASTIC brand capsules (Dow Corning, Midland, MI) containing 10 mg DES to increase granulosa cell number. After 4 d 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), which allowed granulosa cells but not oocytes to pass through (17). The purified granulosa cells were cultured in serum-free McCoy's 5A medium as described previously. The animal protocols were approved by Okayama University Institutional Animal Care and Use Committee.

Measurements of estradiol, progesterone, and cAMP

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 androstenedione, a substrate for P450 aromatase (P450arom). FSH (30 ng/ml) and FSK (10 μM) were added to the culture medium either alone or in combination with indicated concentrations of BMP-2, BMP-4, activin A, SB431542, and SB203580. After 48 h culture, the culture media were collected and stored at −80 C until assay. The levels of estradiol and progesterone 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 IBMX (specific inhibitor of phosphodiesterase activity). After 30 min to 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) after the acetylation of each sample with an assay sensitivity of 0.039 nM.

Cellular RNA extraction, reverse transcription (RT), 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 plates with serum-free McCoy's 5A medium. FSH (30 ng/ml) was added to the culture medium either alone or in combination with indicated concentrations of SB431542, SB203580, BMP-2, BMP-4, and activin A. After 48 h culture, the medium was removed, and total cellular RNA was extracted using TRIzol (Invitrogen). 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 no. M33986); steroidogenic acute regulatory protein (StAR), 557-577 and 787-806 (AB001349); and ribosomal protein L19 (RPL19), 401-421 and 575-595 (J02650). The extracted RNA (1 μg) was subjected to a RT reaction using the First-Strand cDNA Synthesis System (Invitrogen) with random

hexamer (2 ng/μl), reverse transcription (200 U), and deoxynucleotide triphosphate (0.5 mM) at 42 C for 50 min, and 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), and then the expression levels of target genes were standardized by RPL19 level in each sample.

Immunoblot analysis of phosphorylated p38

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 Mc-Coy's 5A medium. After 3 h preculture, cells were treated with activin A and FSH either alone or in combination with BMP-2, BMP-4, follistatin, SB431542, and SB203580 for indicated periods. Cells were then solubilized in 100 μl radioimmunoprecipitation assay lysis buffer (Upstate Biotechnology Inc., Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM NaF, 2% sodium dodecyl sulfate, and 4% β-mercaptoethanol. The cell lysates were then subjected to SDS-PAGE and immunoblotting analysis using antiphospho- and antitotal-p38 MAPK antibody (Cell Signaling Technology, Inc., Danvers, MA). The relative integrated density of each protein band was digitized by National Institutes of Health ImageJ 1.34s (Bethesda, MD).

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 serum-free Mc-Coy's 5A medium. After 3 h preculture, the cells were transiently transfected with 500 ng luciferase reporter plasmid $(CAGA)^9$-Luc and 50 ng cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). After transfection for 12 h, cells were treated with activin A (100 ng/ml) either alone or in combination with SB431542 (0.1–3 μM) and SB203580 (3 μM) for 24 h in serum-free conditions. 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 (Stat-View 5.0 software; Abacus Concepts, Inc., Berkeley, CA). P values less than 0.05 were accepted as statistically significant.

Results

We first investigated effects of BMP-2 and BMP-4 on estradiol and progesterone production in primary granulosa cells. As shown in Fig. 1A, BMP-2 and BMP-4 dose responsively increased FSH-induced estradiol production by granulosa cells, an effect that was further enhanced in the presence of oocytes. Interestingly, BMP-2 and BMP-4 did not alter FSK-induced estradiol production (Fig. 1B), although activin enhanced both FSH- and FSK-induced estradiol synthesis (Fig. 1). On the contrary, BMP-2 and BMP-4 inhibited FSH as well as FSK-induced progesterone production (Fig. 2), the effects of which were not affected by oocyte coculture. Activin increased FSH-induced progesterone production (Fig. 2A); however, activin had no
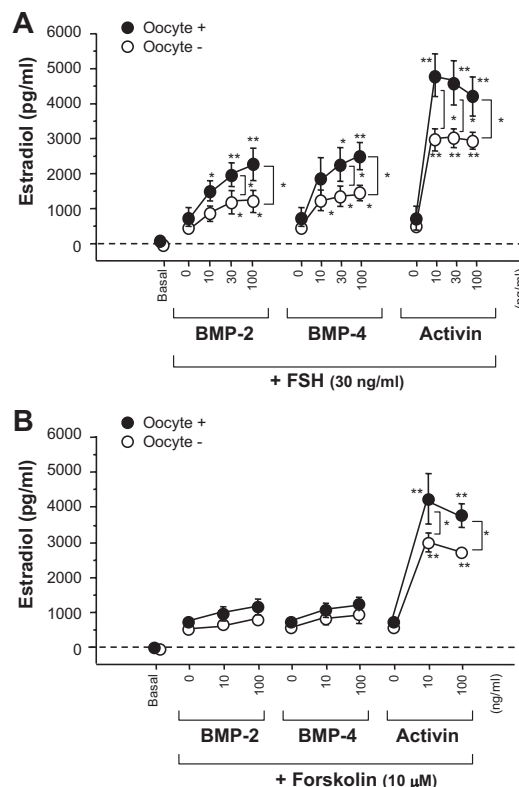

FIG. 1. Effects of BMP-2, BMP-4, and activin on FSH-induced estradiol production by granulosa cells. Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured with or without oocytes (100 oocytes/ml) in serum-free McCoy's 5A medium containing 100 nM androstenedione (a substrate for P450arom). FSH (30 ng/ml) (A) or FSK (10 μM) (B) was added to the culture medium either alone or in combination with BMP-2, -4, and activin A (10–100 ng/ml). After 48 h culture, the levels of estradiol 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. control or between the indicated groups.

significant effects on FSK-induced progesterone production by granulosa cells (Fig. 2B).

