A Novel Antagonistic Effect of the Bone Morphogenetic Protein System on Prolactin Actions in Regulating Steroidogenesis by Granulosa Cells

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To investigate the mechanism by which prolactin (PRL) regulates follicular steroidogenesis in the ovary, we examined the functional roles of PRL in steroidogenesis using rat oocyte/granulosa cell coculture and focusing on the bone morphogenetic protein (BMP) system. The expression of long and short forms of PRL receptor (PRLR) were detected in both oocytes and granulosa cells, and PRL effectively up-regulated PRLR expression in granulosa cells in the presence of FSH. PRL suppressed FSH-induced estradiol production and increased FSH-induced progesterone production in granulosa cells. The PRL effects on FSH-induced progesterone were blocked by coculture with oocytes, implying roles of oocyte-derived factors in suppression of progesterone production in PRL-exposed granulosa cells. In accordance with the data for steroids, FSH-induced aromatase expression was suppressed by PRL, whereas FSH-induced steroidogenic acute regulatory protein, P450scc (P450 side-chain cleavage enzyme), and 3β-hydroxysteroid dehydrogenase type 2 levels were amplified by PRL. However, forskolin- and N^6,O^2-dibutyryl cAMP-induced steroid levels and FSH- and forskolin-induced cAMP were not affected by PRL, suggesting that PRL action on FSH-induced steroidogenesis was not due to cAMP-protein kinase A regulation. Treatment with a BMP-binding protein, noggin, facilitated PRL-induced estradiol reduction, and noggin increased PRL-induced progesterone production in FSH-treated granulosa cells cocultured with oocytes, suggesting that endogenous BMPs reduce progesterone but increase estradiol when exposed to high concentrations of PRL. PRL increased the expression of BMP ligands in oocyte/granulosa cell coculture and augmented BMP-induced phosphorylated mothers against decapentaplegic 1/5/8 signaling by reducing inhibitory phosphorylated mothers against decapentaplegic 6 expression through the Janus kinase/signal transducer and activator of transcription (STAT) pathway. In addition to STAT activation, PRL enhanced FSH-induced MAPK phosphorylation in granulosa cells, in which ERK activation was preferentially involved in suppression of FSH-induced estradiol. Furthermore, noggin treatment enhanced PRLR signaling including MAPK and STAT. Considering that BMPs suppressed PRLR in granulosa cells, it is likely that the BMP system in growing follicles plays a key role in antagonizing PRLR signaling actions in the ovary exposed to high concentrations of PRL. (***Endocrinology*** **151: 5506–5518, 2010)**

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Abbreviations: ALK, Activin receptor-like kinase; BMP, bone morphogenetic protein; BMPRII, BMP type I and type II receptor; BtcAMP, N^6,O^2-dibutyryl adenosine-3′,5′-cyclic monophosphate monosodium salt; CLIA, chemiluminescent immunoassay; DES, diethylstilbestrol; FSHR, FSH receptor; FSK, forskolin; βgal, β-galactosidase; GDF, growth differentiation factor; 3βHSD, 3β-hydroxysteroid dehydrogenase; IBMX, 4-androstene-3,17-dione, 3-isobutyl-1-methylxanthine; JAK, tyrosine kinase Janus kinase; JNK, c-Jun NH2-terminal kinase; L-PRLR, long PRLR form; p, phosphorylated; P450arom, P450 aromatase; PLSD, protected least significant difference; P450scc, P450 side-chain cleavage enzyme; PI3K, phosphatidylinositol 3-kinase; PRL, prolactin; PRLR, PRL receptor; RPL19, ribosomal protein L19; SAPK, stress-activated protein kinase; Smad, phosphorylated mothers against decapentaplegic; S-PRLR, short PRLR form; StAR, steroidogenic acute regulatory protein; STAT, signal transducer and activator of transcription; t, total.

Elevation of serum prolactin (PRL) level is associated with various physiological and/or pathological conditions and is a major cause of amenorrhea. There has been accumulating evidence indicating that PRL exerts a direct inhibitory effect on gonadotropin actions in the ovary (1, 2), although it is recognized that PRL directly inhibits secretion of gonadotropins from the anterior pituitary. In granulosa cells, PRL inhibits estradiol production (1, 3–5) and stimulates progesterone production (6–8) by activating distinct signaling pathways in a differentiation-dependent manner (9). The mechanism by which excess PRL inhibits FSH-induced estradiol secretion in a variety of preovulatory follicle models has been reported to be due to the reduction of aromatase activity (4, 6, 10, 11). PRL also interferes with FSH action by suppressing LH receptor expression at sites downstream of cAMP synthesis in granulosa cells (12). However, the precise mechanism by which PRL alters the gonadotropin-induced steroidogenic capacity of granulosa cells remains unknown.

PRL regulates many functions in diverse target tissues through multiple PRL receptor (PRLR) isoforms of membrane-bound receptors (13, 14). These isoforms are alternative-splice variants of the primary transcript. PRLR is a member of the class I cytokine receptor superfamily that includes receptors for GH, leptin, erythropoietin, and several ILs. The two major PRLR isoforms in rodent ovaries and decidua are short (S-PRLR) and long (L-PRLR) forms, which differ in length and composition of the cytoplasmic tail (13, 14). Hormonal stimulation of L-PRLR has been shown to induce Janus kinase (JAK)-2 activation, PRLR phosphorylation, and the association and phosphorylation of signal transducer and activator of transcription (STAT) transcription factors (15, 16). This triggers STAT dimerization and nuclear translocation, which are necessary for PRL-dependent functions. In addition to the JAK/STAT pathway, PRL has been shown to activate other signaling pathways, including those of protein kinase C, phosphatidylinositol 3-kinase (PI3K), MAPK, and the Src family of tyrosine kinases (17, 18). However, the detailed mechanism by which PRL modulates steroidogenic activity remains uncertain.

Ovarian follicle growth and maturation occur as a result of complex interactions between pituitary gonadotropins and numerous autocrine/paracrine growth factors produced within the ovary. Recent studies have established the concept that members of the TGF-β superfamily, including bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activins, and inhibins, play key roles as autocrine/paracrine factors in female fertility in mammals (19–24). The regulation of FSH responsiveness in granulosa cells is critical for the establishment of dominant follicles and subsequent ovulation in mammals. FSH receptor (FSHR) signaling in granulosa cells is required for follicular selection and dominant follicle formation. The FSH-regulated follicle selection and dominant follicle formation are precisely modulated by autocrine/paracrine factors within the follicles (21) in cooperation with estrogen (25). BMPs play a key role in female fertility by regulating steroidogenesis and mitosis in granulosa cells. Furthermore, BMP ligands suppress FSH-induced progesterone production as a luteinizing inhibitor (20, 22). The major regulatory process of BMPs in folliculogenesis is control of FSHR signaling in granulosa cells.

In the present study, to elucidate the mechanism by which PRL controls follicular functions in the ovary, we investigated roles of PRL in ovarian steroidogenesis using rat oocyte/granulosa cell coculture and focusing on the interaction between PRL and the BMP system. We found that PRL up-regulates the activity of the endogenous BMP system including oocyte-derived BMP ligands and that PRL actions are in turn negatively regulated by BMPs. The BMP system in growing follicles may play a key role in neutralizing PRLR signaling in various physiological and/or pathological conditions due to hyperprolactinemia.

Materials and Methods

Reagents and supplies

Female Sprague Dawley rats were purchased from Charles River Laboratories (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), genistein, BSA, penicillin-streptomycin solution, and recombinant human luteotropic hormone (PRL) were from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human BMP-2, -4, -6, and -7 and mouse noggin were purchased from R&D Systems Inc. (Minneapolis, MN); the ERK inhibitor U0126, the P38-MAPK inhibitor SB203580 and the PI3K inhibitor LY294002 were from Promega Corp. (Madison, WI); the stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK) inhibitor SP600125 was from Biomol Laboratories Inc. (Plymouth Meeting, PA); and the JAK family tyrosine kinase inhibitor AG490 and the AKT inhibitor SH-5 were from Calbiochem (San Diego, CA). Plasmid of Id-1-Luc was kindly provided by Drs. Tetsuro Watabe and Kohei Miyazono (Tokyo University, Tokyo, Japan).

