A unique preovulatory expression pattern plays a key role in the physiological functions of BMP-15 in the mouse

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Mutations in the bone morphogenetic protein 15 (BMP-15) gene cause female infertility in the monoovulatory human and sheep; however, in the polyovulatory mouse, loss-of-function of BMP-15 results only in reduced ovulation rate. To elucidate the cause of these species-specific differences, we investigated the functional role of BMP-15 in the mouse ovary. Here, we found that the functional mature form of BMP-15 is barely detectable in the mouse oocytes until just before ovulation, when it is markedly increased. Further, we found that BMP-15 induces cumulus expansion in mouse cumulus–oocyte complexes. The oocyte culture medium from immature mice primed with pregnant mare serum gonadotropin followed by human chorionic gonadotropin also stimulated cumulus expansion, and this activity was attenuated by BMP-15 antibody. Interestingly, the oocyte culture medium from mice treated with pregnant mare serum gonadotropin alone had no effect. Moreover, BMP-15 stimulated the expression of EGF-like growth factors in cumulus cells as well as a series of molecules downstream of EGF-like growth factor signaling, including cyclooxygenase 2, hyaluronan synthase 2, tumor necrosis factor-stimulated gene 6, and pentraxin 3, all of which are necessary for normal cumulus expansion. An antagonist of the EGF receptor completely abolished the effect of BMP-15 in inducing cumulus expansion. These results are consistent with the phenotype of BMP-15-null mice, which exhibit normal folliculogenesis but have defects in the ovulation process. The species-specific differences in the phenotypes caused by BMP-15 mutations may thus be attributed to the temporal variations in the production of the mature form of BMP-15.

bone morphogenetic protein | cumulus expansion enabling factor | oocyte | ovary | ovulation | folliculogenesis

Bone morphogenetic protein 15 (BMP-15) is an oocyte-specific growth factor that plays a crucial role in determining ovulation quota in mammals (1). Genetic studies have shown that mutations in the *Bmp15* gene in ewes cause increased ovulation rates and fertility in heterozygotes, yet infertility in the homozygous carriers (2, 3). Studies by our laboratory have shown that these mutations are manifested through defects in the processing of the proproteins of the factors (4, 5). A recent study has shown that a mutation in the *Bmp15* gene also causes infertility in humans (6), demonstrating a similar critical role of BMP-15 for fertility in women. Importantly, because this mutation occurs in the proregion of the BMP-15 protein, rather than in the functional mature region, the human BMP-15 mutation must be manifested by causing a defect in the posttranslational processing of the human BMP-15 proprotein, similar to the mutations in sheep (4, 5). However, in contrast to monoovulatory ewes and humans, BMP-15 does not seem to be necessary for folliculogenesis in the polyovulatory mouse (7). Specifically, BMP-15-null mice exhibit no obvious defects in folliculogenesis, completing all stages of follicle development and having multiple corpora lutea (7). Nevertheless, their litter size is smaller compared with the wild-type mice, two-thirds of the normal range. Interestingly, when these null mice were subjected to pharmacological superovulation, the ovaries had denuded oocytes that were trapped within the follicles and not released. Moreover, these mice demonstrated a reduction in the ability of oocytes to develop into viable embryos. Therefore, defects in the BMP-15-null mice are confined to the ovulation process and subsequent fertilization. Thus, there is a clear species-specific difference in the role of BMP-15 in female reproduction (8). The question is: What mechanisms elicit the difference?

Our recent study has provided significant potential for breakthroughs in this issue. Although the recombinant human BMP-15 proprotein is readily processed into the bioactive mature form, the recombinant mouse BMP-15 proprotein is resistant to proteolytic cleavage (9). These results were obtained by using the *in vitro* transfection system of 293 human embryonic kidney and Chinese hamster ovary cell lines. However, if the endogenous BMP-15 proprotein is not processed into its bioactive form *in vivo* in the mouse, a fairly normal folliculogenesis in BMP-15-null mice may be anticipated. Furthermore, the defects in the ovulation and subsequent fertilization processes observed in the null mice may be attributed to the lack of bioactive BMP-15 that is likely to be produced during, and not before, the ovulation process.