In accordance with the effects on estradiol production, FSH-induced P450arom mRNA expression was marginally increased by BMP-2 and BMP-4 in oocyte-free granulosa cells (Fig. 3A). In the presence of oocytes, BMP-2 and BMP-4 significantly enhanced the mRNA levels of FSH-induced P450arom. Similar to the effects on progesterone production, FSH-induced StAR mRNA levels were suppressed by BMP-2 and BMP-4. The effect on StAR was not affected by oocyte coculture (Fig. 3B). Concordant with activin effects on FSH-induced steroidogenesis, activin increased FSH-induced aromatase and StAR expression (Fig. 3). The activin effects on FSH-induced aromatase expression were further augmented by coculture with oocytes ($P < 0.05$) (Fig. 3A). In contrast, StAR mRNA expression induced by FSH in combination with activin was not affected by oocyte actions (Fig. 3B).

FSH-induced cAMP production was increased time dependently by granulosa cells, in which the FSH effects were saturated after 14 h culture (Fig. 4A). At early time points of 30–120 min culture, BMP-2 and BMP-4 enabled to reduce the FSH-induced cAMP production, whereas activin enhanced the FSH-induced cAMP production for 120 min culture. Furthermore, the cAMP accumulation induced by FSH and FSK for 48 h culture was also
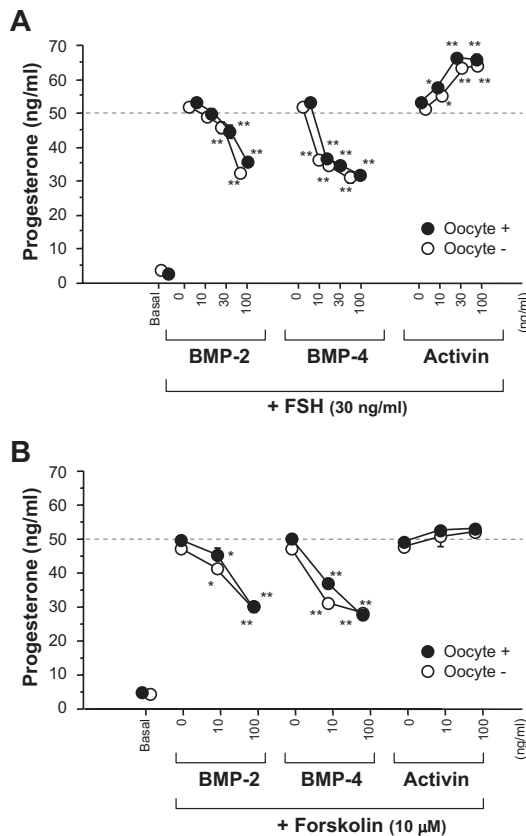
FIG. 2. Effects of BMP-2, BMP-4, and activin on FSH-induced progesterone production. Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured with or without oocytes (100 oocytes/ml) in serum-free McCoy's 5A medium. FSH (30 ng/ml) (A) or FSK (10 μM) (B) was added to the culture medium either alone or in combination with BMP-2, -4, and activin A (10–100 ng/ml). After 48 h culture, the levels of progesterone 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.* control or between the indicated groups.

FIG. 3. Effects of BMP-2, BMP-4, and activin on FSH-induced aromatase and StAR mRNA expression. Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured with or without oocytes (100 oocytes/ml) with serum-free McCoy's 5A medium. FSH (30 ng/ml) was added to the culture medium either alone or in combination with BMP-2, -4, and activin A (100 ng/ml). After 48 h culture, total cellular RNA was extracted. For the quantification of P450arom (A) and StAR (B) mRNA levels, real-time PCR was performed, 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.

decreased by BMP-2 and BMP-4, regardless of the presence of oocytes (Fig. 4B). Activin enhanced FSH-induced but not FSK-induced cAMP production, regardless of oocytes, suggesting that activin up-regulates FSH receptor actions in granulosa cells.

Recently, it has been revealed that the MAPK pathway is involved in regulating FSH-induced steroidogenesis in granulosa cells (12, 18–25). To characterize roles of p38 MAPK in regulation of steroidogenesis, we examined activin effects on p38 MAPK phosphorylation in granulosa cells. Granulosa cells were stimulated with activin A in serum-free culture conditions after 3 h preculture. As shown in Fig. 5A, activin directly stimulated phosphorylation of p38 pathway time dependently, with the highest activation at 60 min after stimulation. The activin effects on p38 phosphorylation were decreased by a selective activin receptor-like kinase (ALK)-4, -5, and -7 inhibitor, SB431542 (3 μM), and a specific p38 inhibitor, SB203580 (3 μM). SB203580 is a specific inhibitor for p38 MAPK but not for the upstream p38 MAPK kinases such as MKK3 or MKK6 (26). Among the isoforms of p38 MAPK, including p38α, β, γ, and δ, it is recognized that SB203580 specifically prevents the phosphorylation of p38α/p38β.