Primary culture of granulosa cells and coculture with oocytes

SILASTIC brand capsules (Dow Corning Corp., Midland, MI) containing 10 mg of DES were implanted in 22-d-old female Sprague Dawley rats 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 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 an oocyte/granulosa cell suspension that 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 an additional 40-μm nylon mesh (BD Falcon) that allowed granulosa cells but not oocytes to pass through (25, 26). 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.

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 of androstenedione, a substrate for P450 aromatase (P450arom). FSH (30 ng/ml), FSK (10 μM), or BtcAMP (1 mM) was added to the culture medium either alone or in combination with indicated concentrations of BMP-2, BMP-4, BMP-6, BMP-7, PRL, noggin, and various inhibitors. 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 a chemiluminescent immunoassay (CLIA) using Architect estradiol and progesterone kits (Abbott Co., Ltd., Tokyo, 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 the indicated treatments, the conditioned medium was collected and stored at −80 C until assay. The extracellular contents of cAMP were determined by an enzyme immunoassay (Assay Designs, Ann Arbor, MI) after acetylation of each sample with an assay sensitivity of 0.039 nM.

Cellular 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 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 FSH, PRL, BMPs, noggin, and AG490. 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 the sample was stored at −80 C until assay. Primer pairs for BMP-2, BMP-4, BMP-6, BMP-7, BMP-15, activin receptor-like kinase (ALK)-2, ALK-3, ALK-6, activin type II receptor, BMP type II receptor (BMPRII), phosphorylated mothers against decapentaplegic (Smad)-1 through Smad8, and ribosomal protein L19 (RPL19) were selected as reported previously (27–30). PCR primer pairs were selected from different exons of the corresponding genes as follows: FSHR, 229-249 and 439-458 (from GenBank accession no. NM_000145); P450arom, 1180-1200 and 1461-1481 (M33986); P450 side-chain cleavage enzyme (P450scc), 147-167 and 636-655 (J05156); S-PRLR, 815-835 and 938-958 (NM_001034111); L-PRLR, 815-835 and 1042-1062 (NM_001034111); steroidogenic acute regulatory protein (StAR), 395-415 and 703-723 (AB001349); 3β-hydroxysteroid dehydrogenase (3βHSD) type 2, 317-336 and 822-841 (M38179); and RPL19, 401-421 and 575-595 (J02650). The extracted RNA (1 μg) was subjected to a reverse transcriptase reaction using a first-strand cDNA synthesis system (Invitrogen) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (0.5 mM) at 42 C for 50 min and at 70 C for 10 min. Hot-start PCR was performed using $MgCl_2$ (1.5 mM), deoxynucleotide triphosphate (0.2 mM), and Taq DNA polymerase (2.5 U) (Invitrogen). The aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of each mRNA expression, real-time PCR was performed using a LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostics 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 for each product were analyzed by the second derivative method after melting-curve analysis (Roche Diagnostic), and then after assay validation by calculating each amplification efficiency, the expression levels of target genes were quantified on the basis of standard curve analysis for each product. For each transcript, all treatment groups were quantified simultaneously in a single LightCycler run (Roche Diagnostics). To correct for differences in RNA quality and quantity between samples, the expression levels of target gene mRNA were normalized by dividing the quantity of target gene by the quantity of RPL19 in each sample. The raw data of each target mRNA level (/RPL19) were statistically analyzed as indicated and then shown as fold changes in the figures.

Western immunoblot analysis

Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured in 12-well plates in serum-free McCoy's 5A medium. After preculture, cells were treated with BMPs either alone or in combination with PRL for 60 min. In another sets of experiments, cells were treated with FSH either alone or in combination with PRL for 15–60 min after preculture with noggin for 8 h. Cells were solubilized in 100 μl radioimmunoprecipitation assay lysis buffer (Upstate Biotechnology, 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/immunoblotting analysis using anti-phosphorylated (p) Smad1/5/8 antibody (Cell Signaling Technology, Inc., Beverly, MA), antiactin antibody (Sigma-Aldrich), anti-p- and anti-total (t) ERK1/2 MAPK antibody (Cell Signaling Technology), anti-p- and anti-t P38-MAPK antibody (Cell Signaling Technology), anti-p- and anti-tSAPK/JNK MAPK antibody (Cell Signaling Technology), and anti-p- and anti-tSTAT3/STAT5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For the detection of Smad6 protein expression, cells were treated with PRL either alone or in combination with AG490 for 48 h. Cells were then solubilized and the cell lysates were subjected to immunoblotting analysis using anti-Smad6 antibody (Cell Signaling Technology). The relative integrated density of each protein band was digitized by NIH image J (version 1.34s; National Institutes of Health, Bethesda, MD), and for evaluating the protein phosphorylation, ratios of the band intensities of phosphorylated proteins per total proteins or an internal control actin were calculated.

Transient transfection and luciferase assay

Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured in 12-well plates in serum-free McCoy's 5A medium. After 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 using FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN). After transfection for 12 h, cells were treated with PRL (100 ng/ml) either alone or in combination with BMPs (100 ng/ml) 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 are shown as ratio of luciferase to βgal activity.

Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The data of steroids and cAMP levels, luciferase activity, real-time PCR analysis, and immunoblot densities were subjected to ANOVA to determine differences (StatView 5.0 software; Abacus Concepts, Inc., Berkeley, CA). If differences were detected, Fisher's protected least significant difference (PLSD) test was used to determine which means differed (StatView 5.0 software). The mRNA data of BMP ligands (see Fig. 2D) were subjected to unpaired *t* test to determine differences (StatView 5.0 software). $P < 0.05$ was accepted as statistically significant.

Results

We first examined PRL effects on regulation of FSH-induced steroidogenesis by rat granulosa cells. PRL treatment significantly suppressed FSH-induced estradiol production by granulosa cells, whereas PRL increased FSH-induced progesterone production in a concentration-dependent manner (Fig. 1A). In accordance with the data for steroids, FSH-induced P450arom mRNA expression was suppressed by addition of PRL. FSH-induced StAR, P450scc, and 3βHSD mRNA levels were in turn amplified by PRL (Fig. 1B). By two-way factorial ANOVA (Fig. 1B), primary effects of FSH and PRL were individually significant ($P < 0.01$) in P450arom, StAR, P450scc, and 3βHSD mRNA levels, and there were significant interactions between FSH and PRL in mRNA levels of P450arom ($P < 0.01$), StAR ($P < 0.05$), P450scc ($P < 0.01$), and 3βHSD ($P < 0.01$). FSK- and BtcAMP-induced steroid levels were not affected by PRL (Fig. 1C). Primary effects of FSK and BtcAMP, but not PRL, on estradiol and progesterone levels were significant ($P < 0.01$) by two-way ANOVA, in which there was no significant interaction between FSK, BtcAMP, and PRL in the steroid production (Fig. 1C). In addition, cAMP production induced by FSH and FSK was not altered by PRL treatment (Fig. 1D), suggesting that PRL modulation of FSH-induced steroidogenesis was not due to cAMP-protein kinase A regulation.

We next used RT-PCR to detect expression of L-PRLR and S-PRLR in rat granulosa cells and oocytes, (Fig. 2A). FSH increased L-PRLR, S-PRLR, and FSHR mRNA expression by granulosa cells. PRL enhanced the expression of both forms of PRLR but did not affect FSHR expression. By two-way ANOVA (Fig. 2A), primary effect of FSH was significant ($P < 0.01$) in FSHR mRNA expression, but there was no significant interaction between FSH and PRL in the FSHR level. Notably, L-PRLR expression was effectively up-regulated by PRL in the presence of FSH. Regarding the L-PRLR mRNA level, primary effects of FSH and PRL and the interaction between FSH and PRL were significant ($P < 0.01$) by two-way ANOVA. In contrast, FSH and PRL did not show additive effects on S-PRLR expression (Fig. 2A). Regarding the S-PRLR mRNA level, primary effects of FSH and PRL were significant ($P < 0.05$), whereas the interaction between FSH and PRL was insignificant by two-way ANOVA. Bioassays further revealed that inhibitory effects of PRL on FSH-induced estradiol production were reversed in the presence of oocytes (Fig. 2B) and that the increase in FSH-induced progesterone production by PRL was blocked by coculture with oocytes, implying roles of oocyte-derived factors in suppression of progesterone production in PRL-exposed granulosa cells (Fig. 2B). Notably, treatment with a BMP-binding protein, noggin, which antagonizes the effects of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, GDF-5 and GDF-6, and vegetally localized protein-1 (31), facilitated PRL-induced estradiol reduction. Noggin also increased PRL-induced progesterone production in FSH-treated granulosa cells cocultured with oocytes in a dose-responsive manner (Fig. 2C), suggesting that endogenous BMPs act to reduce progesterone but increase estradiol production, especially when exposed to high concentrations of PRL. We next examined the impact of PRL on the expression of BMP ligands in oocyte/granulosa coculture. As shown in Fig. 2D, the expression levels of BMP ligands, including BMP-4, -6, and -15 but not BMP-2 and -7, were significantly increased by addition of PRL.