Taken together, we hypothesize that a marked species-specific difference in the phenotype caused by the BMP-15 mutations is attributed to differences in the level of functional BMP-15 mature protein, which may result from proprotein processing. Therefore, the overall aim of the current study is to elucidate the physiological relevance of BMP-15 in the mouse ovary. Specifically, we first investigated whether functional BMP-15 mature protein is produced in the mouse oocytes during the ovulation process *in vivo*. We then examined whether BMP-15 induces cumulus expansion in mouse cumulus–oocyte complexes. Molecular mechanisms underlying the ability of BMP-15 in inducing cumulus expansion were also investigated in this study. Our discovery that the level of functional mature form of BMP-15 is regulated during the ovulation process in the mouse ovary and that BMP-15 induces cumulus expansion provides a fundamental mechanism accounting for the observed phenotype of the BMP-15-null mice.

Results

BMP-15 Protein and mRNA Expression in Mouse Oocytes. In view of the defects in the ovulation process of BMP-15-null mice, we began with a study of the developmental pattern of mouse BMP-15 protein expression in the oocyte *in vivo* during the ovulation process. As presented in Fig. 1*A*, the mature form of mouse BMP-15 was barely detectable in the oocytes collected from all mice

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Abbreviations: ALK, activin receptor-like kinase; C.E.I., cumulus expansion index; COCs, cumulus–oocyte complex; COX-2, cyclooxygenase 2; FSH, follicle-stimulating hormone; GDF-9, growth and differentiation factor 9; HAS-2, hyaluronan synthase 2; hCG, human chorionic gonadotropin; LH, luteinizing hormone; PMSG, pregnant mare serum gonadotropin; TSG-6, tumor necrosis factor-stimulated gene 6; Ptx-3, pentraxin 3; BMP-15, bone morphogenetic protein 15; EGF-R, EGF receptor.

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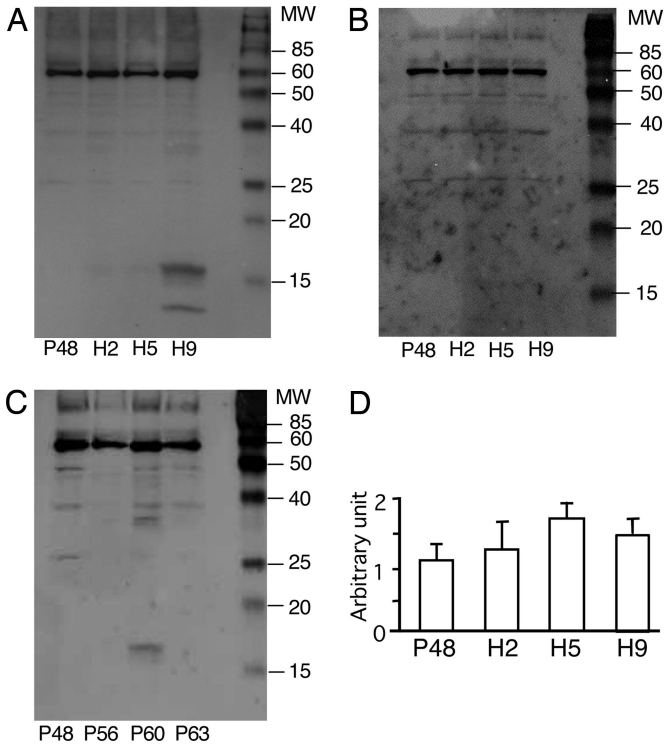

Fig. 1. Expression of mouse BMP-15 in the oocytes during the ovulation process. Oocyte lysates were collected from 24-day-old mice that had been primed with PMSG (5 units) for 48 h (designated as P48) and PMSG for 48 h followed by hCG (5 units) for 2, 5, and 9 h (H2, H5, and H9, respectively) (*A*) or from mice treated with PMSG alone (5 units) for 48, 56, 60, or 63 h (*C*). Six micrograms of each oocyte lysate was subjected to Western immunoblotting analysis by using the anti-mouse BMP-15 antibody (*A* and *C*) or anti-mouse BMP-15 antibody preincubated with an excess amount of antigen to block the binding sites on the antibody (*B*). The data presented are representative of five independent experiments. The steady-state mRNA expression levels in the oocytes were determined by using real-time PCR analysis (*D*). Data are normalized to L19 mRNA levels, combined from five independent experiments and represented as the mean ± SEM ($P > 0.18$).

except for those treated with pregnant mare serum gonadotropin (PMSG) for 48 h followed by human chorionic gonadotropin (hCG) treatment for 9 h. The higher and lower mature protein bands most likely represent the glycosylated and nonglycosylated forms, respectively, similar to our previous data from recombinant mouse BMP-15 mature protein (9). Both the bands were completely blocked by the antibody preincubated with antigen that was used to generate the antibody (Fig. 1*B*). However, the band detected at ≈60 kDa was nonspecific because it remained unchanged after the antigen treatment. Additionally, the antibody had no crossreactivity with its closest homolog, growth and differentiation factor 9 (GDF-9) (Fig. 6, which is published as supporting information on the PNAS web site). It is well known that mice treated with hCG after PMSG injection ovulate at around 12 h after hCG treatment (10). Therefore, the functional mature form of BMP-15 is produced just before ovulation in the oocytes undergoing luteinizing hormone (LH)/hCG-induced meiotic maturation.