On the other hand, SB431542 is originally characterized as a specific inhibitor of ALK-5. Because the kinase domains of ALK-4 and ALK-7 are si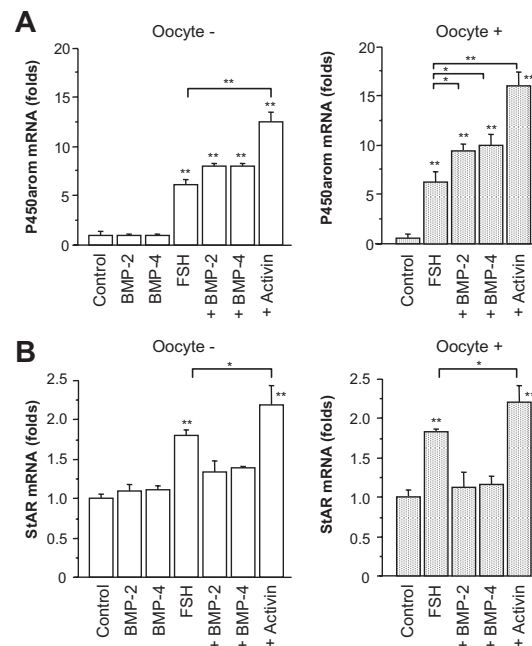milar to that of ALK-5, the inhibitor is later tested and confirmed as a specific inhibitor of ALK-4, -5, and -7 (27). Up to 10 μM, SB431542 has no significant effect on the kinase activities of ALK-1, -2, and the components of the ERK1/2, stress-activated protein kinase/c-Jun N-terminal kinase, or MAPK signaling pathways. ALK-3 and -6 and the p38 MAPK signaling pathways were weakly affected only when using SB431542 at a concentration of 10 μM (28). Therefore, SB431542 is specific for ALK-4, -5, and -7 when used at less than 10 μM. To confirm the inhibitory effects of SB431542 on activin signaling in granulosa cells, the activity of Smad2/3-responsive (CAGA)[9]-Luc, which contains nine tandemly repeated CAGA boxes (29), was examined (Fig. 5B). Activin readily increased (CAGA)[9]-Luc activity, which was significantly suppressed by cotreatment with SB431542 in a concentration-responsive manner. On the contrary, 3 μM SB203580 did not affect the activin induction of (CAGA)[9]-Luc activity in granulosa cells.

FSH also induced p38 MAPK phosphorylation in granulosa cells for 60 min (Fig. 5C). Unlike activin, the FSH-induced p38 activation was not affected by SB431542 but abolished by SB203580. Treatments with cAMP compounds such as BtcAMP and FSK failed to activate p38 phosphorylation in granulosa cells for 60 min (Fig. 5C). The time-course change of p38 phosphorylation induced by BtcAMP was also less effective than that induced by FSH (Fig. 5D), suggesting that cAMP-protein kinase A (PKA) signaling is not directly involved in the FSH-induced p38 activation. Furthermore, FSH-induced p38 activation was not affected by follistatin or follistatin plus SB431542, whereas
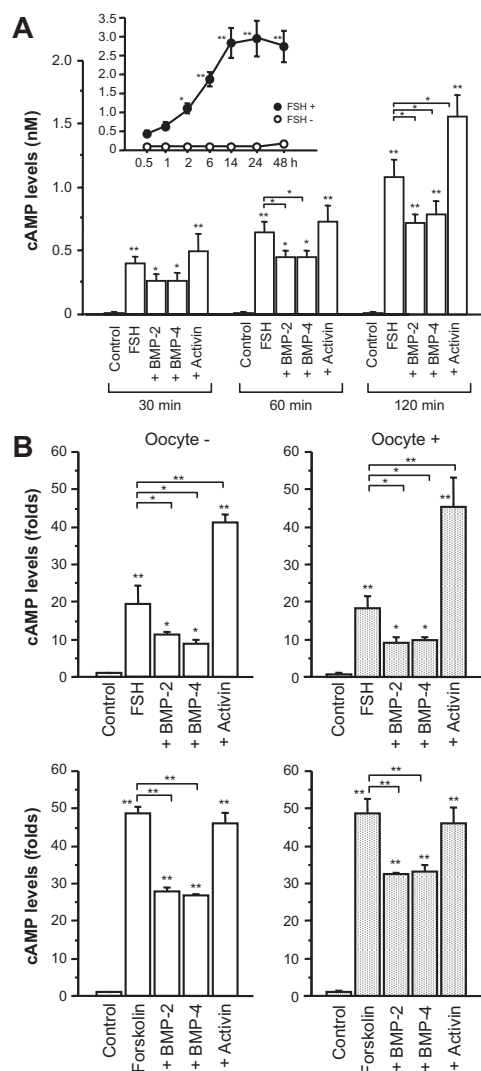
FIG. 4. Effects of BMP-2, BMP-4, and activin on FSH-induced cAMP production. A, Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured with serum-free McCoy's 5A medium containing 0.1 mM of IBMX. FSH (30 ng/ml) was added to the culture medium either alone or in combination with BMP-2, -4, and activin A (100 ng/ml). After 30 min to 48 h culture, the extracellular contents of cAMP in the conditioned medium 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. "FSH +" levels at 0.5 h, each control or between the indicated groups. B, 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 IBMX. FSH (30 ng/ml) or FSK (10 μM) was added to the culture medium either alone or in combination with BMP-2, -4, and activin A (100 ng/ml). After 48 h culture, 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. each control or between the indicated groups.

treatment with follistatin in combination with SB203580 abolished the FSH-induced p38 activation (Fig. 5E). Activin additively enhanced FSH-induced p38 phosphorylation, in which the increment elicited by activin was reversed by treatment with SB431542 (Fig. 5F). Thus, activin and FSH independently stimulate the p38 pathway in granulosa cells.

To elucidate functional roles of ALK-4, -5, and -7, which are mainly activated by endogenous activin in granulosa cell culture,

the effects of SB431542 on ALK-4, -5, and -7 inhibition of FSH-induced steroidogenesis were examined. As shown in Fig. 6A, treatment with SB431542 caused a potent reduction of FSH-induced estradiol production, whereas it did not affect FSH-induced progesterone levels. In accordance with the results of steroid production, P450arom expression induced by FSH was suppressed by SB431542, whereas FSH-induced StAR mRNA levels were not changed by SB431542. FSH-induced cAMP production was moderately reduced by SB431542, possibly due to the blocking of endogenous activin actions (Fig. 6B). Exogenously added activin effectively up-regulated FSH-induced cAMP levels, and SB431542 restored the activin-induced up-regulation of cAMP synthesis. These data suggest that endogenous activin plays a key role in FSH-induced estradiol production via ALK-4, -5, and -7, partly through the cAMP-PKA pathway.