We next examined PRL effects on BMP-induced Smad1/5/8 signaling activity in granulosa cells. It was of interest that PRL augmented Smad1/5/8 phosphorylation induced by BMP-2, -4, -6, and -7 as revealed by Western immunoblots (Fig. 3A). By two-way ANOVA (Fig. 3A), primary effects of BMPs and PRL were significant ($P < 0.01$) in pSmad1/5/8(/actin) level, and there was significant interaction ($P < 0.05$) between BMPs and PRL in pSmad1/5/8 activation. The promoter activity of BMP target gene *Id-1*, which is stimulated by BMP-2, -4, -6, and -7, was also enhanced in the presence of PRL by granulosa cells (Fig. 3B). Primary effects of BMPs and PRL were significant ($P < 0.01$) in Id-1-Luc(/gal) level by two-way

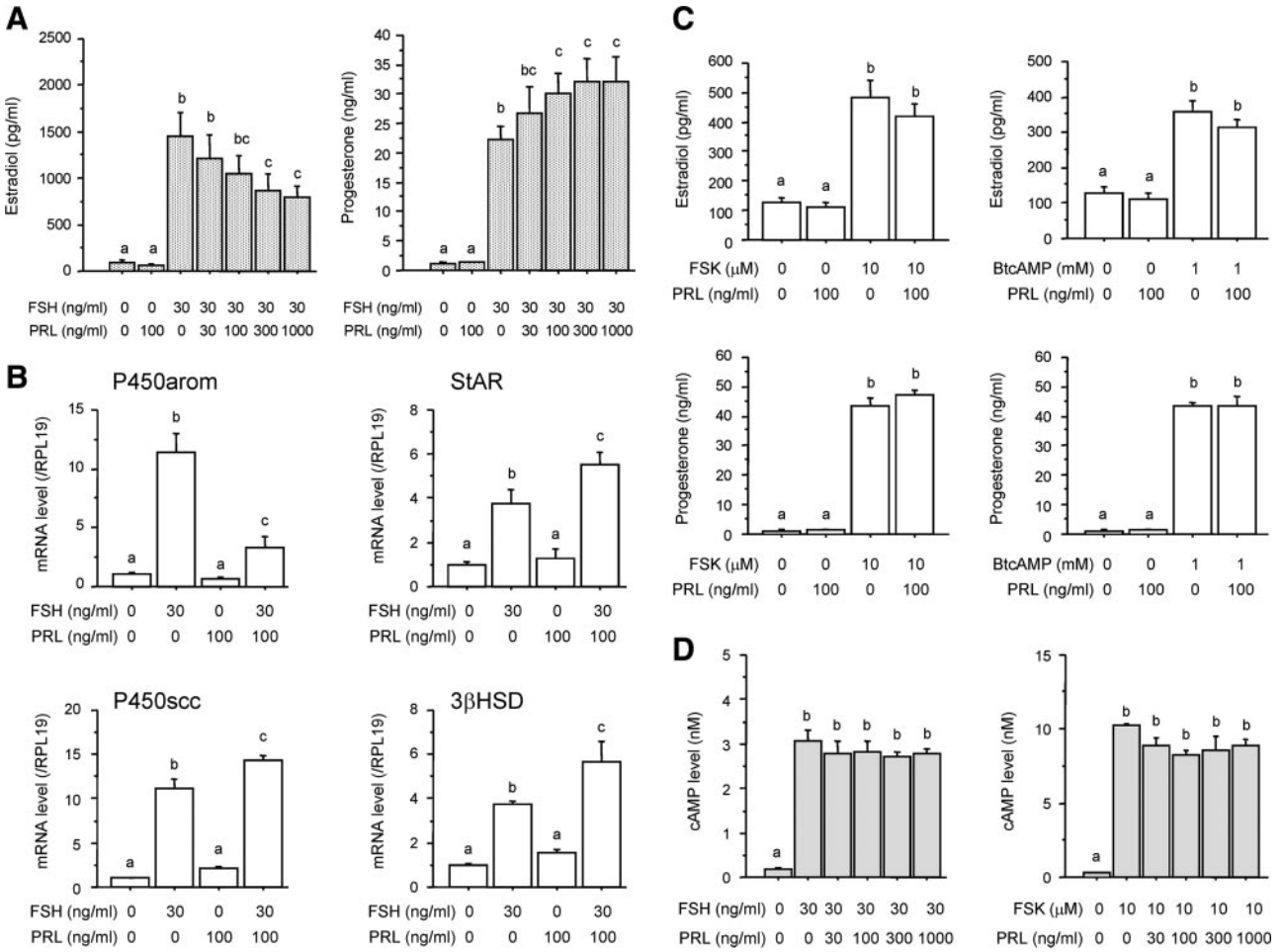

FIG. 1. Effects of PRL on FSH-induced steroidogenesis by rat granulosa cells. A, Effects of PRL on estradiol and progesterone production. Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured (without oocytes) in serum-free McCoy's 5A medium containing 100 nM of androstenedione (a substrate for aromatase). FSH (30 ng/ml) was added to the culture medium either alone or in combination with PRL (30–1000 ng/ml). After 48 h culture, the levels of estradiol and progesterone in the medium were determined by CLIA. B, Effects of PRL on steroidogenetic enzymes expression. Total cellular RNA was extracted from granulosa cells (5×10^5 viable cells in 1 ml, without oocytes) treated with FSH (30 ng/ml) and PRL (100 ng/ml) for 48 h, in which aromatase, StAR, P450scc, and 3βHSD mRNA levels were determined by quantitative real-time RT-PCR. The expression levels of target gene mRNA were standardized by RPL19 level in each sample, and then levels of mRNA of each target gene were expressed as fold changes. Note that there were significant interactions between FSH and PRL in the levels of P450arom ($P < 0.01$), StAR ($P < 0.05$), P450scc ($P < 0.01$), and 3βHSD ($P < 0.01$) by ANOVA. C, Effects on PRL on FSK- and BtcAMP-induced steroidogenesis. Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured (without oocytes) in serum-free McCoy's 5A medium containing 100 nM of androstenedione. FSK (10 μM) or BtcAMP (1 mM) was added to the culture medium either alone or in combination with PRL (100 ng/ml). After 48 h culture, the levels of estradiol and progesterone in the medium were determined by CLIA. Note that there was no significant interaction between FSK, BtcAMP, and PRL in steroid production by ANOVA. D, Effects of PRL on cAMP production induced by FSH and FSK. Granulosa cells (1×10^5 viable cells in 200 μl) were cultured (without oocytes) in serum-free McCoy's 5A medium containing 0.1 mM of IBMX. FSH (30 ng/ml) or FSK (10 μM) was added to the culture medium either alone or in combination with PRL (30–1000 ng/ml). After 48 h culture, the levels of cAMP in the medium were determined by enzyme immunoassay after acetylation of each sample. Results in all panels are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by two-way factorial ANOVA, and when a significant effect due to treatment was observed ($P < 0.05$), subsequent comparisons of groups means were conducted using Fisher's PLSD. For each result within a panel, values with *different superscript letters* are significantly different at $P < 0.05$.

