Human BMP8A suppresses luteinization of rat granulosa cells via the SMAD1/5/8 pathway

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Abstract

Bone morphogenetic proteins (BMPs) are known to play an indispensable role in preventing the precocious luteinization of granulosa cells within growing ovarian follicles. In this study, we found that the transcripts of *BMP8* genes are enriched in the ovaries of humans and rodents. When analyzing transcriptomic datasets obtained from human mature granulosa cells, we further found that the *BMP8* transcripts not only show the highest abundance among the searchable BMP-related ligands but also decrease significantly in women of advanced age or women with polycystic ovarian syndrome. The correlation between the *BMP8* levels in granulosa cells and the decline in ovarian function in these subjects suggests that BMP8 protein may be involved in the regulation of granulosa cell function(s). Using a rat model, we demonstrated that human BMP8A protein activates the SMAD1/5/8 and the SMAD2/3 pathways simultaneously in both immature and mature granulosa cells. Furthermore, the expression of potential type I and type II receptors used by BMP8 in rat granulosa cells was characterized. We found that BMP8A treatment can significantly inhibit gonadotropin-induced progesterone production and steroidogenesis-related gene expression in granulosa cells. Pathway dissection using receptor inhibitors further revealed that such inhibitory effects occur specifically through the BMP8-activated SMAD1/5/8, but not SMAD2/3, pathway. Taken together, considering its abundance and possible functions in granulosa cells, we suggest that BMP8 may act as a novel luteinization inhibitor in growing follicles.

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Introduction

Successful ovulation is governed by cooperation between gonadotropins and various local factors. During folliculogenesis, follicle-stimulating hormone (FSH) mediates follicular transition from antral follicles to preovulatory follicles. The evoking signal promotes not only proliferation but also estradiol production in granulosa cells. By the end of the follicular phase, the luteinizing hormone (LH) surge triggers ovulation, which is followed by the terminal differentiation of residual follicular cells into luteal cells that then produce progesterone (Richards *et al.* 1995). Of interest, while gonadotropins elicit their functions for follicle maturation and ovulation, the involvement of local factors, namely luteinization inhibitors, is required to selectively attenuate progesterone biosynthesis before ovulation in order to prevent premature luteinization (Shimasaki *et al.* 1999, Oktem & Urman 2010).

In mice, it has been shown that premature luteinization can block ovulation by trapping the enclosed oocytes within luteinizing granulosa cells (Pangas *et al.* 2006, Grasa *et al.* 2016). In humans, although it has not been clearly linked to a similar phenotype, premature luteinization is one of the common phenomena observed in older women with poor reproductive performance

and has been hypothesized to be one of the causes of anovulation in women with polycystic ovarian syndrome (PCOS) (Willis *et al.* 1998, Wu *et al.* 2015). In women of advanced age, for example, their granulosa cells, which are collected from follicular fluid, tend to undergo earlier luteinization, as reflected by increases in the expression of LH receptor (*LHCGR*), *CYP11A1* and progesterone receptor (*PGR*); this is consistent with the facts of declining numbers in retrieved oocytes and embryos and lower pregnancy rates in these individuals (Wu *et al.* 2015). Intriguingly, a study indicates that granulosa cells from follicles of PCOS patients express higher levels of *LHCGR* and *CYP11A1* than those from size-matched control follicles of regularly cycling women; this suggests that granulosa cells in these selected PCOS subjects may undergo premature luteinization (Jakimiuk *et al.* 2001). In addition, in patients who undergo *in vitro* fertilization (IVF), premature luteinization has been associated not only with decreased oocyte recovery, poor oocyte maturation and a lower fertilization rate (Stanger & Yovich 1985, Lejeune *et al.* 1986, Silverberg *et al.* 1991), but also with a lower rate of pregnancy and a higher incidence of spontaneous abortion (Schoolcraft *et al.* 1991, Fanchin *et al.* 1993, Manzi *et al.* 1995). Therefore, preventing premature granulosa cell luteinization may greatly improve IVF outcomes.

The molecular mechanisms that prevent the precocious luteinization of granulosa cells are still poorly understood. However, previous studies have indicated that many members of the bone morphogenetic protein (BMP) subfamily have an indispensable role in guiding this event (Knight & Glistler 2006). Indeed, several BMP members secreted by the ovary, including BMP2, BMP4, BMP7 and BMP15, have been tested individually *in vitro* and shown to prevent luteinization of human granulosa cells efficiently (Chang *et al.* 2013, 2015, Shi *et al.* 2010, 2011). However, whether other BMP candidates are involved in preventing premature luteinization and whether differential expression of BMPs can reflect the luteinization status of human mature granulosa cells remain unclear.