Because a specific inhibitor of p38 activation, SB203580, potently suppressed FSH- and activin-induced p38 phosphorylation in granulosa cells (Fig. 5, A, C, E, and F), the functional roles of the p38 pathway were investigated. As shown in Fig. 7A, SB203580 efficaciously inhibited FSH-induced estradiol production concentration dependently. In contrast, SB203580 had negligible effects on FSH-induced progesterone production (Fig. 7A) and FSH-induced cAMP production (Fig. 7B). Likewise, P450arom expression induced by FSH was suppressed by SB203580, whereas FSH-induced StAR mRNA levels were not changed by SB203580 (Fig. 7A). The cAMP enhancement by activin in combination with FSH was not influenced by treatment with SB203580 (Fig. 7B). Together, activation of the p38 pathway is directly involved in FSH-induced estradiol production independently of cAMP-PKA pathway.

We next investigated the effects of BMP-2 and BMP-4 on FSH-induced p38 activation. As shown in Fig. 8A, BMP-2 and BMP-4 alone had no significant effect on p38 phosphorylation, although FSH stimulated p38 phosphorylation in granulosa cells. Importantly, BMP-2 and BMP-4 significantly enhanced the FSH-induced p38 phosphorylation (Fig. 8). The effects induced by FSH, in combination with either BMP-2 or BMP-4, were further increased in the presence of oocytes (Fig. 8B).

Discussion

In the present study, it is revealed that BMP-2 and BMP-4 differentially regulate FSH-induced steroidogenesis not only by stimulating FSH-induced p38 MAPK signaling but also by suppressing the FSH-induced cAMP pathway in granulosa cells (Fig. 9). A FSH-receptor signaling, p38 pathway is tightly linked to estradiol production, and the effects are enhanced by oocyte actions. This "oocyte-granulosa cell communication" plays a key role for differential regulation of FSH-induced steroidogenesis by BMP-2 and BMP-4 in the process of normal follicular development (Fig. 9).

It has been known that activin plays a pivotal role in the acquisition of responsiveness to FSH by granulosa cells of preantral follicles. *In vitro* studies involving undifferentiated or partially differentiated granulosa cells have shown that activin enhances FSH-stimulated aromatase activity and estradiol
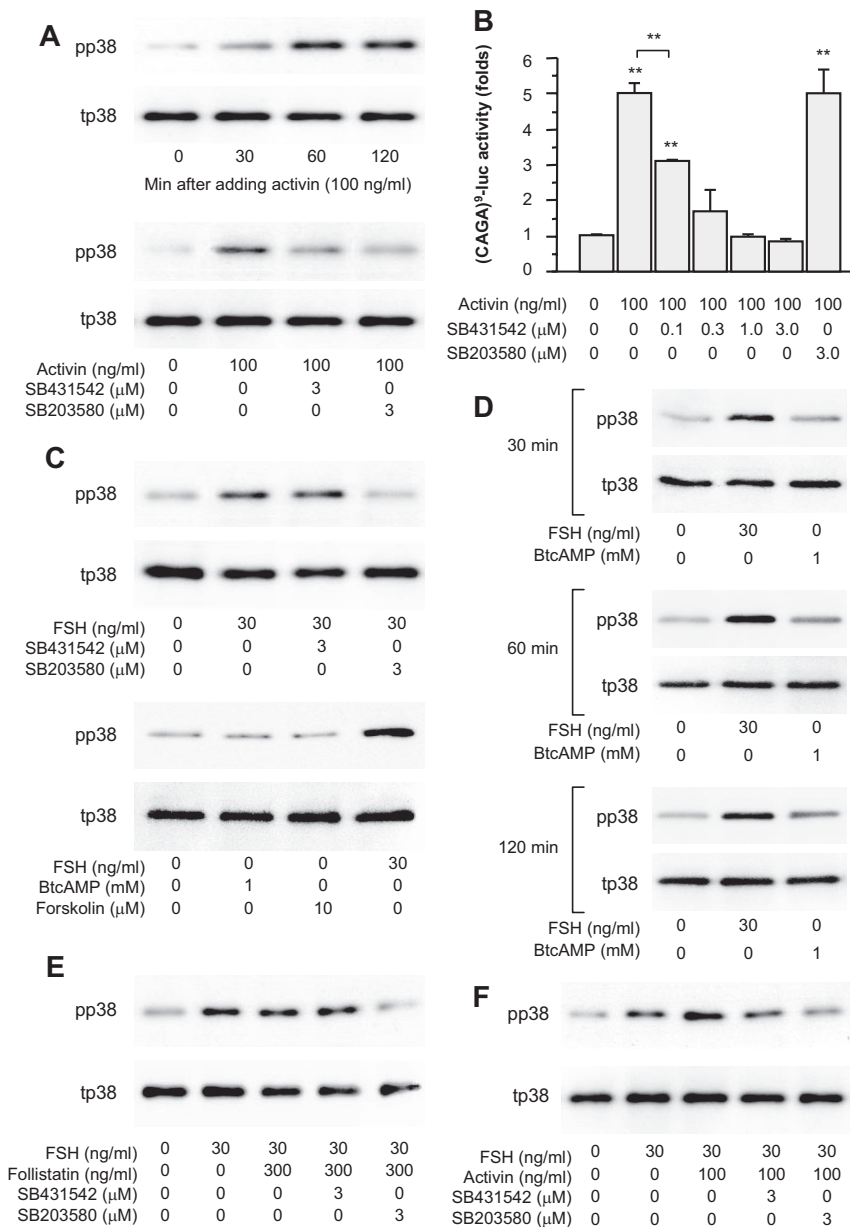
FIG. 5. Effects of activin and FSH on p38 MAPK phosphorylation by granulosa cells. A, Rat granulosa cells (5 × 10⁵ viable cells in 1 ml) were cultured in serum-free McCoy's 5A medium. After 3 h preculture, cells were treated with activin A (100 ng/ml) either alone or in combination with SB431542 (3 μM) and SB203580 (3 μM) for 30–120 min. Cell lysates were subjected for p38 MAPK analysis. B, Granulosa cells (5 × 10⁵ viable cells in 1 ml) were cultured in McCoy's 5A medium. After 3 h preculture, the cells were transiently transfected with 500 ng luciferase reporter plasmid (CAGA)⁹-Luc and 50 ng pCMV-β-gal. After transfection for 12 h, cells were treated with activin A (100 ng/ml) either alone or in combination with SB431542 (0.1–3 μM) and SB203580 (3 μM) for 24 h. Luciferase activity and βgal activity of the cell lysate were measured by luminescencer. 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 *vs.* control or between the indicated groups. C, Granulosa cells were treated with FSH (30 ng/ml), BtcAMP (1 mM), or FSK (10 μM) either alone or in combination with SB431542 (3 μM) and SB203580 (3 μM) for 60 min. D, Granulosa cells were treated with FSH (30 ng/ml) or BtcAMP (1 mM) for 30–120 min. E and F, Cells were treated with FSH (30 ng/ml) either alone or in combination with SB431542 (3 μM), SB203580 (3 μM), follistatin (300 ng/ml), and activin A (100 ng/ml) for 60 min. Cells were then lysed and subjected to SDS-PAGE/immunoblotting analysis using antiphospho- and antitotal-p38 MAPK antibodies.