ANOVA, in which there was significant interaction ($P < 0.01$) between BMPs and PRL in Id-1-Luc activity (Fig. 3B). To elucidate the mechanism by which PRL up-regulated BMP-Smad signaling by granulosa cells, the expression levels of BMP receptors and Smads were examined by quantitative PCR analysis. The expression levels of all BMP types 1 and 2 receptors were not significantly altered by PRL or FSH treatment (Fig. 3C). The expression levels of Smad1, -4, and -5 were not changed by PRL, whereas the level of Smad8 mRNA was increased by FSH but not affected by PRL (Fig. 3D). As for inhibitory Smads, the mRNA level of Smad7 was reduced by FSH but not by PRL treatment (Fig. 3E), whereas the Smad6 mRNA level was significantly reduced by PRL in granulosa cells. By two-way ANOVA (Fig. 3, D and E), the primary effect of FSH was significant in Smad8 ($P < 0.05$), Smad6 ($P < 0.01$), and Smad7 ($P < 0.01$) mRNA levels, and the primary effect of PRL was also significant in Smad6 level ($P <$

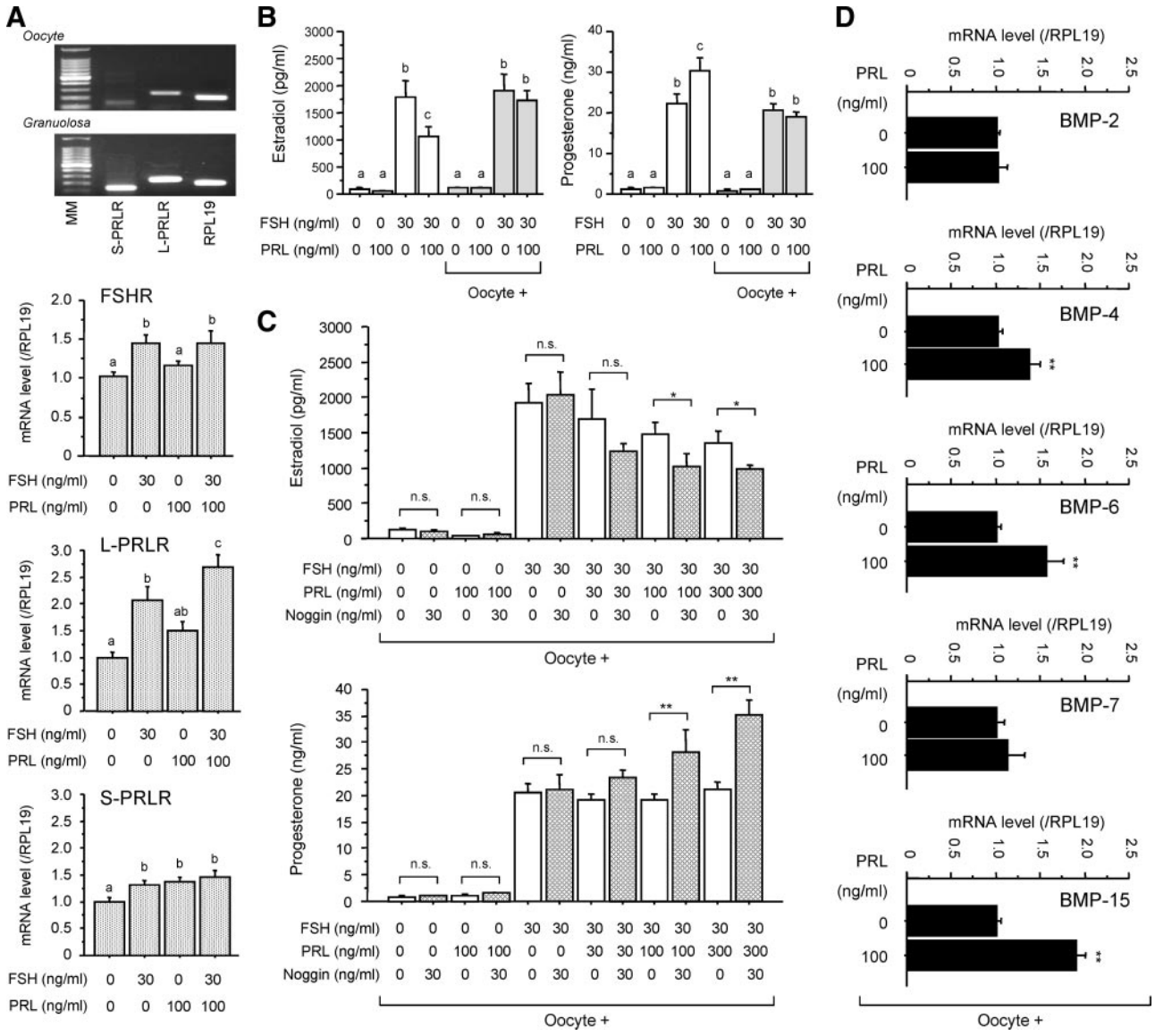

FIG. 2. Involvement of the endogenous BMP system in regulation of PRL effects on ovarian steroidogenesis. A, Expression of PRLRs in oocytes and granulosa cells. Total cellular RNAs extracted from oocytes and granulosa cells were subjected to RT-PCR for detecting the expression of S-PRLR and L-PRLR, respectively, and RPL19. Total cellular RNA was extracted from granulosa cells (5×10^5 viable cells in 1 ml, without oocytes) treated with FSH (30 ng/ml) and PRL (100 ng/ml) for 48 h, in which FSHR, L- PRLR, and S-PRLR mRNA levels were determined by quantitative real-time RT-PCR. The expression levels of target gene mRNA were standardized by RPL19 level in each sample, and then levels of mRNA of each target gene were expressed as fold changes. Note that there was significant interaction between FSH and PRL in the L-PRLR level ($P < 0.01$) but not in the FSHR or S-PRLR level by ANOVA. MM, molecular weight marker. B, Effects of oocytes on PRL regulation of FSH-induced steroids 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 of androstenedione (a substrate for aromatase). FSH (30 ng/ml) was added to the culture medium either alone or in combination with PRL (100 ng/ml). After 48 h culture, the levels of estradiol and progesterone in the medium were determined by CLIA. Note that there was significant interaction between FSH and PRL in estradiol ($P < 0.05$) and progesterone ($P < 0.01$) levels by ANOVA. C, Effects of noggin on PRL regulation on FSH-induced steroids production by oocyte/granulosa coculture. Rat granulosa cells (1×10^5 viable cells in 200 μl) were cultured with oocytes (100 oocytes/ml) in serum-free McCoy's 5A medium containing 100 nM of androstenedione (a substrate for aromatase). FSH (30 ng/ml) was added to the culture medium either alone or in combination with PRL (30–300 ng/ml) and noggin (30 ng/ml). After 48 h culture, the levels of estradiol (*upper panel*) and progesterone (*lower panel*) in the medium were determined by CLIA. D, Effects of PRL on expression of BMP ligands in oocyte/granulosa cell coculture. Total cellular RNA was extracted from oocyte/granulosa coculture (5×10^5 viable granulosa cells/ml with 100 oocytes/ml) treated with PRL (100 ng/ml) for 48 h, in which BMP-2, -4, -6, -7, and -15 mRNA levels were determined by quantitative real-time RT-PCR. The expression levels of target gene mRNA were standardized by RPL19 level in each sample, and then levels of mRNA of each target gene were expressed as fold changes. Results in all panels are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by two-way factorial ANOVA or unpaired *t* test, and when a significant effect due to treatment was observed by ANOVA ($P < 0.05$), subsequent comparisons of groups means were conducted using Fisher's PLSD. For each result within A and B, values with *different superscript letters* are significantly different at $P < 0.05$ and in C and D. *, $P < 0.05$ and **, $P < 0.01$ between the indicated groups. n.s., Not significant.