BMP8 is encoded by a pair of genes, *BMP8A* and *BMP8B* (Zhao & Hogan 1996). In this study, we initially found that the two *BMP8* genes have a differential tissue expression profile in various tissues of humans and rats. Among the reproductive organs, the transcripts of both *BMP8A* and *BMP8B* were enriched in the ovary. We previously have demonstrated that both recombinant BMP8A protein and BMP8B protein are functional to activate canonical BMP signaling in different cells, including spermatogonia, P19 and 293T cells (Wu *et al.* 2017). As mentioned above, BMP signaling is indispensable for the prevention of the precocious luteinization of granulosa cells. In this study we therefore are particularly interested in the evaluation of the BMP8 roles in this event. To clarify the expression profiles of BMP8, the ovaries from superovulated rats and the transcriptomic datasets of granulosa cells derived from women at different ages or women with PCOS were used. To evaluate the effects of BMP8 protein on preventing luteinization, granulosa cells collected from a superovulated rat model were used. In addition, the underlying mechanisms whereby BMP8 protein is able to suppress the gonadotropin induction of progesterone production and steroidogenic enzyme expression were further characterized.

Materials and methods

Animals and ethics

Sprague–Dawley rats were obtained from the laboratory animal center (National Yang-Ming University, Taipei, Taiwan). In the superovulation model, immature female rats (26 days old) were intraperitoneally injected with 15 international units (IU) pregnant mare serum gonadotropin (PMSG) in the morning (09:00–10:00 h). Rats were then anesthetized and killed using CO₂ at indicated time points. All animals were housed under a controlled humidity, temperature, and light regimen. Animal care and treatments were approved by the Institutional Animal Care and Use Committee of the National Yang-Ming University (Permit No. 1021229).

Hormones and reagents

L-15 Leibovitz medium, McCoy's 5A medium, fetal bovine serum, and penicillin-streptomycin-glutamine were obtained from Invitrogen. PMSG, hCG and human FSH were obtained from Calbiochem. Human BMP4 (314-BP), activin A (338-AC) and human BMP8A (1073-BPC) proteins were purchased from R&D systems. SB431542, dorsomorphin, hyaluronidase, forskolin and other chemicals unless noted were purchased from Sigma-Aldrich.

Regarding generation of bioactive recombinant human BMP8A protein, we previously have demonstrated that BMP10 prodomain, known to facilitate the production and folding of the mature domain of some BMP molecules (Mazerbourg *et al.* 2005), greatly helps the production of functional BMP8A mature protein than BMP8A prodomain itself in the 293T expression system (Wu *et al.* 2017). Thus, this strategy was applied in this study. Briefly, recombinant DNAs that encode human BMP10 prodomain and human BMP8A mature domain or N-tagged His₆-human BMP8A mature domain were fused and cloned into pcDNA3.1 vector. The 293T cell conditioned medium containing recombinant non-tagged BMP8A protein was collected and concentrated 20-fold by filtration with 3-kD molecular cutoff filters (Amicon Ultra, Millipore). The 293T cell conditioned medium containing His₆-tagged BMP8A protein was used for recombinant protein purification by Ni Sepharose excel (GE Healthcare). Purified recombinant His₆-tagged BMP8A protein was subjected to Western blotting and the band intensities specific to the mature domain were quantified using the ImageJ program for concentration conversion using a standard curve established by a serial dilution of a commercially available BMP8A mature domain (R&D Systems; 1073-BP). Any effect of the BMP10 prodomain on SMAD signaling was excluded based on the results in Supplementary Fig. 1E and F shown in our previous study (Wu *et al.* 2017).

Cultures of primary granulosa cells

Immature granulosa cells were isolated from early antral follicles from immature rats primed with diethylstilbestrol for 3 days, whereas mature granulosa cells were isolated from large antral follicles from rats primed with PMSG for 2 days (Hung *et al.* 2012). In brief, the ovaries were punctured in L-15 Leibovitz medium with a 26 gauge needle to release granulosa cells. The cell suspensions were filtered through 40 µm cell strainer to remove large tissue clumps and oocytes. The purity of granulosa cells was confirmed based on the differential expression of *Fshr* and *Lhcgr*, which was determined by real-time PCR relative to *Actb* (cells before PMSG injection: *Fshr*, 0.0030; *Lhcgr*, 0.0022; cells at 48 h after PMSG injection: *Fshr*, 0.0045; *Lhcgr*, 0.0358) (Luo *et al.* 2004). The cells were cultured in McCoy's 5A supplemented with penicillin, streptomycin, glutamine and androstenedione (10⁻⁷ M) and adjusted to appropriate cell number for subsequent experiments (Sun *et al.* 2010). For transcript quantification and progesterone measurement, 2 × 10⁵ cells/mL were used. For immunoblotting against phosphorylated SMADs, 9 × 10⁵ cells/mL were used.