In comparison with the critical roles of activin in increasing the FSH sensitivity in the early growing follicles, we demonstrated a novel cellular mechanism in the regulation of granulosa steroidogenesis involving interactions between oocytes, FSH, BMP-2, and BMP-4 (Fig. 9). Previous studies with sheep granulosa cells have shown that BMP-2 stimulates FSH-induced estradiol and inhibin A production (33). BMP-2 also stimulates both the expression of the inhibin/activin βB-subunit and secretion of dimeric inhibin B in cultures of human granulosa-lutein cells (34). In addition, theca-derived BMP-4 suppresses FSH-induced progesterone production by rat and sheep granulosa cells (10, 35). These data suggest a possible role of BMP-2 and -4 in regulating the luteinizing process. In the present study, we discovered a new functional paradigm involving BMP-2 and BMP-4 enhancement of estradiol production by granulosa cells (Fig. 9). Notably, BMP-2 and BMP-4 up-regulated FSH-induced estradiol production, which was further enhanced in the presence of oocytes. BMP-2 and BMP-4 differentially regulate FSH-induced steroidogenesis by stimulating FSH-induced p38 MAPK activation as well as suppressing cAMP production in granulosa cells (Fig. 9).

Although the importance of BMPs in the ovary is now well established (36), specific BMP signaling pathways and their particular regulatory roles in granulosa cells have yet to be elucidated completely. In granulosa cells, BMP-2, -6, -7, and -15 have stimulated the phosphorylation of Smad1/5/8 in human and rat granulosa cells (37, 38). The receptors for TGF-β superfamily members consist of type I and II receptors, each of which exhibits 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 BMP type II receptors (BMPRIIs) and the appropriate type I receptor (39). The combinations of BMP ligands and receptors are not completely definitive, in which BMP-2 and -4 can readily bind to ALK-2 and/or ALK-6 (40–42) with having a high affinity to BMPRII. In this regard, we recently reported that FSH preferentially up-regulates the expression of type I receptors, ALK-3 and ALK-6, as well as type II receptors, activin type II receptor and BMPRII (43). FSH further increased expression levels of Smad1/5/8 and decreased expression of inhibitory Smads, Smad6/7, suggesting
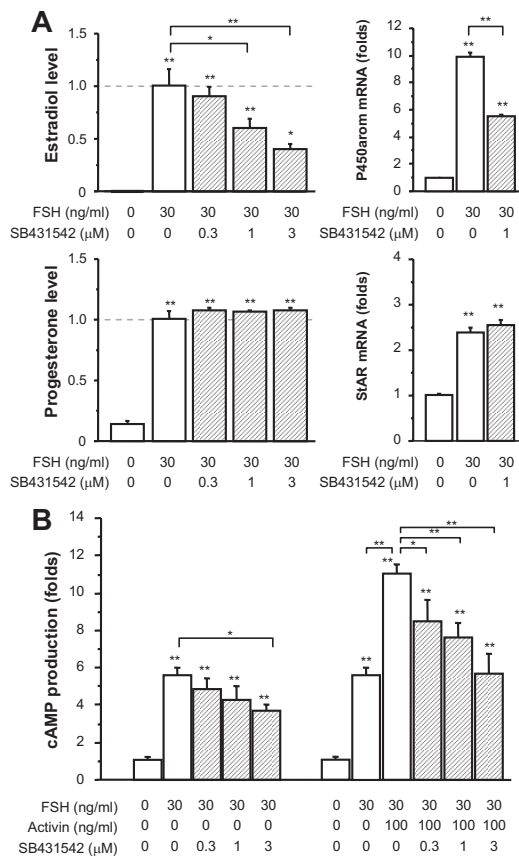
production regardless of the developmental stage. However, activin promotes FSH-stimulated progesterone production by undifferentiated granulosa cells, but not by partially differentiated granulosa cells (30–32). These findings indicate that activin specifically stimulates the early differentiation of granulosa cells.

