Biological Function and Cellular Mechanism of Bone Morphogenetic Protein-6 in the Ovary*

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The process of ovarian folliculogenesis is composed of proliferation and differentiation of the constitutive cells in developing follicles. Growth factors emitted by oocytes integrate and promote this process. Growth differentiation factor-9 (GDF-9), bone morphogenetic protein (BMP)-15, and BMP-6 are oocyte-derived members of the transforming growth factor-β superfamily. In contrast to the recent studies on GDF-9 and BMP-15, nothing is known about the biological function of BMP-6 in the ovary. Here we show that, unlike BMP-15 and GDF-9, BMP-6 lacks mitogenic activity on rat granulosa cells (GCs) and produces a marked decrease in follicle-stimulating hormone (FSH)-induced progesterone (P4) but not estradiol (E2) production, demonstrating not only the first identification of GCs as BMP-6 targets in the ovary but also its selective modulation of FSH action in steroidogenesis. This BMP-6 activity resembles BMP-15 but differs from GDF-9 activities. BMP-6 also exhibited similar action to BMP-15 by attenuating the steady state mRNA levels of FSH-induced steroidogenic acute regulatory protein (StAR) and P450 side-chain cleavage enzyme (P450scc), without affecting P450 aromatase mRNA level, supporting its differential function on FSH-regulated P4 and E2 production. However, unlike BMP-15, BMP-6 inhibited forskolin- but not 8-bromo-cAMP-induced P4 production and StAR and P450scc mRNA expression. BMP-6 also decreased FSH- and forskolin-stimulated cAMP production, suggesting that the underlying mechanism by which BMP-6 inhibits FSH action most likely involves the down-regulation of adenylate cyclase activity. This is clearly distinct from the mechanism of BMP-15 action, which causes the suppression of basal FSH receptor (FSH-R) expression, without affecting adenylate cyclase activity. As assumed, BMP-6 did not alter basal FSH-R mRNA levels, whereas it inhibited FSH- and forskolin- but not 8-bromo-cAMP-induced FSH-R mRNA accumulation. These studies provide the first insight into the biological function of BMP-6 in the ovary and demonstrate its unique mechanism of regulating FSH action.

One major direction of current research in the investigation of mechanisms controlling folliculogenesis is the identification of the biological functions of autocrine/paracrine factors that are produced in the ovary. Some of these molecules are synthesized and secreted by the oocyte (1) and act as morphogens to control follicle growth as well as differentiation (2). There is a large body of evidence indicating that oocyte-derived factors modulate ovarian function (1–3). In vitro experiments have shown that these factors can act to inhibit progesterone (P4) production (4), follicle-stimulating hormone (FSH)-induced expression of P450 side-chain cleavage enzyme (P450scc) (4), and luteinizing-hormone receptor (LH-R) mRNA (5), while acting to stimulate estradiol (E2) production (6) and granulosa cell (GC) mitosis (7). Three oocyte-derived members of the transforming growth factor-β (TGF-β) superfamily, namely growth differentiation factor-9 (GDF-9), bone morphogenetic protein-15 (BMP-15), and BMP-6 are, in particular, potentially involved in mediating these important biological consequences triggered by the putative oocyte factors.

Recently, there have been a number of studies published on the role of GDF-9 and BMP-15 in the ovary. With regard to GDF-9, female mice lacking GDF-9 have resulted in an early block in folliculogenesis leading to infertility, suggesting that GDF-9 is obligatory for normal folliculogenesis and female fertility (8–10). In vitro studies using rat and mice GCs have demonstrated that GDF-9 regulates GC mitogenesis and steroidogenesis as well as cumulus expansion (11–14). As for BMP-15, which is most closely related in structure to GDF-9, an intriguing finding has been reported recently by Galloway et al. (15), in which they identified a causative point mutation in the bmp-15 gene in Inverdale sheep that has been known to exhibit aberrant follicle development and ovulation rate. Of interest, the homozygous Inverdale female sheep are infertile because follicle growth is arrested at the primary preantral stage, whereas the heterozygous females exhibit increased ovulation rate. Thus this mutation causes increased ovulation and infertility in a dosage-sensitive manner, suggesting an important role for BMP-15 in folliculogenesis and ovulation. By in vitro studies with rat GCs, we have shown that BMP-15 stimulates mitosis independent of FSH and regulates steroidogenesis by inhibiting FSH receptor (FSH-R) expression (16, 17).