cDNA preparation and real-time PCR quantification

For preparation of cDNA from granulosa cells, total RNA from each sample was extracted using the TRIzol reagent (Thermo Fisher Scientific) and then reverse-transcribed using the High-capacity cDNA RT kit (Applied Biosystems) with the oligo-dT primer. For subsequent quantitative real-time PCR, the Power SYBR Green Master Mix reagent (Applied Biosystems) was used. The primer pairs for each gene were listed as follows: rat *Bmp8a* forward, CATGGGGTTTGTGGGCTATC; rat *Bmp8a* reverse, GACTGAGGATGGCCAAGGAC (accession ID: NM_001109432.1; amplicon: 155 bp). Rat *Bmp8b* forward, TGTGGCAGAGGATTGGTTCA; rat *Bmp8b* reverse, CAGGGACTGTACTGCCACA (accession ID: XM_002729526.5; amplicon: 145 bp). Rat *Star* forward, AGATGAAGTGCTAAGTAAGGTGGTG; rat *Star* reverse, CCAGTTCTTCATAGAGTCTGTCCAT (accession ID: NM_031558.3; amplicon: 98 bp). Rat *Hsd3b* forward, AGACCATCCTAGATGTCAATCTGAA; rat *Hsd3b* reverse, CAGGATGATCTTCTGTAGGAGT (accession ID: NM_017265.4; amplicon: 134 bp). Rat *Cyp11a1* forward, CCAAGTTCAACCTCCTCTGA; rat *Cyp11a1* reverse, GTGTGACTGCAGCCTGCAAT (accession ID: NM_017286.3; amplicon: 127 bp). Rat *Id1* forward, TGGACGAACAGCAGGTGAAC; rat *Id1* reverse, ATCTCCACCTTGCTCACTTTC (accession ID: NM_012797.2; amplicon: 112 bp). Rat *Smad7* forward, CGATACAAAAACGGGAAGCAA; rat *Smad7* reverse, ACACACTGACAACTGAAATGCTGAT (accession ID: NM_030858.1; amplicon: 100 bp). Rat *Actb* forward, CTCTGTGTGGATTGGTGGCTC; rat *Actb* reverse, CTGCTTGCTGATCCACATCTG (accession ID: NM_031144.3; amplicon: 70 bp). For *Bmp8a* and *Bmp8b*, single products were confirmed by melting curves, agarose gel electrophoresis and sequencing. The products of other genes have been verified previously (Luo et al. 2004).

Immunoblotting

To detect the existence of endogenous BMP8 protein, the ovaries collected from superovulated rats at indicated time points were homogenized in PBS supplemented with protease inhibitor (Roche, 04693132001) using a tissue grinder (Shinoteh Inc) on ice and centrifuged at 12,000 *g* for 30 min, whereas the ovarian cells were lysed in reducing sample buffer directly. The derived supernatants from ovary homogenates were quantified by BCA protein assay (Pierce), a total of 20 µg protein was loaded per lane. To detect phosphorylated SMAD proteins in primary granulosa cells, the cells seeded overnight in 12-wells plates were changed to fresh serum-free medium and treated with indicated ligands for 1 h. The cells were lysed in reducing sample buffer. Protein samples were boiled to denature and analyzed by SDS-PAGE in 10–12% slab gels. Western blotting was performed using goat antibody against BMP8 (1:1000; R&D Systems, AF-1073), rabbit antibody recognizing phosphorylated SMAD1/5/8 (1:1000; Cell Signaling, 9511) or phosphorylated SMAD2/3 (1:1000; Cell Signaling, 9510). Detailed protocols were described previously (Wu et al. 2017).

Progesterone ELISA

To assess the production of progesterone, granulosa cells were pretreated with or without SB431542 or dorsomorphin for 30 min before being incubated with PBS control, FSH, hCG, and/or BMP8 for 48 h as indicated. The supernatant was collected and the amount of progesterone was measured by competitive ELISA. Briefly, 96-well microtiter plates pre-coated with progesterone-BSA (10 ng/well) were incubated with specific anti-progesterone antibody (Sigma, P1922) in the presence of the above supernatant or serially diluted progesterone-containing standards. The plates were then washed and incubated with horseradish peroxidase-conjugated secondary antibody, and the luminescent signals were read by adding the enhanced chemiluminescence substrate. The within-assay coefficient of variation and between-assay coefficient of variation were less than 5%. The sensitivity was less than 100 pg/mL (Vitt et al. 2000).