FIG. 6. Effects of an ALK-4, -5, and -7 inhibitor, SB431542, on FSH-induced steroidogenesis. A, Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured with serum-free McCoy's 5A medium containing 100 nM androstenedione (a substrate for P450arom). FSH (30 ng/ml) was added to the culture medium either alone or in combination with SB431542 (0.3–3 μM). After 48 h culture, the levels of estradiol and progesterone in the media were determined by RIA. Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured with serum-free McCoy's 5A medium. Total cellular RNA was also extracted in 48 h culture after treating cells with FSH (30 ng/ml) in combination with SB4321542 (1 μM). For the quantification of P450arom and StAR mRNA levels, real-time PCR was performed, and the expression levels of target genes were standardized by RPL19 level in each sample. B, Granulosa cells (1×10^5 viable cells in 200 μl) were cultured with serum-free McCoy's 5A medium containing 0.1 mM IBMX. FSH (30 ng/ml) was added to the culture medium either alone or in combination with activin A (100 ng/ml) and SB431542 (0.3–3 μM). After 48 h culture, the extracellular contents of cAMP in the conditioned medium were determined by enzyme immunoassay after the acetylation of each sample. Results show the mean $\pm$ SEM of data performed with triplicate treatments. **, $P < 0.01$ and *, $P < 0.05$ vs. control or between the indicated groups.

FIG. 7. Effects of a p38 inhibitor SB203580 on FSH-induced steroidogenesis. A, Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured with serum-free McCoy's 5A medium containing 100 nM androstenedione (a substrate for P450arom). FSH (30 ng/ml) was added to the culture medium either alone or in combination with SB203580 (0.3–10 μM). After 48 h culture, the levels of estradiol and progesterone in the media were determined by RIA. Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured with serum-free McCoy's 5A medium. Total cellular RNA was also extracted in 48 h culture after treating cells with FSH (30 ng/ml) in combination with SB203580 (3 μM). For the quantification of P450arom and StAR mRNA levels, real-time PCR was performed, and the expression levels of target genes were standardized by RPL19 level in each sample. B, Granulosa cells (1×10^5 viable cells in 200 μl) were cultured with serum-free McCoy's 5A medium containing 0.1 mM IBMX. FSH (30 ng/ml) was added to the culture medium either alone or in combination with activin A (100 ng/ml) and SB203580 (0.3–3 μM). After 48 h culture, the extracellular contents of cAMP in the conditioned medium were determined by enzyme immunoassay after the acetylation of each sample. Results show the mean $\pm$ SEM of data performed with triplicate treatments. **, $P < 0.01$ and *, $P < 0.05$ vs. control or between the indicated groups.

that BMP-Smad signaling can be facilitated by FSH actions in granulosa cells (12, 43).

In addition to the Smad1/5/8 pathway induced by BMP ligands, involvement of MAPK signaling has also been implicated in granulosa cells (12, 18–25). As for the functional link between MAPK and BMP system in the ovary, it was reported that inhibitors of ERK1/2 phosphorylation suppress granulosa cell mitosis induced by BMP-15 (38) and cumulus cell expansion induced by GDF-9 (44). 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 (45). Considering that ERK inhibition increased estradiol (19) and simultaneously decreased progesterone and cAMP production induced by FSH (12), ERK activation leads to inhibition
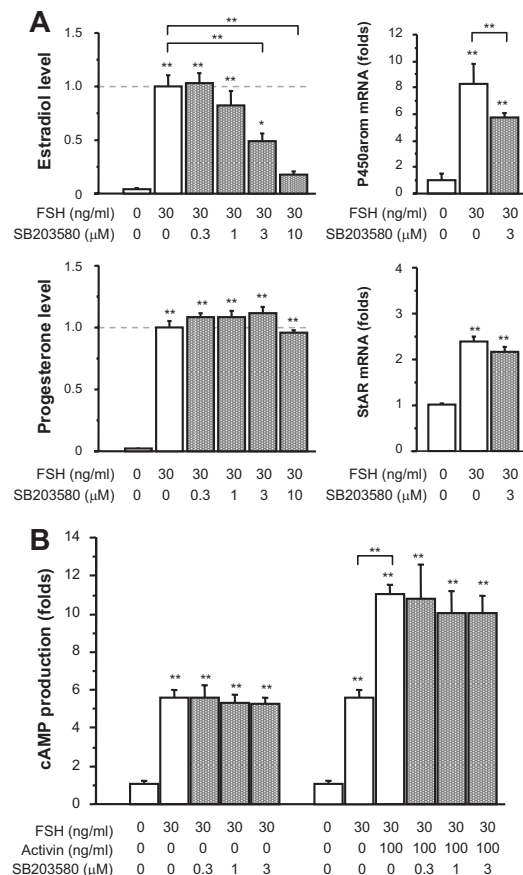
of estradiol synthesis and amplification of cAMP. Furthermore, we recently reported that FSH-induced ERK1/2 phosphorylation is suppressed by BMP-7 but not affected by BMP-6, leading to the enhancement of FSH-induced estradiol production by BMP-7 (12).