Because it is reported that PMSG alone can induce the endogenous LH surge and subsequent ovulation in immature mice, with 95% of mice ovulating at 63 h after PMSG injection (11), we also analyzed oocyte lysates from mice treated with PMSG alone for 48, 56, 60, or 63 h for expression of BMP-15 to confirm our findings using exogenously administered hCG. As shown in Fig. 1*C*, BMP-15 mature protein was detected only at 60 h after PMSG injection. Subsequent careful examination of the oviducts for ovulated oocytes indicated that most of the mice treated with PMSG for 63 h had ovulated, whereas no oocytes were found in the oviducts at 60 h or earlier. Thus, the BMP-15 mature protein is produced just before ovulation in response to both the endogenous LH surge and exogenously administered LH.

To investigate whether changes in the mature protein production during the PMSG/hCG time-course treatment are due to differences in BMP-15 mRNA expression, we measured steady-state mRNA levels of BMP-15 by quantitative real-time PCR in the oocytes from mice treated with PMSG for 48 h and PMSG for 48 h followed by hCG for 2, 5, and 9 h. As presented in Fig. 1*D*, BMP-15 mRNA levels were not significantly changed during the PMSG/hCG time course.

BMP-15 Induces Cumulus Expansion. To investigate whether BMP-15 can induce cumulus expansion *in vitro*, cumulus–oocyte complexes (COCs) were incubated with increasing doses of recombinant human BMP-15 or follicle-stimulating hormone (FSH), followed by analysis for the degree of cumulus expansion. Addition of either BMP-15 or FSH induced cumulus expansion in a dose-dependent manner (Fig. 2). As expected (12), FSH induced cumulus expansion with a cumulus expansion index (C.E.I.) of 1.5 at a concentration of 3 ng/ml (Fig. 2*B*). A similar degree of cumulus expansion was observed with 10 ng/ml of BMP-15 (Fig. 2*C*). However, no significant difference in the degree of cumulus expansion between BMP-15 and FSH was observed at and above 30 ng/ml (Fig. 2 *B* and *C*). Moreover, addition of BMP-15 and FSH together at a concentration of 30 ng/ml each failed to induce greater expansion than that caused by either of the proteins alone (data not shown).

Cumulus Expansion Is Induced by Factor(s) Secreted from Oocytes of Preovulatory Follicles After the LH Surge. We next investigated whether endogenous mouse BMP-15 is also able to induce cumulus expansion. Oocytes were collected from mice treated with PMSG for 48 h, or with PMSG followed by hCG for 9 h, and cultured for 24 h before collecting the conditioned medium. The collected medium was added to the COCs obtained from immature mice treated with PMSG for 48 h, and cumulus expansion was evaluated after the 20 h culture. Interestingly, the degree of cumulus expansion markedly increased when COCs were cultured with oocyte-conditioned medium from PMSG/hCG-treated mice, whereas as reported (12) the medium from oocytes of mice treated with PMSG alone had no effect (Fig. 3*A*). These findings strongly suggest that a stimulatory factor(s) for cumulus expansion is secreted from the oocytes only after the LH/hCG surge, which coincides with the timing of the production of BMP-15 mature protein.

Endogenous BMP-15 Induces Cumulus Expansion. To ascertain that the increase in the degree of cumulus expansion by the oocyte culture medium obtained from PMSG/hCG-treated mice was attributed to endogenous BMP-15, an antibody specific for mouse BMP-15 was added to the culture medium. Addition of BMP-15 antibody (20 μg/ml) attenuated the increased cumulus expansion evoked by the oocyte-conditioned medium obtained from PMSG/hCG-treated mice (Fig. 3*B*). The degree of suppression of bioactivity by the BMP-15 antibody was similar to that by follistatin. Follistatin is known to have the ability to block the bioactivity of some members of the TGF-β superfamily, including BMP-15 (13).