Characterization of transcriptomic datasets and statistical analyses

Normalized data from each human transcriptomic dataset provided by the submitters were retrieved from the Gene Expression Omnibus (GEO) repository. The group differences between two independent samples were examined by Mann–Whitney test. For other data analyses, Student's *t*-test was used to compare differences between two groups, whereas one-way ANOVA followed by Bonferroni post test was used to compare differences among multiple groups. For progesterone assay, three independent experiments were performed and gave similar trends. Data are expressed as means ± s.d. for one of the experiments due to batch variation.

Results

The expression profiles of BMP8 in diverse mammalian tissues

BMP8 consists of a pair of genes, *BMP8A* and *BMP8B*. Importantly, the expression profiles of these two genes in diverse mammalian tissues have not yet been clarified. We therefore, as a first step, analyzed the expression profiles of these two *BMP8* genes in humans and rodents in a whole tissue spectrum manner. Regarding the *BMP8* profiles in humans, we browsed NCBI's Gene Expression Omnibus (GEO) database and targeted a transcriptomic dataset that contains 42 normal human tissues (GSE14938) (She et al. 2009). By analyzing this dataset, we identified that the transcript level in the ovary of either *BMP8A* or *BMP8B* ranked third among 42 human tissue types analyzed (Fig. 1A and B). Specifically, their expression in the human ovary was much higher than in the testis.

To further evaluate the expression profiles of these two *Bmp8* genes in rodents, we performed real-time PCR quantification in 22 different types of rat tissues. Consistently, our results indicated that the transcript level of either *Bmp8a* or *Bmp8b* in the rat ovary was also

Figure 1 Expression profiles of *BMP8A* and *BMP8B* in various mammalian tissues. In humans, the relative expression levels of *BMP8A* (A) and *BMP8B* (B) were compared across 42 human tissue samples. This data was extracted from a public microarray dataset (GSE14938) and only 20 tissues with the highest abundance levels for expression of the two *BMP8* genes are shown. RNAs of each tissue were pooled from multiple donors for a microarray assay and the signal intensity was normalized against a pooled reference comprising about 20 normal adult tissue pools. No data variation was shown (She *et al.* 2009). In rats, the transcript levels of *Bmp8a* (C) and *Bmp8b* (D) in 22 rat tissue samples were evaluated by real-time PCR. Depending on the tissue size, tissues from four to six adult rats were pooled for RNA extraction. The ovaries and testes were collected from four rats. Data were normalized against *Actb* amounts are expressed as means $\pm$ s.d. from three independent quantification replicates.

relatively higher than in the testis (Fig. 1C and D), where BMP8 signaling has been reported to be essential for the maintenance of postnatal spermatogenesis in rodents (Zhao & Hogan 1996, Zhao *et al.* 1998, Wu *et al.* 2017).

Next, the amounts of BMP8 protein in the rodent gonads were assessed. The BMP8 antibody can only detect total BMP8 protein due to high sequence similarity between BMP8A and BMP8B protein. In mice, mature BMP8 protein can indeed be detected in the mature ovary and testis, with the ovary showing a higher abundance (Fig. 2A). In the rat model, the amounts of BMP8 protein in the pre-pubertal ovaries and the mature ovaries were similar (Fig. 2B). We next investigated the expression profiles of the two *Bmp8* genes during folliculogenesis using a superovulated rat model. Quantitative real-time PCR analysis showed that the transcript levels of both *Bmp8a* and *Bmp8b* were attenuated after PMSG injection (Fig. 2C and D). Taken together, our findings suggest that the two *BMP8* genes are expressed at relatively high levels in the ovary of diverse mammals and that such expression is regulated during folliculogenesis.

Expression and regulation of the BMP8 genes in granulosa cells

As mentioned in the introduction, in this study we are particularly interested in characterizing the molecular candidates for their involvement in the prevention of precocious luteinization of granulosa cells, an event known to be guided by SMAD1/5/8 signaling (Knight & Glister 2006). By exploring public transcriptomic databases derived from human granulosa cells, we intriguingly found that BMP8 is potentially of importance to this event. For example, when analyzing a RNA-