In contrast, although BMP-6 mRNA is highly expressed in both immature and mature oocytes of mice (10, 18), there have

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The abbreviations used are: P4, progesterone; AC, adenylate cyclase; BMP-6, bone morphogenetic protein-6; BMP-15, bone morphogenetic protein-15; DES, diethylstilbestrol; E2, estradiol; FSH, follicle-stimulating hormone; FSH-R, follicle-stimulating hormone receptor; GC, granulosa cell; GDF-9, growth differentiation factor-9; LH-R, luteinizing hormone receptor; P450arom, P450 aromatase; P450scc, P450 side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; TGF-β, transforming growth factor-β; 8-Br-cAMP, 8-bromo-cAMP; IBMX, 3-isobutyl-1-methylxanthine; RT-PCR, reverse transcription-polymerase chain reaction.
been few studies designed to investigate the biological functions and target cells of BMP-6 in the ovary. Mice with homozygous loss-of-function mutations in the bmp-6 gene by targeted deletion are fertile with normal sized litters (19), which may suggest that bmp-6 is dispensable for fertility. The lack of perturbative reproductive phenotype seen in these mutant mice could be the reflection of compensation by related BMPs (1) expressed in the ovary by virtue of a redundant safety system to maintain homeostasis. Alternatively, it is possible that BMP-6 evokes no biological effects in the ovary.

Because no information is available at present from any species about the biological function of BMP-6 in the ovary, its role in the ovary remains beyond speculation. In the present study, we report the identification of GCs as a target cell type for BMP-6 in the rat ovary. Furthermore, we report that BMP-6 evokes biological activities that are distinct from other TGF-β superfamily members and that it utilizes novel cellular mechanism in GCs.

**EXPERIMENTAL PROCEDURES**

**Reagents and Supplies—**Ovine FSH (NIDDK-dFSH-520) was produced by the National Hormone and Pituitary Program (Rockville, MD). Diethylstilbestrol (DES), forskolin, 8-bromo-cAMP (8-Br-cAMP), 3-isobutyl-1-methylxanthine (IBMX), and 4- androstenedion-3,17-dione (androstenedione) were purchased from Sigma and female Harlan Sprague-Dawley (SD) rats from Charles River Laboratories (Wilmington, MA). Recombinant human BMP-15 tagged with a FLAG epitope (BMP-15) was produced by 293 cells and purified using anti-FLAG monoclonal antibody as reported previously (16).

**Primary Cell Culture—**Twenty three-day-old female SD rats were implanted with silastic capsules containing 10 mg of DES to increase GC number. After 4 days of DES exposure, GCs were collected from the ovaries and cultured in serum-free McCoy’s 5a medium supplemented with 2 mM l-glutamine and antibiotics at 37 °C in an atmosphere of 5% CO₂ in air. The animal protocols were approved by the University of California at San Diego Institutional Animal Care and Use Committee.

**Analysis of Steroid and cAMP Production—**GCs (10⁵ viable cells) were cultured in a 96-well plate with 200 μl of medium containing one or a combination of the following: 0–10 ng/ml FSH, 0–300 ng/ml BMP-6, 10 μM forskolin, and 1 mM 8-Br-cAMP. For the assessment of steroids, 100 nM androstenedione, a substrate for P450 aromatase (P450arom), was added to the media. After 48 h culture, the supernatant of culture media was collected and stored at −80 °C until assay for steroids and CAMP. The levels of P₄ and E₂ in the media were measured by a radioimmunoassay (16). The extracellular content of cAMP in the medium was determined by cAMP enzyme immunoassay kit (Sigma) after the incubation of each sample.

**RNA Extraction and Analysis by Quantitative Competitive RT-PCR—**GCs (2 × 10⁵ viable cells) were cultured in a 6-well plate with 2 ml of McCoy’s 5a medium containing one or a combination of the following: 10 ng/ml FSH, 100 ng/ml BMP-6, 10 μM forskolin, and 1 mM 8-Br-cAMP. For the assessment of steroids, 100 nM androstenedione, a substrate for P450 aromatase (P450arom), was added to the media. After 48 h culture, total RNA was extracted by guanidinium acid/isothiocyanate/H₉₂₅₁ (16). The extracellular content of cAMP in the medium was determined by cAMP enzyme immunoassay kit (Sigma) after the incubation of each sample.