FSH also stimulates the rapid phosphorylation of p38 MAPK in granulosa cells (46). The early stimulation with cAMP compounds was less effective on p38 phosphorylation compared with FSH effects in our time-course study. It is also reported that FSH-responsive activation of p38 MAPK is dependent on PKA based on an inhibition study with H89 (25, 47). However, the PKA target that regulates p38 MAPK activity has not been identified. Based on inhibition by the p38 MAPK inhibitor SB203580, FSH-stimulated activation of p38 MAPK leads to
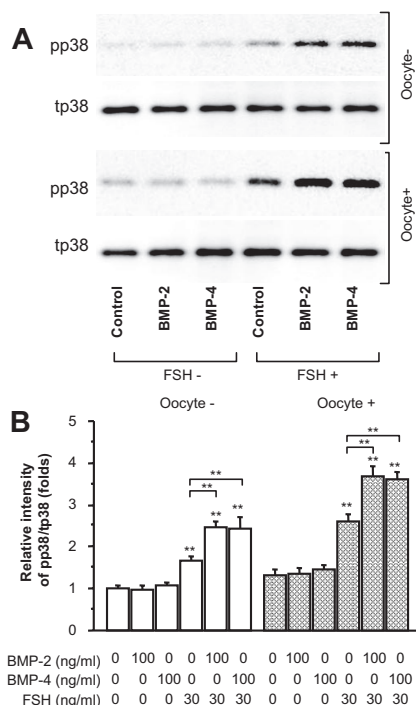
FIG. 8. Effects of BMP-2 and BMP-4 on FSH-induced p38 phosphorylation. A, Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured with or without oocytes (100 oocytes/ml) in serum-free McCoy's 5A medium. After 3 h preculture, cells were treated with FSH (30 ng/ml) either alone or in combination with BMP-2 and BMP-4 (100 ng/ml) for 1 h. The cells were then lysed and subjected to SDS-PAGE/immunoblotting analysis using antiphospho- and antitotal-p38 MAPK antibodies. B, The bands on the x-ray film were scanned, digitized, and the signal intensities were numerically converted. Results show the mean ± SEM of data performed with triplicate treatments. **, $P < 0.01$ *vs.* control or between the indicated groups.

FIG. 9. A possible mechanism by which BMP-2, BMP-4, and activin differentially regulate FSH-induced steroidogenesis in granulosa cells through the p38 pathway. The pathway of p38 MAPK activation is directly linked to stimulation of estradiol synthesis. Enhancement of FSH-induced p38 phosphorylation by BMP-2 and BMP-4 is likely to be a key process for up-regulating estradiol production. Activin not only increases FSH-receptor sensitivity but also directly activates p38 phosphorylation in granulosa cells. The presence of oocytes further facilitates FSH-induced p38 signaling in granulosa cells. In addition, BMP-2 and BMP-4 also commonly suppress cAMP synthesis, which is linked to progesterone suppression as a luteinizing inhibitor. Thus, BMP-2 and BMP-4 differentially regulate FSH-induced steroidogenesis by stimulating FSH-induced p38 MAPK activation as well as suppressing cAMP production in granulosa cells. The former mechanism is involved in estradiol production and enhanced by oocyte actions, whereas the latter leads to reduction of progesterone synthesis.

phosphorylation of the actin-capping protein HSP-27 in granulosa cells (47). This implies that phosphorylation of HSP-27 through the p38 pathway may be associated with the cytoskeletal reorganization of granulosa cells induced by FSH.

The regulation of FSH responsiveness in granulosa cells is critical for the establishment of dominant follicles and subsequent ovulation in mammals. FSH receptor signaling in granulosa cells is required for follicular selection and dominant follicle formation, which is precisely modulated by autocrine/paracrine factors within the follicles (36) in cooperation with estrogen (48). In this regard, we earlier discovered that oocyte action is required for estrogen to amplify FSH-dependent granulosa differentiation (48). Because the presence of oocytes also facilitates BMP-Smad signaling in granulosa cells regardless of FSH (12), the reciprocal effects between the BMP system, FSH receptor signaling, and oocyte actions may be crucial for regulating the development of growing follicles under the influence of estrogen produced by granulosa cells *per se*.

Possible oocyte factors that are responsible for the activation of p38 MAPK have yet to be elucidated in this study. Vanderhyden *et al.* (49–51) demonstrated a key role for oocytes in enhancing estradiol and suppressing progesterone production induced by FSH using mouse granulosa cells. In the present study, oocytes activated FSH induction of the p38 signaling pathway, leading to efficacious estrogen production by granulosa cells. Thus, oocytes modulate the signaling interaction between the BMP system and
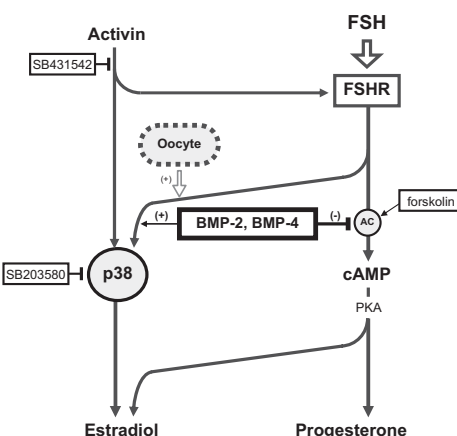
p38 signaling in granulosa cells, leading to effective induction of estrogen synthesis in response to FSH. Future profiling would be necessary to determine such oocyte-derived factors that regulate steroidogenesis in the developing follicles. However, the detailed mechanism of oocyte-somatic cell interaction for steroidogenesis remains uncertain.

Collectively, the present data demonstrate a novel "oocyte-granulosa cell communication" (Fig. 9). The pathway of p38 MAPK activation is directly linked to increase in estradiol synthesis. Enhancement of FSH-induced p38 phosphorylation by granulosa-derived BMP-2 and theca-derived BMP-4 is likely to be a key process for up-regulating estradiol production, in which oocytes further activate FSH induction of the p38 signaling pathway. In contrast, BMP-2 and BMP-4 simultaneously suppress cAMP synthesis, leading to progesterone reduction as a luteinizing inhibitor. Thus, oocytes modulate the signaling interaction between the BMP system and MAPK signaling in granulosa cells, which may be crucial for optimal regulation of FSH-induced steroidogenesis in developing follicles.