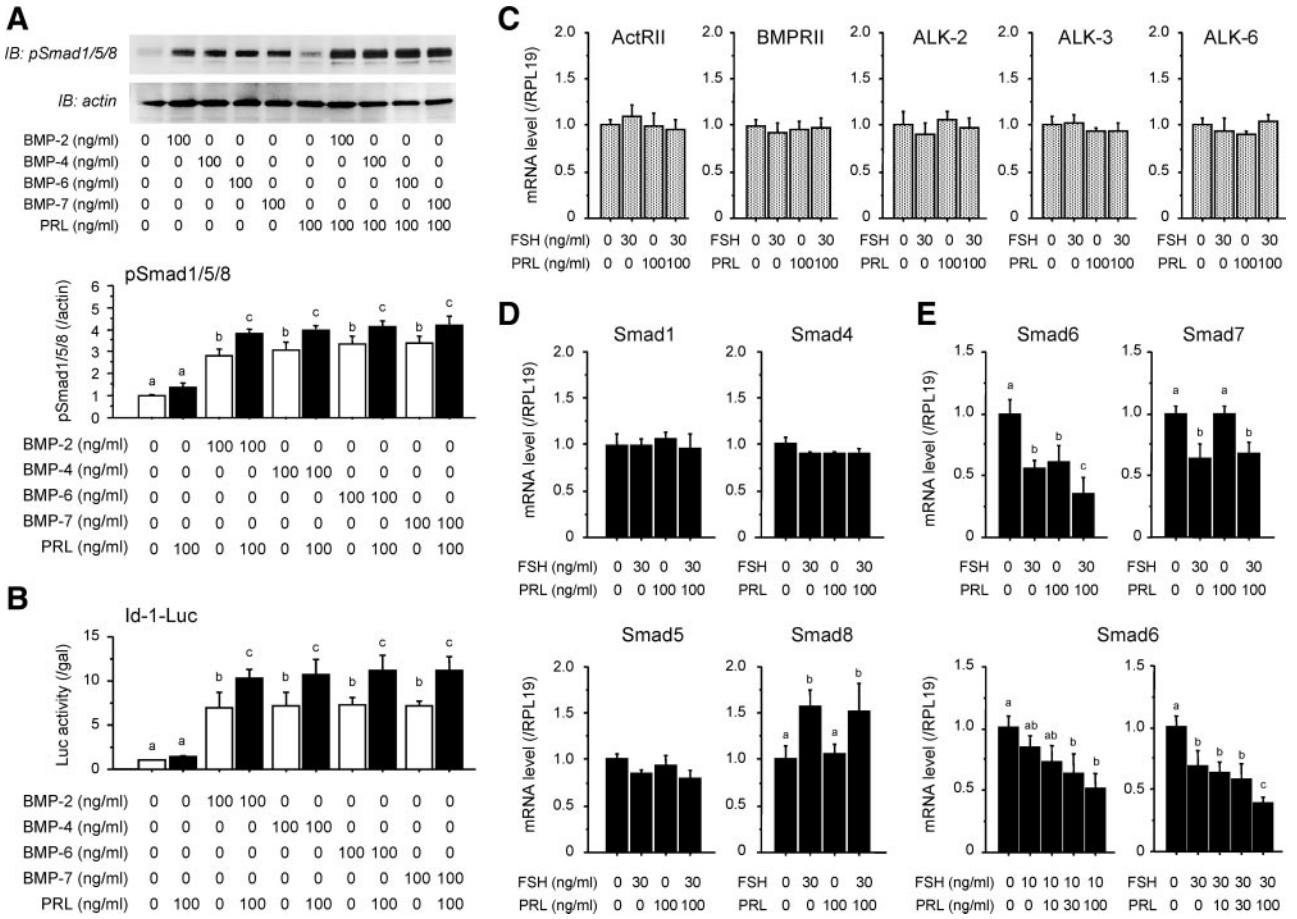

FIG. 3. Effects of PRL on BMP-Smad signaling in granulosa cells. A, Effects of PRL on pSmad1/5/8 induced by BMPs. Rat granulosa cells (5×10^5 viable cells in 1 ml, without oocytes) were cultured in serum-free McCoy's 5A medium. After preculture, BMP-2, -4, -6, and -7 (100 ng/ml) were added to the cultured media in combination with PRL (100 ng/ml) for 60 min. Cell lysates were subjected to SDS-PAGE/immunoblotting analysis for pSmad1/5/8 analysis. The results shown are representative of those obtained from three independent experiments. The relative integrated density of each protein band was digitized, pSmad1/5/8 levels were normalized by actin level in each sample, and then pSmad1/5/8 levels were expressed as fold changes. Note that there was significant interaction ($P < 0.05$) between BMPs and PRL in pSmad1/5/8 (/actin) level by ANOVA. B, Effects of PRL on Id-1-Luc activity induced by BMPs. After preculture, granulosa cells (5×10^5 viable cells in 1 ml, without oocytes) were transiently transfected with 500 ng of Id-1-Luc reporter plasmid and 50 ng cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal). After transfection, cells were treated with BMP-2, -4, -6, and -7 (100 ng/ml) in combination with PRL (100 ng/ml) for 24 h, and then luciferase activity and βgal activity of the cell lysate were measured. The data are shown as the ratio of luciferase to βgal activity and Id-1-Luc levels were expressed as fold changes. Note that there was significant interaction ($P < 0.01$) between BMPs and PRL in Id-1-Luc (/gal) activity by ANOVA. C–E, Effects of PRL on the expression of BMP receptors and Smads. Rat granulosa cells (5×10^5 viable cells in 1 ml, without oocytes) were cultured in serum-free McCoy's 5A medium with FSH (30 ng/ml) and PRL (100 ng/ml). After 48 h culture, total cellular RNA was extracted and activin type II receptor (ActRII), BMPRII, ALK-2, ALK-3, ALK-6 (C), Smad1, Smad4, Smad5 and Smad8 (D), and Smad6 and Smad7 (E) mRNA levels were determined by quantitative real-time RT-PCR. The expression levels of target gene mRNA were standardized by RPL19 level in each sample, and then levels of mRNA of each target gene were expressed as fold changes. Note that there was significant interaction ($P < 0.05$) between FSH and PRL in Smad6 mRNA level. Results in all panels are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by two-way factorial ANOVA, and when a significant effect due to treatment was observed ($P < 0.05$), subsequent comparisons of groups means were conducted using Fisher's PLSD. For each result within a panel, values with *different superscript letters* are significantly different at $P < 0.05$.

0.01), in which the interaction between FSH and PRL was significantly detected ($P < 0.05$). In addition, the PRL effects on Smad6 suppression were augmented in the presence of FSH in a concentration-responsive manner ($P < 0.05$) (Fig. 3E).

PRL is known to activate intracellular signaling such as JAK/STAT, MAPK, and PI3K-AKT pathways via activation of multiple isoforms of membrane-bound receptors including alternative splice variants of the short (S-PRLR) and long (L-PRLR) forms (18). As shown in Fig. 4A, PRL stimulated pSTAT3/5 that was detected at 60 min after the administration, but not at 15 min. FSH had no effect on pSTAT3/5. Of note, pSTAT3/5 induced by PRL was enhanced by pretreatment with noggin (Fig. 4A). By three-way ANOVA (Fig. 4A), primary effects of PRL and noggin, but not FSH, were significant ($P < 0.01$) in pSTAT3/tSTAT3 level, in which the interaction between FSH and PRL was significant ($P < 0.05$). The primary effects of