**Results**

We have first assumed that GCs are target cells for BMP-6 because rat GCs express BMP receptor type IB (BMPR-IB, also known as ALK-6) and type II (BMPR-II) at lower levels BMPR-IA (ALK-3) (28) as well as activin type II and type IIB receptors (29), all to which BMP-6 can bind (30). We have, therefore, tested whether GCs would respond to BMP-6. Similar to our previous studies on BMP-4, BMP-7, and BMP-15 (16, 28), we examined the effect of BMP-6 on P₄ and E₂ production induced by FSHI using primary rat GCs cultured in serum-free medium. As observed earlier (16, 28), FSH alone increased P₄ and E₂ production in a dose-dependent manner (Fig. 1). By comparison, BMP-6 alone (up to 300 ng/ml) did not affect basal levels of P₄ and E₂ production. However, co-treatment of GCs with a saturated dose of FSH (10 ng/ml) and increasing doses of BMP-6 (10–300 ng/ml) caused marked inhibition (80%) of the FSH-induced P₄ production (ED₅₀ = 48 ng/ml). On the contrary, BMP-6 had no effect on the FSH-induced E₂ production. These findings indicate that rat GCs express a functional type I and type II receptor set for BMP-6 and that P₄ synthesis induced by FSH is selectively down-regulated by virtue of BMP-6.

Based on these findings, we hypothesized that BMP-6 may be specifically involved in modulating the expression of steroi...
dogenic genes in response to FSH stimulation. To elucidate this hypothesis, we analyzed mRNA levels for three key regulators of GC steroidogenesis StAR, P450scс, and P450arom in the GCs treated with FSH and/or BMP-6 by a quantitative competitive RT-PCR. We have carefully validated and established this technique in our laboratory as an accurate technique for the quantification of RNA expression as reported in our previous studies (17). As shown in Fig. 2, treatment of GCs for 48 h with a saturating dose (100 ng/ml) of BMP-6 alone had no effect on the steady state mRNA levels for StAR, P450scс, P450arom and a housekeeping gene L19. Treatment with FSH, in contrast, markedly increased their mRNA levels except for L19. Interestingly, BMP-6 reduced FSH-stimulated StAR and P450scс mRNA levels to the basal levels but had no effect on P450arom mRNA level. L19 mRNA level showed no difference at all among these four different treatments. Given the fact that StAR and P450scс are major rate-limiting factors of P4 synthesis in GCs (31), the suppression of their mRNA expressions are likely to reflect the selective inhibition of FSH-induced P4 production. On the other hand, the failure of BMP-6 to inhibit FSH-stimulated P450arom (catalytic enzyme to convert androstenedione to E2) mRNA expression is consistent with that to inhibit FSH-induced E2 production.

To elucidate further the mechanism by which BMP-6 suppresses FSH-induced expression of StAR and P450scс, we utilized forskolin and 8-Br-cAMP that mimic FSH action in steroid synthesis by bypassing FSH-R and G proteins. Forskolin is a direct activator of adenylate cyclase (AC) and 8-Br-cAMP is a stable analog of cAMP. As shown in Fig. 3, forskolin (10 μM) stimulated the expression of StAR and P450scс mRNA, whereas BMP-6 suppressed the forskolin-induced mRNA levels of StAR and P450scс, similar to those had seen with the FSH treatment. However, in striking contrast to the FSH and forskolin results, BMP-6 failed to change the 8-Br-cAMP-induced mRNA levels of these steroidogenic factors. The mRNA level of the control housekeeping gene, L19, did not change in response to treatment with any of the indicated reagents and their combinations.

We further compared the effects of BMP-6 on FSH-, forskolin-, and 8-Br-cAMP-induced P4 production by GCs (Fig. 4). Consistent with the results shown in Fig. 1, FSH-induced P4 was significantly suppressed by BMP-6 up to ~80%. As expected, BMP-6 also suppressed forskolin-induced P4 by ~80% but did not significantly inhibit 8-Br-cAMP-induced P4 production. These data reinforce our findings that BMP-6 exerts its biological activity by inhibiting FSH signaling at a site downstream of the FSH-R and upstream of cAMP signaling.