Figure 2 Evaluation of the protein amount and the RNA regulatory profile of BMP8 in the rodent ovary. (A and B) Detection of BMP8 protein. The ovaries and the testes from mature mice (8 weeks old) (A) or the ovaries from rats at 26 days and 8 weeks old (B) were homogenized. The derived supernatants (S) and pellets (P) (20 μ g protein per lane) were subjected to immunoblotting using the antibody against BMP8. Blots are representative of three independent experiments. (C and D) The ovaries collected from immature rats at the indicated time points after PMSG injection and were used for real-time PCR quantification of the transcripts of *Bmp8a* (C) and *Bmp8b* (D). The ovaries from three to five rats were collected and pooled for each time point. Data were normalized against *Actb* amounts and are expressed as means $\pm$ s.d. from three independent quantification replicates. ** P < 0.01; *** P < 0.001; one-way ANOVA, followed by Bonferroni post test.

sequencing transcriptomic dataset derived from human mature granulosa cells ($n=12$; GSE62093) (Yu *et al.* 2015), *BMP8B* exhibited the highest expressional abundance among the searchable ligands capable of activating SMAD1/5/8 signaling (Fig. 3A); unfortunately, *BMP8A* was not included in this dataset. In addition, as compared with young individuals (age 26 ± 2.2 years; $n=6$), a significant decrease in the expression of *BMP8B*, as well as that of *AMH*, *GDF7*, *BMP6* and *BMP4*, was observed in older women (age 40 ± 2.3 years; $n=6$) (Fig. 3A); this suggests that there might be a decline in SMAD1/5/8 signaling in the mature granulosa cells as the age of woman increases.

Moreover, using a transcriptomic dataset of granulosa cells collected from PCOS women (age less than 35 years; $n=7$) and matched controls (age less than 35 years; $n=3$) (GSE34526) (Kaur *et al.* 2012), we also observed that, among all searchable ligands capable of activating SMAD1/5/8 signaling, only the expression of *BMP8B* showed a significant suppression in women with PCOS (Fig. 3B). Taken together, the above findings derived from re-analysis of human transcriptomic data

suggest that *BMP8* transcripts are abundant in granulosa cells, where their levels correlated with the decline in ovarian function in these selected subjects. However, the functions of BMP8 protein in granulosa cells have never been explored.

Therefore, to investigate the above questions, we initially used a rat granulosa model based on the fact that high expression levels of the *BMP8* genes in the mammalian ovaries seem to be common across species (Figs 1 and 2). Firstly, the protein amounts of BMP8 were evaluated in granulosa cells isolated from immature follicles (before PMSG injection) and mature follicles (48 h after PMSG injection) of superovulated rats (Fig. 3C). Consistent with the mRNA profiles assessed during the ovarian cycle (Fig. 2C and D), Western blotting results indicated that the protein amounts of BMP8 in granulosa cells encountered a significant drop after maturation, suggesting that BMP8 signaling will be reduced upon initiation of ovulation and luteinization.

BMP8 activates the SMAD1/5/8 and SMAD2/3 pathways simultaneously in rat granulosa cells

We previously have demonstrated that BMP8 can activate the SMAD1/5/8 and SMAD2/3 pathways simultaneously via different receptor complexes in spermatogonia (Wu *et al.* 2017). Here we further characterized BMP8 signaling in granulosa cells. Human BMP8A and BMP8B differ in only two amino acids in their mature domain and have been shown to activate similar downstream signaling (Zhao & Hogan 1996, Wu *et al.* 2017). Therefore, we chose only human BMP8A for subsequent experiments. Indeed, we found that the purified recombinant His₆-tagged BMP8A protein not only induced the phosphorylation of SMAD1/5/8 but also the phosphorylation of SMAD2/3 in both immature (Fig. 4A and B) and mature rat granulosa cells (Fig. 4C and D). In addition to the detection of phosphorylated SMADs, induction of SMAD downstream genes was evaluated. Besides, bioactivities of pure human BMP8A protein obtained from a commercial source and the conditioned medium containing non-tagged recombinant BMP8A protein generated from our designed construct were also compared. As compared with the commercial BMP8A protein that showed negligible effect, both non-tagged and His₆-tagged recombinant BMP8A proteins produced from transfected 293T cells can significantly induce the expression of *Id1*, an early response gene of SMAD1/5/8 signaling, and the expression of *Smad7*, an early response gene of SMAD2/3 signaling, in treated immature rat granulosa cells (Fig. 4E and F); this may further exclude the potential effect of His₆ tag on affecting BMP8 bioactivity. Thus, we used purified His₆-tagged recombinant BMP8A proteins, hereafter referred to simply as BMP8A, for subsequent treatments.