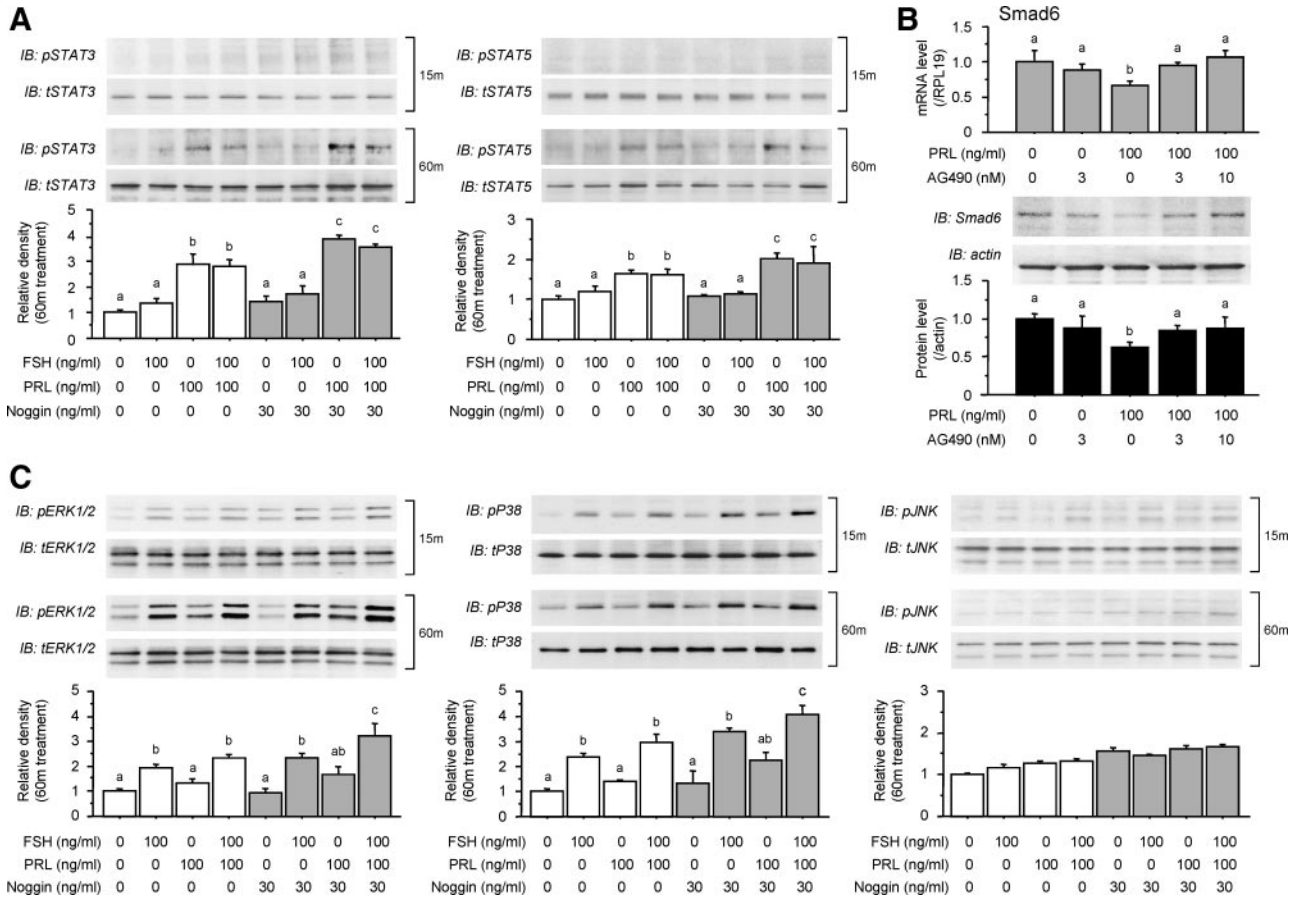

FIG. 4. Involvement of PRLR signaling and BMP system in steroidogenesis by granulosa cells. A, Effects of PRL and FSH on JAK/STAT phosphorylation. Rat granulosa cells (5×10^5 viable cells in 1 ml, without oocytes) were cultured in serum-free McCoy's 5A medium. After preculture with noggin (30 ng/ml), PRL (100 ng/ml) and FSH (100 ng/ml) were added to the cultured media for 15 and 60 min. Cell lysates were subjected to SDS-PAGE/immunoblotting (IB) for pSTAT3 and pSTAT5 analysis. The results shown are representative of those obtained from three independent experiments. The relative integrated density of each protein band was digitized, pSTAT3/5 levels were normalized by tSTAT3/5 level in each sample, and then phosphorylated STAT levels 60 min after stimulation were expressed as fold changes. Note that there was significant interaction between FSH and PRL ($P < 0.05$) in the pSTAT3/tSTAT3 level, and there were also significant interactions between FSH and noggin ($P < 0.05$) and between PRL and noggin ($P < 0.01$) in pSTAT5/tSTAT5 levels by ANOVA. B, Effects of JAK inhibition on Smad6 expression induced by PRL. Rat granulosa cells (5×10^5 viable cells in 1 ml, without oocytes) were cultured in serum-free McCoy's 5A medium with PRL (100 ng/ml) and AG490 (3–10 nM). After 48 h culture, total cellular RNA and protein were extracted and Smad6 levels were determined by quantitative real-time RT-PCR and immunoblots, respectively. For mRNA analysis, the expression levels of Smad6 mRNA were standardized by RPL19 level in each sample, and then levels of mRNA of each target gene were expressed as fold changes. For protein analysis, cell lysates were subjected to Western immunoblotting for Smad6. The results shown are representative of those obtained from three independent experiments. The relative integrated density of each protein band was digitized, Smad6 level was normalized by actin level in each sample, and then Smad6 levels were expressed as fold changes. C, Effects of PRL and FSH on MAPK phosphorylation. Rat granulosa cells (5×10^5 viable cells in 1 ml, without oocytes) were cultured in serum-free McCoy's 5A medium. After preculture with noggin (30 ng/ml), PRL (100 ng/ml) and FSH (30 ng/ml) were added to the cultured media for 15 and 60 min. Cell lysates were subjected to SDS-PAGE/immunoblotting for pERK1/2, P38-MAPK (pP38), and pJNK analysis. The results shown are representative of those obtained from three independent experiments. The relative integrated density of each protein band was digitized, pMAPK levels were normalized by tMAPK (including tERK1/2, tP38, and tJNK) levels in each sample, and then phosphorylated protein levels after 60 min stimulation were expressed as fold changes. Note that there were significant interactions between FSH and noggin ($P < 0.05$) in the pERK/tERK level and between FSH and noggin ($P < 0.05$) in the pP38/tP38 level, and no significant interaction was detected between FSH, PRL, and noggin in the pJNK/tJNK level by ANOVA. Results in all panels are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by three-way and two-way factorial ANOVA, and when a significant effect due to treatment was observed ($P < 0.05$), subsequent comparisons of groups means were conducted using Fisher's PLSD. For each result within a panel, values with *different superscript letters* are significantly different at $P < 0.05$.

FSH, PRL, and noggin were also significant ($P < 0.01$) in pSTAT5/tSTAT5 level, and the significant interactions were shown between FSH and noggin ($P < 0.05$) and between PRL and noggin ($P < 0.01$). Furthermore, the inhibition of Smad6 mRNA and Smad6 protein expression induced by PRL was reversed by cotreatment with AG490, a JAK family tyrosine-kinase inhibitor (Fig. 4B). In addition to the STAT activation, PRL enhanced FSH-induced phosphorylation of ERK1/ERK2 and P38-MAPK, in granulosa cells; however, PRL alone had no significant effects on MAPK activation (Fig. 4C). PRL had no effects on SAPK/JNK phosphorylation both alone and