Therefore, we next examined the possible effect of BMP-6 on AC activity with which cellular AMP is converted to cAMP, leading to the activation of protein kinase A. For this analysis, GCs were treated with FSH (10 ng/ml) or forskolin (10 μM) in the presence or absence of increasing doses of BMP-6 (0–100 ng/ml) in the presence of androstenedione (100 nm) for 48 h after which total RNA was extracted and then subjected to quantitative competitive RT-PCR analysis as described under “Experimental Procedures.” The PCR products are shown in the upper panel, and the ratios of PCR products (target/internal control) are graphed. Bars with different letters indicate that group means are significantly different at p < 0.05, i.e., internal control.
IBMX should be in response to decreased synthesis of cAMP by AC rather than increased metabolic activity of phosphodiesterase. These data suggest that BMP-6 inhibits AC activity enhanced by FSH and forskolin, and the attenuation of FSH- and forskolin-induced StAR and P450scc mRNA expression by BMP-6 seems to be, at least in part, attributed to the decreased activity of AC.

Since FSH-R expression in GCs is known to be stimulated by FSH, forskolin (34), or 8-Br-cAMP (35), the effect of BMP-6 on FSH-R mRNA expression was also examined (Fig. 6). Control untreated cells spontaneously expressed basal level of FSH-R mRNA, and BMP-6 did not alter the level by itself. FSH treatment increased FSH-R mRNA level up to 2-fold, and BMP-6 abolished its effect. Stimulation of FSH-R mRNA expression by forskolin was significantly suppressed by BMP-6 but that by 8-Br-cAMP at two different concentrations was unchanged. Furthermore, virtually identical activity of BMP-6 was obtained from the experiments in which we tested the effect of BMP-6 on the expression of several other genes (Fig. 7).

DISCUSSION

In the present study, we first provided evidence for the biological activity of BMP-6 in GCs. Experiments in which BMP-6 was added to cultured GCs indicated that BMP-6 is potent in suppressing FSH-induced P4 production without affecting FSH-induced E2 production and that BMP-6 does not exhibit any mitogenic activity in GCs. Our data demonstrate that the biological effects of BMP-6 on GCs are distinct from those of other TGF-β superfamily members, including inhibin (36, 37), activin (37–39), TGF-β (36, 40–43), BMP-4 (28),
The biological effects of FSH on GCs by inhibiting FSH-induced P\(^4\) production without affecting FSH-induced E\(_2\) synthesis. The maximal efficacy of BMP-6 inhibition of FSH-induced P\(^4\) was \(~30\%\) more than that of BMP-15, but the ED\(_{50}\) of both factors were similar, \(~10^{-10}\) M, which is within a physiological range. The biological effects of GDF-9, a closely related factor also secreted by oocytes, are clearly different from BMP-6. GDF-9 stimulates E\(_2\) production by cultured primary rat GCs in the absence of FSH yet suppresses FSH-induced both E\(_2\) and P\(^4\) production (14).

The ability of BMP-6 to regulate specifically FSH-induced P\(^4\) biosynthesis without affecting FSH-induced E\(_2\) synthesis indicates that BMP-6 must preferentially inhibit the biochemical pathways that lead to P\(^4\) production. The present studies revealed that BMP-6 reduced the steady state levels of FSH-induced mRNAs involved in P\(^4\) synthesis, including StAR and P450scc. However, when BMP-6 was administered without FSH, no changes in the expression of these molecules were observed, indicating that BMP-6 must be acting only by regulating FSH activity. In this regard, our previous studies showed that BMP-15 regulated the sensitivity of GCs to FSH by inhibiting the expression of FSH-R (16). The present finding that BMP-6 alone had no effect on the steady state levels of FSH-R mRNA indicates that BMP-6 must work by a mechanism that is different from BMP-15 and is downstream of the FSH-R.