Our previous study has characterized the possible combinations of receptor complexes for BMP8 protein

Figure 3 Abundance and regulation of BMP8 in mammalian granulosa cells. (A) The transcript abundances of BMP-related ligands were analyzed in a RNA-sequencing transcriptomic dataset derived from mature granulosa cells harvested from older and young women (GSE62093 (Yu *et al.* 2015)). The normalized RNA-seq data (RPKM, reads per kilobase per million) is represented by a box-plot. Data are means $\pm$ s.d. * $P < 0.05$; ** $P < 0.01$; Mann–Whitney test. (B) Changes in the expression of BMP-related ligands were compared in granulosa cells from normal (matched control subjects) and PCOS patients (GSE34526 (Kaur *et al.* 2012)). Data are means $\pm$ s.d. * $P < 0.05$; Mann–Whitney test. (C) Isolated granulosa cells collected from superovulated rats at indicated time points were lysed (three individual rats per group). The derived supernatant fractions (20 μ g protein per lane) were subjected to immunoblotting using the antibody against BMP8 (left panel). The blots were assessed by densitometer using β -actin as a loading control. Data are shown as means $\pm$ s.d. (right panel). * $P < 0.05$; ** $P < 0.01$. Data are means $\pm$ s.d. * $P < 0.05$; Student's *t* test. $n=3$ individual animals.

Figure 4 BMP8A activates both the SMAD1/5/8 and SMAD2/3 pathways in rat granulosa cells. Granulosa cells from immature rats primed with PMSG for 0 h (A and B) or 48 h (C and D) were treated with PBS control, BMP4 (1 nM), activin A (1 nM), or His₆-tagged BMP8A (1 or 3 nM) for 1 h. The cell lysates were then subjected to immunoblotting using antibody against phosphorylated SMAD1/5/8 (A and C) or phosphorylated SMAD2/3 (B and D). The signals of phosphorylated-SMAD were quantified and then normalized against β -actin signals. Real-time qPCR analysis of the transcripts of *Id1* (E) and *Smad7* (F), the SMAD target genes, in immature rat granulosa cells treated with indicated proteins for 1 h. Data are expressed as means $\pm$ S.E.M. n.s., not significant; * P < 0.05, ** P < 0.01, *** P < 0.001 compared to no-treatment control; one-way ANOVA, followed by Bonferroni post test. n = 3 independent biological replicates.

using overexpression and knockdown strategies in 293T cells (Wu *et al.* 2017). Specifically, in 293T cells, BMP8 can activate SMAD1/5/8 signaling potentially through receptor complexes formed by the type I receptor ALK3 or ALK6 and the type II receptor BMPR2 or ACVR2A, whereas it is also able to activate SMAD2/3 signaling potentially through receptor complexes formed by the type I receptor ALK4 or ALK5 and the type II receptor ACVR2A, ACVR2B or TGFBR2. Based on the above findings, the expression levels of these receptor candidate genes were quantified in granulosa cells in order to evaluate which can form the possible receptor complexes for BMP8. The results indicated that *Alk3*, *Alk5* and *Alk6*, which are the type I receptor genes, and *Acvr2a* and *Bmpr2*, which are the type II receptor genes, are stably expressed and relatively abundant in both immature (Fig. 5A) and mature granulosa cells (Fig. 5B). In granulosa cells, promiscuous interactions between the type I and the type II receptors have been found and this

can lead to crosstalk of SMAD pathways. For example, GDF9 can mediate SMAD2/3 signaling via the BMPR2/ALK5 complex (Mazerbourg *et al.* 2004). Taken together, these findings suggest that BMP8 can act on granulosa cells as an autocrine factor. It activates SMAD1/5/8 signaling and SMAD2/3 signaling simultaneously through different receptor complexes potentially formed by the type I receptor ALK3, ALK5 or ALK6 and the type II receptor BMPR2 or ACVR2A (Fig. 5C). However, the exact receptor combination responsible for BMP8-mediated SMAD signaling needs more studies.

BMP8 inhibits gonadotropin-induced progesterone production in granulosa cells via the SMAD1/5/8 pathway

BMPs are known to inhibit premature luteinization of granulosa cells by suppressing gonadotropin-driven progesterone synthesis (Knight & Glister 2006,

Figure 5 The transcript levels of the type I and type II receptors linked to BMP8 signaling. The transcript levels of various type I receptors (*Alk3*, *Alk4*, *Alk5* and *Alk6*) and various type II receptors (*Acvr2a*, *Acvr2b*, *Bmpr2* and *Tgfb2*) were evaluated by real-time PCR in granulosa cells from immature rats primed with PMSG for 0 h (A) or 48 h (B). Data were normalized against *Actb* amounts and are expressed as means $\pm$ S.E.M. $n = 3$ biological replicates. (C) Proposed model of BMP8-mediated signal transduction in rat granulosa cells.