References

1. Xiao S, Robertson DM, Findlay JK 1992 Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology 131:1009–1016

2. Shimasaki S, Moore RK, Erickson GF, Otsuka F 2003 The role of bone morphogenetic proteins in ovarian function. Reprod Suppl 61:323–337

3. Shimasaki S, Moore RK, Otsuka F, Erickson GF 2004 The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72–101

4. Hasegawa Y, Miyamoto K, Abe Y, Nakamura T, Sugino H, Eto Y, Shibai H, Igarashi M 1988 Induction of follicle stimulating hormone receptor by erythroid differentiation factor on rat granulosa cell. Biochem Biophys Res Commun 156:668–674

5. Guo Q, Kumar TR, Woodruff T, Hadsell LA, DeMayo FJ, Matzuk MM 1998 Overexpression of mouse follistatin causes reproductive defects in transgenic mice. Mol Endocrinol 12:96–106

6. Nishimori K, Matzuk MM 1996 Transgenic mice in the analysis of reproductive development and function. Rev Reprod 1:203–212

7. 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

8. 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

9. 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

10. 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

11. 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

12. Miyoshi T, Otsuka F, Inagaki K, Otani H, Takeda M, Suzuki J, Goto J, Ogura T, Makino H 2007 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. Endocrinology 148:337–345

13. 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

14. Eppig JJ 2001 Oocyte control of ovarian follicular development and function in mammals. Reproduction 122:829–838

15. Matzuk MM, Burns KH, Viveiros MM, Eppig JJ 2002 Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296:2178–2180

16. Inouye S, Guo Y, DePaolo L, Shimonaka M, Ling N, Shimasaki S 1991 Recombinant expression of human follistatin with 315 and 288 amino acids: chemical and biological comparison with native porcine follistatin. Endocrinology 129:815–822

17. 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

18. 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

19. 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

20. 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

21. 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

22. 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

23. 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

24. 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

25. 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

26. Cuenda A, Rousseau S 2007 p38 MAP-kinases pathway regulation, function and role in human diseases. Biochim Biophys Acta 1773:1358–1375

27. Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ, Hill CS 2002 SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol 62:65–74

28. Gilchrist RB, Ritter LJ, Myllymaa S, Kaivo-Oja N, Dragovic RA, Hickey TE, Ritvos O, Mottershead DG 2006 Molecular basis of oocyte-paracrine signalling that promotes granulosa cell proliferation. J Cell Sci 119:3811–3821

29. Kusanagi K, Kawabata M, Mishima HK, Miyazono K 2001 α-helix 2 in the amino-terminal mad homology 1 domain is responsible for specific DNA binding of Smad3. J Biol Chem 276:28155–28163

30. Miro F, Smyth CD, Hillier SG 1991 Development-related effects of recombinant activin on steroid synthesis in rat granulosa cells. Endocrinology 129:3388–3394

31. Shukovski L, Findlay JK, Robertson DM 1991 The effect of follicle-stimulating hormone-suppressing protein or follistatin on luteinizing bovine granulosa cells *in vitro* and its antagonistic effect on the action of activin. Endocrinology 129:3395–3402

32. Hillier SG, Miro F 1993 Inhibin, activin, and follistatin. Potential roles in ovarian physiology. Ann NY Acad Sci 687:29–38

33. Souza CJ, Campbell BK, McNeilly AS, Baird DT 2002 Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry. Reproduction 123:363–369

34. Jaatinen R, Bondestam J, Raivio T, Hilden K, Dunkel L, Groome N, Ritvos O 2002 Activation of the bone morphogenetic protein signaling pathway induces inhibin β(B)-subunit mRNA and secreted inhibin B levels in cultured human granulosa-luteal cells. J Clin Endocrinol Metab 87:1254–1261

35. Mulsant P, Lecerf F, Fabre S, Schibler L, Monget P, Lanneluc I, Pisselet C, Riquet J, Monniaux D, Callebaut I, Cribiu E, Thimonier J, Teyssier J, Bodin L, Cognie Y, Chitour N, Elsen JM 2001 Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Mérino ewes. Proc Natl Acad Sci USA 98:5104–5109

36. 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

37. 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

38. Moore RK, Otsuka F, Shimasaki S 2003 Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. J Biol Chem 278:304–310

39. Miyazono K, Kusanagi K, Inoue H 2001 Divergence and convergence of TGF-β/BMP signaling. J Cell Physiol 187:265–276

40. 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

41. 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

42. 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

43. 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

44. Su YQ, 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

45. **Liao WX, Moore RK, Otsuka F, Shimasaki S** 2003 Effect of intracellular interactions on the processing and secretion of bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9. Implication of the aberrant ovarian phenotype of BMP-15 mutant sheep. J Biol Chem 278:3713–3719

46. **Salvador LM, Park Y, Cottom J, Maizels ET, Jones JC, Schillace RV, Carr DW, Cheung P, Allis CD, Jameson JL, Hunzicker-Dunn M** 2001 Follicle-stimulating hormone stimulates protein kinase A-mediated histone H3 phosphorylation and acetylation leading to select gene activation in ovarian granulosa cells. J Biol Chem 276:40146–40155

47. **Maizels ET, Cottom J, Jones JC, Hunzicker-Dunn M** 1998 Follicle stimulating hormone (FSH) activates the p38 mitogen-activated protein kinase pathway, inducing small heat shock protein phosphorylation and cell rounding in immature rat ovarian granulosa cells. Endocrinology 139:3353–3356

48. **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

49. **Vanderhyden BC, Cohen JN, Morley P** 1993 Mouse oocytes regulate granulosa cell steroidogenesis. Endocrinology 133:423–426

50. **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

51. **Vanderhyden BC** 1996 Oocyte-secreted factors regulate granulosa cell steroidogenesis. Zygote 4:317–321