To elucidate the mechanism of BMP-6 regulation of FSH signaling, we investigated whether BMP-6 would also suppress the effects of forskolin and 8-Br-cAMP on StAR and P450scc mRNA levels in GCs. When added alone, both forskolin and 8-Br-cAMP exhibited similar activities to FSH in GCs. BMP-6 did not inhibit forskolin-induced responses, which further supports the hypothesis that BMP-6 is acting downstream of the FSH-R, but did not inhibit 8-Br-cAMP-induced responses, which indicates that BMP-6 must be acting upstream of cAMP signaling. This action of BMP-6 was broadly observed in a battery of other cell types. Experiments using a bone marrow-derived stromal cell line (ST2) and osteoblast-like cells (MC3T3-E1) showed that BMP-2 and BMP-4 had a synergistic effect on increasing parathyroid hormone-induced production of cAMP (44). In contrast, BMP-6, even when added at pharmacological doses, did not cause an increase in parathyroid hormone-induced cAMP levels. These data imply that BMP-6 may have different effects on AC than other BMPs.

FIG. 6. Effects of FSH (10 ng/ml), forskolin (10 \(\mu\)M), 8-Br-cAMP (0.2 and 1 \(\mu\)M), and BMP-6 (100 ng/ml) on the expression of FSH-R mRNA. FSH-R mRNA levels in GCs with the indicated treatments were analyzed by a semi-quantitative RT-PCR and are presented together with those of L19 mRNA. Bars with different letters indicate that group means are significantly different at \(p < 0.05\), i.e., internal control.

FIG. 7. Effects of FSH (10 ng/ml), forskolin (10 \(\mu\)M), 8-Br-cAMP (1 \(\mu\)M), and BMP-6 (100 ng/ml) on the expression of inhibin/activin subunits and LH-R mRNA. Target mRNA levels in GCs with the indicated treatments were analyzed by a semi-quantitative RT-PCR analysis using specific primer pairs. Two distinct bands of LH-R are due to alternative spliced transcripts. The representative data are shown from three independent experiments.

BMP-7 (28), and GDF-9 (14). However, it is intriguing that the function of BMP-6 with respect to GC steroidogenesis has a strong resemblance to that of BMP-15, another oocyte-secreted factor (16). Both BMP-6 and BMP-15 selectively modulate the
What is the physiological relevance of BMP-6 in the ovarian function? Our current in vitro studies may suggest the role for BMP-6 in steroidogenesis. After increases in circulating FSH during the follicular phase of the ovarian cycle, dominant follicles are selected and grow rapidly, resulting in a marked increase in E\textsubscript{2} synthesis/secretion by GCs (48). In striking contrast, GCs in these follicles do not respond to FSH to synthesize P\textsubscript{4} in vivo. However, once the GCs from these dominant follicles are removed and cultured with FSH in vitro, they spontaneously secrete copious amounts of both E\textsubscript{2} and P\textsubscript{4}. These findings thus led to the proposition that there should be inhibitor(s) of FSH-stimulated P\textsubscript{4} production present in the ovary in vivo. By using rabbit dominant follicles in situ, El-Fouly et al. (49) demonstrated that removal of the oocyte caused granulosa and theca cells to luteinize spontaneously and secrete large quantities of P\textsubscript{4} equivalent to that produced by normal corpora lutea, suggesting the presence of such inhibitory molecule(s) in the oocytes. They may be present in developing follicles and function to prevent GCs from secreting P\textsubscript{4}. Our current findings that oocyte-derived BMP-6 inhibits FSH-induced P\textsubscript{4} production by GCs suggest that BMP-6 may contribute to preventing the premature luteinization of the dominant follicles. In this regard, we have previously reported that theca cell-derived BMP-4 and -7 can regulate FSH-dependent steroid synthesis in GCs (28). In contrast to activins that theca cell-derived BMP-4 and -7 can regulate FSH-deconjugative steroid synthesis, BMP-4 and -7 are putative (theca cell-derived) luteinization inhibitors (28). Collectively, our findings suggest that factors that inhibit premature luteinization of GCs in dominant follicles may come from two directions within the follicle, namely BMP-4 and -7 from the theca cells and BMP-6 and BMP-15 from the oocyte. Given that each of these factors has similar, yet distinct, biological functions and mechanisms of action in GCs, one can imply that the intrafollicular regulation of luteinization is controlled by complex and redundant mechanisms.