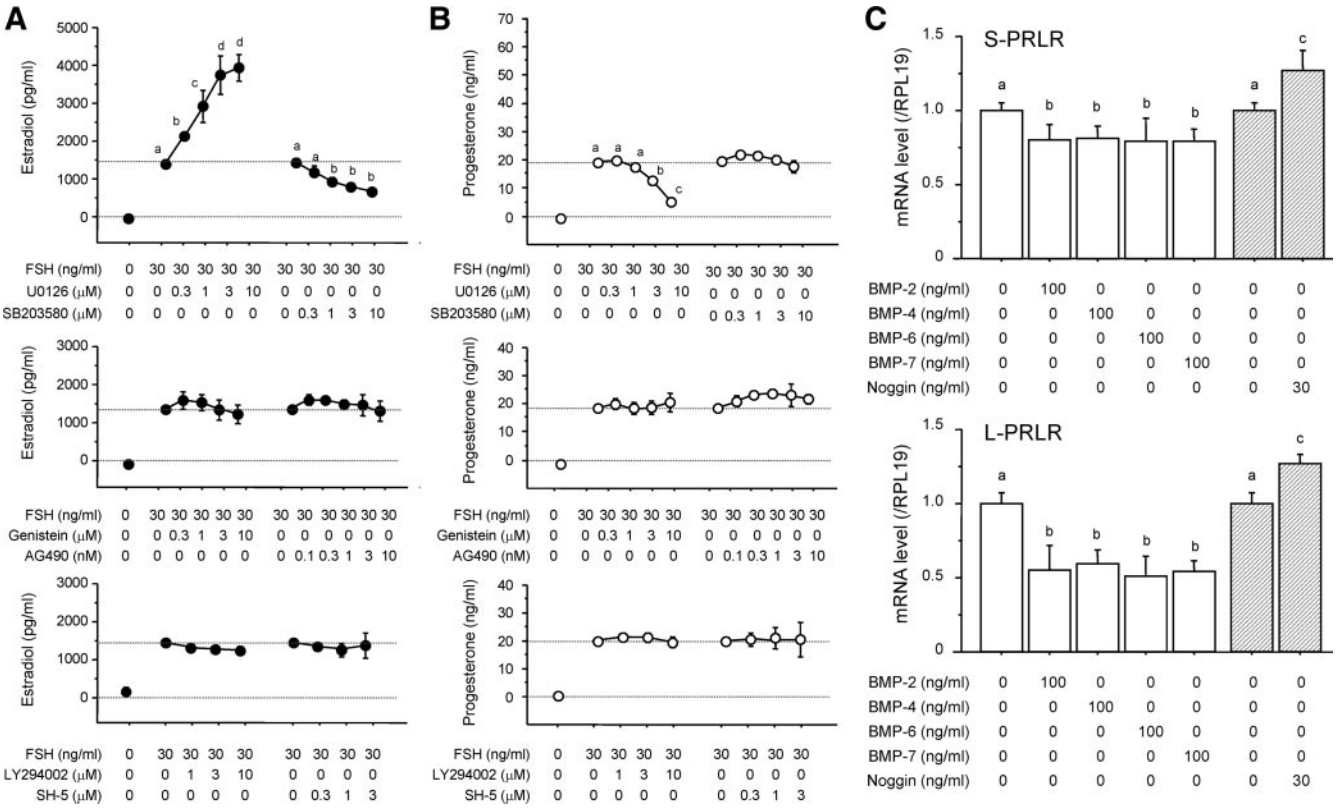

FIG. 5. Involvement of the MAPK pathway on FSH-induced steroidogenesis and BMP actions on the PRLR expression in granulosa cells. A and B, Inhibitory effects of the MAPK, JAK, and PI3K-AKT pathways on FSH-induced steroidogenesis. Rat granulosa cells (1×10^5 viable cells in 200 μl, without oocytes) were cultured in serum-free McCoy's 5A medium containing 100 nM of androstenedione. FSH (30 ng/ml) was added to the culture medium either alone or in combination with U0126 (0.3–10 μM), SB203580 (0.3–10 μM), genistein (0.3–10 μM), AG490 (0.1–10 nM), LY294002 (1–10 μM), and SH-5 (0.3–3 μM). After 48 h culture, the levels of estradiol (A) and progesterone (B) in the medium were determined by the CLIA method. C, Effects of BMPs and noggin on PRLR expression in granulosa cells. Rat granulosa cells (5×10^5 viable cells in 1 ml, without oocytes) were cultured in serum-free McCoy's 5A medium with BMP-2, -4, -6, and -7 (100 ng/ml) and noggin (30 ng/ml). After 48 h culture, total cellular RNA was extracted and S-PRLR and L-PRLR mRNA levels were determined by quantitative real-time RT-PCR. The expression levels of PRLR mRNA were standardized by RPL19 level in each sample, and then levels of mRNA of each target gene were expressed as fold changes. Results in all panels are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by two-way factorial ANOVA, and when a significant effect due to treatment was observed ($P < 0.05$), subsequent comparisons of groups means were conducted using Fisher's PLSD. For each result within a panel, values with *different superscript letters* are significantly different at $P < 0.05$.

in the presence of FSH. In accordance with the pSTAT, the effects of PRL on MAPK activation were also most detectable at 60 min after administration as compared with the levels of phosphorylation at 15 min. Noggin treatment also enhanced PRLR-signal activities including those of MAPKs (ERK1/ERK2 and P38-MAPK) especially in the presence of FSH stimulation (Fig. 4C). By three-way ANOVA (Fig. 4C), primary effects of FSH ($P < 0.01$), PRL ($P < 0.01$), and noggin ($P < 0.05$) were significant in the pERK/tERK level, in which the interaction between FSH and noggin was significant ($P < 0.05$). Regarding P38 signaling, primary effects of FSH, PRL, and noggin were significant ($P < 0.01$), and the interaction between FSH and noggin was significant ($P < 0.05$). Regarding the SAPK/JNK pathway, primary effects of FSH and PRL were significant ($P < 0.05$), although there was no significant interaction between FSH, PRL, and noggin.

Next, the involvement of the ERK1/ERK2 and P38-MAPK in steroidogenesis was examined using specific inhibitors for MAPKs. As seen in Fig. 5, A and B, FSH-induced estradiol production was increased by the ERK inhibitor U0126 but suppressed by the P38-MAPK inhibitor SB203580, whereas FSH-induced progesterone production was suppressed by U0126. In contrast, the JAK inhibitor and the tyrosine kinase inhibitors, AG490 and genistein, respectively, had no significant effects on the FSH-induced steroidogenesis by granulosa cells (Fig. 5, A and B). The PI3K inhibitor LY294002 and the AKT inhibitor SH-5 also did not affect the estradiol or progesterone production induced by FSH (Fig. 5, A and B). Thus, the PRL regulation on FSH-induced steroidogenesis was most likely modulated by ERK1/ERK2 signaling.

The impact of the BMP system on PRL sensitivity was also evaluated by quantifying PRLR mRNA expression levels in granulosa cells. As shown in Fig. 5C, the expression levels of both S-PRLR and L-PRLR were reduced by treatments with BMP-2, -4, -6, and -7. In contrast, the

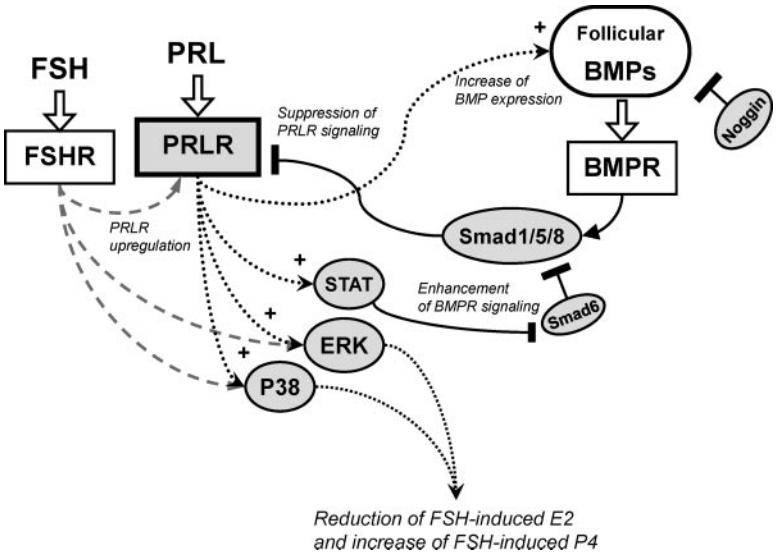

FIG. 6. Interrelationship between the PRL and the BMP system in regulating ovarian steroidogenesis induced by FSH. PRL suppresses estradiol (E2) production and increases progesterone (P4) production induced by FSH through augmenting FSH-induced MAPK activity with PRLR up-regulation in granulosa cells. PRL also up-regulates the expression of endogenous BMP ligands and Smad1/5/8 signaling activity by inhibiting Smad6 expression via the JAK/STAT pathway. On the other hand, BMPs suppress PRLR expression in granulosa cells. The BMP system in growing follicles may play a key role in antagonizing PRL actions in hyperprolactinemic conditions.

inhibition of endogenous BMP actions by noggin augmented expression levels of both S-PRLR and L-PRLR in granulosa cells, suggesting that the endogenous BMP system may play a key role in repressing PRL actions.

Discussion

PRL, a peptide hormone produced by the pituitary gland and extrapituitary tissues (32), is a key regulator of many reproduction-related physiological functions in mammals. The phenotype of null mutant PRLR mouse models reflect the importance of PRL in reproduction (33). Male and female PRLR-knockout mice exhibit multiple reproductive defects leading to infertility. Homozygous null females of PRLR are sterile and ovulate a reduced number of oocytes that are mostly immature, have low fertilization rates, exhibit an arrest of embryo development, have an altered oviduct microenvironment, and have a uterus that is refractory to implantation (33). Studies on follicular development in female PRLR-null mutant mice have shown that there were no differences in either follicular development or the ovulation and fertilization rate compared with wild-type animals (34). Taken together, analysis of the knockout models has led to a conclusion that PRL is essential for corpus luteum functions (18). Given that corpus luteum plays a central role in maintaining pregnancy by producing progesterone, the PRLR must be a key component for regulating ovarian function and governing the regulation of progesterone secretion. However, the precise mechanism by which PRL regulates gonadotropin-induced steroidogenesis remains unclear.