Shimasaki *et al.* 1999). We here demonstrated that, among various BMP molecules, BMP8 is abundant in granulosa cells (Fig. 3). Therefore, whether and how BMP8 affects progesterone production in granulosa cells needed to be further characterized. Similar to the BMP4 control, we were able to demonstrate that addition of BMP8A significantly dampened gonadotropin-induced progesterone production in a dose-dependent manner in both immature (Fig. 6A) and mature (Fig. 6B) rat granulosa cells.

Interestingly, in both immature (Fig. 7A) and mature (Fig. 7B) granulosa cells, the suppression of gonadotropin-driven progesterone production by BMP8A can be reversed by adding dorsomorphin, an inhibitor of the SMAD1/5/8 pathway, but not by adding SB431542, an inhibitor of the SMAD2/3 pathway, while in the controls these SMAD pathway inhibitors had no effect on gonadotropin-driven progesterone production themselves. Furthermore, changes in the transcriptional

Figure 6 BMP8A suppresses gonadotropin-induced progesterone synthesis in rat granulosa cells. Granulosa cells from immature rats primed with PMSG for 0 h (A) or 48 h (B) were treated with FSH (0.1 nM) or hCG (0.1 nM) in combination with either BMP4 (a positive control; 1 nM) or BMP8A (1 nM or 3 nM). The levels of progesterone in the medium were determined after 48 h incubation. Data are expressed as means $\pm$ S.D. ** $P < 0.01$; *** $P < 0.001$; one-way ANOVA, followed by Bonferroni post test. Three experiments were performed and gave similar results.

levels of steroidogenesis-related genes, including *Star*, *Cyp11a1* and *Hsd3b*, were evaluated. In immature granulosa cells, it seems that only the FSH-elevated level of *Cyp11a1*, but not that of *Star* or *Hsd3b*, was significantly reduced by BMP8A (Fig. 7C, D and E). In contrast, in mature granulosa cells, the levels of *Star*, *Cyp11a1* and *Hsd3b* induced by hCG can all be suppressed by BMP8A (Fig. 7F, G and H). Specifically,

Figure 7 Effects of BMP8A on gonadotropin-induced steroidogenesis-related genes in rat granulosa cells. Granulosa cells from immature rats primed with PMSG for 0 h (A, C, D and E) or 48 h (B, F, G and H) were treated with FSH (0.1 nM) or hCG (0.1 nM) in combination with dorsomorphin (D; 10 μ M), SB431542 (S; 10 μ M) and/or BMP8A (3 nM) as indicated. (A and B) The levels of progesterone in the medium were determined after 48 h incubation. Data are expressed as means $\pm$ S.D. Three experiments were performed and gave similar results. Changes in the transcripts of steroidogenesis-related genes, including *Star* (C and F), *Cyp11a1* (D and G) and *Hsd3b* (E and H), were then evaluated by real-time PCR. Data were normalized against *Actb* amounts and are expressed as means $\pm$ S.E.M. *** P < 0.001; one-way ANOVA, followed by Bonferroni post test. n = 3 biological replicates.

the above suppressive effects of BMP8A can be reversed by the presence of dorsomorphin but not of SB431542 (Fig. 7C, D, E, F, G and H), suggesting that the suppression of gonadotropin-driven progesterone synthesis and steroidogenesis-related genes by BMP8 is mediated via the SMAD1/5/8 pathway only.

Discussion

Using a model of rat granulosa cells, we demonstrated BMP8 is able to inhibit gonadotropin-stimulated progesterone production as well as the expression of various steroidogenesis-related genes, including *Star*, *Cyp11a1* and *Hsd3b*, via activation of SMAD1/5/8 signaling. Interestingly, among the ligands capable of activating the SMAD1/5/8 pathway, we found that BMP8 is relatively abundant in granulosa cells, where both the transcript levels and protein amounts of BMP8 decrease in parallel with follicle maturation. Taken together, our findings suggest that BMP8 signaling can counteract the activities of gonadotropins in granulosa cells and thus potentially prevents their premature luteinization. In other words, regulation of BMP8 expression would be critical for proper follicle development and for the timing control of subsequent ovulation. Thus, BMP8 may act as a novel luteinization inhibitor in the rodent ovary.