Recently, increased attention has been paid to the Booroola strain of Merino ewes which, like the heterozygous Inverdale, is considered highly prolific (50). Heterozygous Booroola ewes exhibit higher litter sizes than wild-type ewes due to increased ovulation rates, similar to Inverdale heterozygotes. However, in contrast to homozygous Inverdale ewes that are infertile, homzygous Booroola ewes have even higher ovulation rates and litter sizes than the heterozygotes. Recently, three independent research groups have identified that the increased ovulation rate seen in the Booroola Merino ewes is associated with a point mutation in the gene encoding BMPR-IB (51–53). Notably, this mutation was located in the highly conserved intracellular kinase signaling domain of the BMPR-IB. To date, the functional ligand that binds to this receptor and the cellular mechanism of how this mutation causes an increase in ovulation rate have not been established; however, several BMP family members including BMP-6, BMP-7, BMP-4, and GDF-5 have been shown to bind to the BMPR-IB in various cell types (30, 54). Our present findings on the biological activities and cellular mechanism of BMP-6 suggest that the Booroola phenotype may in fact be caused by the inability of Booroola GCs to properly elicit BMP-6 signaling. This hypothesis is supported by the earlier observations that, when cultured for 48 h in the presence of FSH and LH, follicles dissected from the ovaries of Booroola ewes produce increased amounts of P\textsubscript{4} than comparable follicles from wild-type ewes, yet there is no change in E\textsubscript{2}, androstenedione, nor testosterone production (55). Also, Booroola follicles were found to be more responsive to FSH and LH stimulation with respect to cAMP production than wild-type follicles (55). Further studies demonstrated that the changes in steroidogenesis and cAMP levels in GCs of the Booroola ewes are not caused by changes in FSH binding capacity of GCs (56). Collectively, the enhanced gonadotropin responsiveness of the follicles from Booroola ewes, compared with those from wild-type ewes, could be explained by the incapable BMPR-IB signaling triggered by endogenous BMP-6. Because of the loss of BMPR-IB signaling in the Booroola ewes, BMP-6 would not be able to inhibit cAMP synthesis, which would result in an increase in the sensitivity of GCs to FSH (and the subsequent selective increase in P\textsubscript{4} production) without affecting the number of FSH-R on the surface of the GCs. Wilson et al. (52) have suggested that BMP-15 could be one of the candidate ligands for the defective BMPR-IB signaling in the Booroola ewes. We assume, however, that BMP-6 is a more likely candidate based on our previous finding that BMP-15 action is dependent on its ability to down-regulate FSH-R expression. This hypothesis can be supported by comparing the phenotypes of Booroola homozygotes to Inverdale homozygotes. Heterozygous Booroola ewes have higher ovulation rates than heterozygous Booroola ewes, whereas Inverdale homozygotes are infertile with a block in folliculogenesis at the primary follicle stage. Our previous investigations demonstrated that BMP-15 is potent in stimulating mitosis of GCs, and we proposed that lack of the mitotic properties of BMP-15 may be the cause of arrested follicle development in Inverdale homozygotes. In Booroola homozygotes the mitotic capacity of the GCs seems to be intact, which is consistent with the lack of BMP-6 exhibiting any effect on GC mitosis.

In summary, these studies provide the first insight into the biological activities of BMP-6 in the ovary. BMP-6 is able to suppress selectively FSH-induced P\textsubscript{4} production and the relevant steroidogenic factors. This physiological effect is similar to BMP-15; however, unlike BMP-15, BMP-6 does not inhibit FSH-R expression; instead BMP-6 has a novel mechanism of

FIG. 8. Effect of BMP-6 on GC mitosis. GCs (~\texttimes10\textsuperscript{5} viable cells) were cultured in serum-free media and treated for 24 h with [\textsuperscript{methyl}\textsuperscript{3}H]-thymidine plus increasing doses of BMP-6 (0–300 ng/ml) or BMP-15 (100 ng/ml) after 24 h of culture. The labeled thymidine incorporated into the cells was counted. Actual GC number was also counted after 24 h of culture with or without BMP-6 (300 ng/ml) or BMP-15 (100 ng/ml). Data are mean ± S.E. *, p < 0.05 compared with control.
modulating FSH signaling, namely the attenuation of FSH-stimulated cAMP production. Also, unlike BMP-15 and GDF-9, BMP-6 does not have proliferative properties in GCs. Taken together our current studies demonstrate that oocyte-derived BMP-6 exerts a distinct function among multiple members of the TGF-β superfamily that are expressed in the ovary and may play an important role in FSH-dependent follicle development.

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