In the present study, we first demonstrated that PRL suppresses FSH-induced estradiol production through a reduction in aromatase expression and that PRL increases progesterone production by augmenting StAR, P450scc, and 3βHSD expression in granulosa cells. Importantly, the ERK1/ERK2 pathway, rather than cAMP-protein kinase A, is likely to be involved in the mechanism by which PRL modulates granulosa steroidogenesis induced by FSH (Fig. 6). We earlier reported the presence of oocyte-granulosa cell communication involving oocyte-derived factors and BMP receptor signaling elicited by BMP-2, -4, and -7 in granulosa cells through the enhancement of FSH-induced MAPK pathways (35, 36). In this regard, FSH-induced ERK1/ERK2 phosphorylation is suppressed by BMP-7, leading to the enhancement of FSH-induced estradiol production by BMP-7 (35). Enhancement of FSH-induced P38-MAPK phosphorylation by granulosa-derived BMP-2 and theca-derived BMP-4 is likely to be a key process for up-regulating estradiol production (36). Because ERK inhibition increased estradiol and simultaneously decreased progesterone production induced by FSH, the enhanced ERK signaling induced by FSH plus PRL may have led to inhibition of estradiol synthesis and amplification of progesterone in granulosa cells. Considering that the stimulatory effects of PRL on FSH-induced progesterone production in granulosa cells were reversed in the presence of oocytes, oocyte-derived factors were most likely involved in the suppression of progesterone production.

It has been shown that several oocyte-derived factors play central roles in the communication network between oocytes and somatic follicular cells, which are crucial for not only oocyte maturation but also growth and differentiation of surrounding granulosa and theca cells (37, 38). Given the finding that noggin reversed the oocyte effects on progesterone control by granulosa cells, it is possible that oocyte-derived BMPs, such as BMP-6 and -15, are involved in the reduction in FSH-induced progesterone levels in granulosa cells exposed to a high concentration of PRL. For instance, BMP-6 inhibits FSH-induced progesterone synthesis by suppressing adenylate cyclase activity

in granulosa cells (39). BMP-6 is expressed in oocytes and granulosa cells of healthy Graafian follicles (40). BMP-6 mRNA expression in granulosa cells rapidly decreases at the time the dominant follicle is selected (40), implying that BMP-6 is linked to the mechanism of dominant follicle selection. Moreover, BMP-15, which is specifically expressed by oocytes in the ovary, suppresses FSH action by inhibiting FSHR expression (41). These findings further support the hypothesis that BMPs are long-sought luteinization inhibitors in growing follicles. BMP ligands, including oocyte-derived BMP-6 and -15, were up-regulated in oocyte/granulosa cell coculture exposed to high concentrations of PRL. Also, PRL activated BMPR-Smad1/5/8 signaling by reducing Smad6 expression by granulosa cells. On the other hand, BMPs suppressed PRLR expression in granulosa cells, whereas noggin treatment increased PRLR expression. Thus, it is likely that the BMP system in growing follicles plays a key role in antagonizing PRL actions in hyperprolactinemic conditions (Fig. 6).

BMP ligands stimulate pSmad1/5/8 in granulosa cells. BMP ligands act together to form a high-affinity complex with BMPRIIs and the appropriate type I receptor (42, 43). FSH augments pSmad1/5/8 induced by BMPs by decreasing the expression of inhibitory Smads, Smad6/7, suggesting that BMP-Smad signaling can be facilitated by FSH actions in granulosa cells (35, 44). In the present study, it was found that PRL also contributes to the enhancement of BMP-Smad1/5/8 signal transduction by inhibiting Smad6 expression in granulosa cells. Furthermore, the expression of BMP-4, -6, and -15 mRNA was significantly up-regulated by PRL treatment in oocyte/granulosa cell coculture. In contrast, the expression levels of BMP-2 and -7 were not altered by PRL. The promoters for BMP-2, -4, and -7 have sequences that are similar but individual (45, 46); thus, the mechanism of differential modulation of BMP ligands expression in our cultures could be associated with the differences in transcriptional regulation of each BMP ligand in response to PRL. BMP-2 and -4/7 are expressed preferentially in granulosa cells and theca cells, respectively, secondary to dominant follicles (40). In our immature follicle cells, the expression levels of BMP-2 and -7 are low compared with that of oocyte-derived BMP-6 and-15 or endothelial-derived BMP-4. Given the present findings and results of previous studies showing PRLR distribution in granulosa cells and oocytes (47, 48), functional PRLR signaling is activated in not only granulosa cells but also oocytes in growing follicles and modulates endogenous BMP activity.

PRL exerts its actions by binding to specific membrane receptors. Long, short, and intermediate isoforms of PRLR (49) encoded by a single gene and produced by alternative splicing (50) have been described, and they are widely expressed in animal tissues. At the surface of the target cell, one PRL molecule binds to two PRLR molecules. However, most experimental evidence has shown that only binding of PRL to homodimers of the long isoform can induce proliferative or differentiative cell responses. It has also been shown that the binding to heterodimers, or to homodimers of the short isoform, may rather silence PRL actions (51, 52). Expression levels of S-PRLR have been reported to be stable during the estrous cycle, whereas the expression of L-PRLR exhibits remarkable changes throughout the estrous cycle affected by gonadotropins (48). Therefore, it is generally accepted that a correlation exists between being a target of PRL actions and the expression of L- PRLR. On the other hand, PRL signaling through S-PRLR remains obscure; however, it has been reported that PRL fails to activate the JAK/STAT pathway through the S-PRLR but can activate MAPK and PI3K pathways (18). In the present study, the expression of L-PRLR in granulosa cells was effectively enhanced by PRL and/or FSH and was also suppressed by BMPs compared with that of S-PRLR, indicating that L-PRLR is likely to be a functioning isoform in granulosa cells.

STAT activation is reported to occur at rapid phases of PRL stimulation in luteal granulosa cells (53). In our primary granulosa cell culture, the phosphorylation of STAT3/5 and MAPKs induced by PRL in combination with FSH was highly detected at 60 min after PRL stimulation compared with 15 min stimulation. This could be due to the expression levels of endogenous L-PRLR in granulosa cells isolated from early antral follicles. The level of L-PRLR expression in granulosa cells of early antral follicles is known to be much lower than that of preovulatory or luteal granulosa cells in the rat ovary (9). PRL also plays a unique role in both the rescue and continued function of the corpus luteum during pregnancy. One well-established function of PRL in the regulation of luteal function is its ability to stimulate estrogen receptor expression, thereby maintaining luteal responsiveness to estrogens. We previously discovered that oocytes are obligatory for estrogen-dependent augmentation of FSH action in rat granulosa cells, leading to efficacious estrogen production by granulosa cells (25). The detailed mechanism of oocyte-somatic cell interaction for steroidogenesis remains uncertain. Nevertheless, it can be postulated that PRL action may be involved in enhancing oocyte-granulosa cell communication by estrogen and BMP actions.

The PRLR is a member of the superfamily of cytokine/hematopoietic receptors. These receptors are characterized by four conserved cysteines, a Trp-Ser-X-Trp-Ser motif in their extracellular domain, and no intrinsic kinase activity (18). It is well established that the PRLR is asso-

ciated with JAK2 and activates JAK2 rapidly upon exposure to PRL (17). Signaling through JAK2 has been shown to be necessary for both PRL-induced proliferation and regulation of gene transcription by transactivation of STAT5 through phosphorylation of both STAT5a and STAT5b on specific tyrosine residues in the C terminus (17, 18). Based on the present results, we conclude that PRL-induced pSTAT3 and/or pSTAT5 is involved in the enhancement of BMP-Smad1/5/8 signal transduction by inhibiting Smad6 expression in granulosa cells. However, JAK/STAT seems not to be directly linked to FSH-induced steroidogenesis because the inhibition of JAK kinases by AG490 had no effect on FSH-induced steroidogenesis. Considering that BMP-Smad1/5/8 signaling is crucial for control of FSHR signaling, PRL action through the JAK/STAT pathway that suppresses Smad6 expression is indirectly, but functionally, involved in the regulation of bioavailability of FSH actions.

Collectively, as summarized in Fig. 6, a novel functional interrelationship between PRL and the BMP system in regulation of ovarian steroidogenesis was uncovered. PRL suppresses FSH-induced estradiol production by augmenting FSH-induced MAPK activity with PRLR up-regulation. PRL also enhances the expression of endogenous BMP ligands and Smad1/5/8 signaling activity by inhibiting Smad6 expression via the JAK/STAT pathway. On the other hand, BMPs suppress PRLR expression in granulosa cells. Therefore, it is likely that the BMP system in growing follicles plays a key role in antagonizing PRLR signaling actions in the ovary exposed to high concentrations of PRL (Fig. 6).

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