Although some physiological differences exist between the primate and rodent ovaries, the expression patterns of BMP-related ligands seem to be conserved in terms of evolution across species. For example, it

has been shown that the expression profiles of several BMPs, such as BMP2, BMP6, BMP7 and BMP15, in the human ovaries are virtually identical with those in the rodent ovary (Yoshino *et al.* 2011). Moreover, most of these factors also play similar and critical roles during ovarian follicle development in both primates and rodents (Knight & Glister 2006). Therefore, our findings in rodents create a further interest in characterizing the expression profiles and physiological roles of these two *BMP8* genes in the human ovary. Although these have not yet been fully characterized, in this study we have preliminarily found that BMP8 exhibited similar expressional characteristics in the ovaries of humans and rodents. For example, expressions of both the *BMP8A* and *BMP8B* genes were enriched in the ovaries of humans and rats (Fig. 1). In terms of granulosa cells, we also identified that *BMP8B* is likely to be the most abundant candidate among all searchable BMP-related ligands in human mature granulosa cells (Fig. 3A). Women of advanced age or women with PCOS often suffer from premature luteinization (Willis *et al.* 1998, Wu *et al.* 2015). Interestingly, our findings also indicated that the level of *BMP8B* in granulosa cells from these women is relatively low compared with that in granulosa cells from the matched normal controls (Fig. 3B). Taken together, these results provide a hint that BMP8 may also act as a luteinization inhibitor in the human ovary to control the timing of ovulation; if this is true, then the levels of the *BMP8* genes in residual granulosa cells may reflect the quality of oocyte retrieved from the same follicle.

It has been reported that deletion of either *Bmp8a* or *Bmp8b* in mice can cause defects in postnatal spermatogenesis (Zhao *et al.* 1996, 1998). Although we found that the ovary exhibits higher levels of transcripts and protein amounts of BMP8 than the testis in rodents (Fig. 1), no phenotypic change regarding the ovary or other female reproductive tracts has been implicated in the above knockout mice. Many BMP-related and TGF β -related ligands have been shown to be expressed and interact in a complex manner in the ovary (Knight & Glister 2006). Also, both *Bmp8a* and *Bmp8b* are expressed in the ovary. Therefore, it is likely that compensation by other ligands limits deleterious effects of functional loss of BMP8A or BMP8B protein in the knockout mouse models.

BMP8 proteins are uniquely marked by an additional 8th cysteine in the mature domains of the TGF β superfamily that normally contain seven conserved cysteines (Ozkaynak *et al.* 1992). Therefore, certain folding machinery in specific cell types and/or conditions may be required for the generation of functional BMP8 mature domains. Using prodomain shuffling, we previously have found that BMP10 prodomain may overcome the above issue and can successfully facilitate the production of bioactive BMP8A protein in 293T cells (Wu *et al.* 2017). In this study we further demonstrated that purified BMP8A protein can activate both the SMAD1/5/8 pathway and the SMAD2/3 pathway in immature and PMSG-primed mature rat granulosa cells (Fig. 4). Of interest, gene depletion models using mice have indicated that both sets of SMAD signaling in granulosa cells are involved in the control of follicle ovulation and luteinization. For example, female mice with granulosa cell-specific deficiency in *Smad4* exhibit prematurely luteinized follicles with trapped oocytes and eventually become infertile by 4–6 months of age (Pangas *et al.* 2006). In contrast to this, although individual disruption of either SMAD1/5/8 signaling or SMAD2/3 signaling in mouse granulosa cells can lead to disorders of multiple ovarian processes or even to development of metastatic granulosa cell tumors, none of each can totally phenocopy the *Smad4*-conditional knockout mice (Li *et al.* 2008, Pangas *et al.* 2008). Taken together, these findings favor the hypothesis that the SMAD1/5/8 and SMAD2/3 pathways may mediate different activities in granulosa cells, while at the same time these two SMAD pathways do also work synergistically in order to control the correct timing of folliculogenesis and luteinization (Pangas 2012). Consistently, our data also indicated that the two SMAD pathways activated by BMP8 in granulosa cells may function in different ways. We showed that blockage of the SMAD1/5/8 pathway, but not blockage of the SMAD2/3 pathway, activated by BMP8A can restore gonadotropin-induced progesterone production in granulosa cells, suggesting that BMP8-driven SMAD2/3 signaling is not involved in the control of steroidogenesis. On the other hand,

activation of the SMAD2/3 pathway by either GDF9 or TGF β has been shown to have pronounced effects on promoting proliferation and survival of granulosa cells (Dorrington *et al.* 1988, Sasseville *et al.* 2010). Therefore, it is worthwhile to further explore whether BMP8 protein can affect other activities of granulosa cells, such as proliferation, differentiation and/or their subsequent survival after luteinization, via activation of the SMAD2/3 pathway.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

F-J W and C-W L designed the research. F-J W and Y-W W did the research and data analysis. F-J W, Y-W W and C-W L wrote the manuscript. All authors read and approved the final manuscript.

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