BMP-15 Increases the mRNA Expression of Genes Regulating Cumulus Expansion. We next examined whether BMP-15 can regulate the mRNA expression of key genes regulating ovulation, including EGF-like growth factors (amphiregulin, betacellulin, and epiregulin), cyclooxygenase 2 (COX-2), hyaluronan synthase 2 (HAS-2), tumor necrosis factor-stimulated gene 6 (TSG-6), and pentraxin 3 (Ptx-3) (14). COCs collected from mice treated with PMSG for 48 h were used in this study. As shown in Fig. 4, the mRNA levels in the control sets were up-regulated with time because of the presence of FBS, although the significance of the up-regulation by FBS is

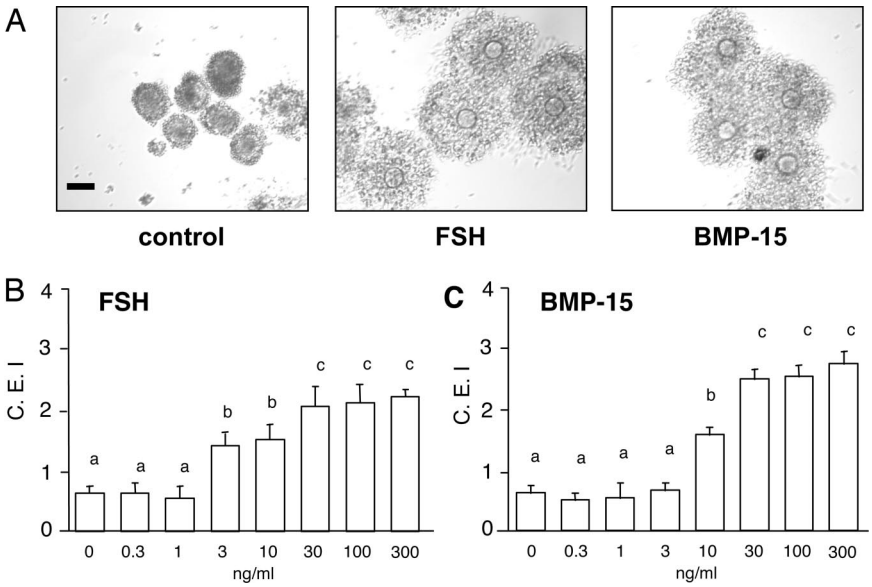

Fig. 2. Effect of BMP-15 on cumulus expansion. Mouse COCs were cultured with control medium, BMP-15 (300 ng/ml), or FSH (300 ng/ml) at 37°C, and the level of cumulus expansion was assessed after 20 h. (Scale bar in *A*: 100 μm.) Ten COCs were cultured with the indicated concentrations of FSH (*B*) or BMP-15 (*C*). The degree of cumulus expansion was assessed after 20 h and expressed as C.E.I. Data from three different experiments were combined and represented as the mean ± SEM. Bars with different letters indicate a significant difference at $P < 0.05$.

unclear because no cumulus expansion was observed when COCs were cultured in the control media containing FBS (Fig. 2). However, as compared with the controls, BMP-15 caused nearly 2- to 3-fold increase in the mRNA expression levels of amphiregulin, betacellulin, epiregulin, COX-2, and HAS-2 after 3 h of the treatment. Also, there was a significant increase in the mRNA levels of TSG-6 and Ptx-3 after 6 h of BMP-15 stimulation.

EGF Receptor (EGF-R) Signaling Mediates the Stimulatory Activity of BMP-15 in Cumulus Expansion. Because BMP-15 stimulates the mRNA expression of EGF-like growth factors (amphiregulin, betacellulin, and epiregulin), we hypothesized that EGF-R signaling is involved in the mechanism by which BMP-15 stimulates cumulus expansion. To test this hypothesis, we examined the effect of AG1478, a selective inhibitor of EGF-R signaling (14), on BMP-15-induced cumulus expansion. Mouse COCs were preincubated for 30 min with AG1478 or DMSO (control), and then BMP-15 (300 ng/ml) or EGF (50 ng/ml) was added to the culture medium. After 20 h of culture, the degree of cumulus expansion was determined. As shown in Fig. 5, EGF and BMP-15 could induce cumulus expansion to nearly the same degree, and these effects were completely abolished by the addition of AG1478, thereby indicating that EGF-R signaling is essential for BMP-15 to stimulate cumulus expansion.

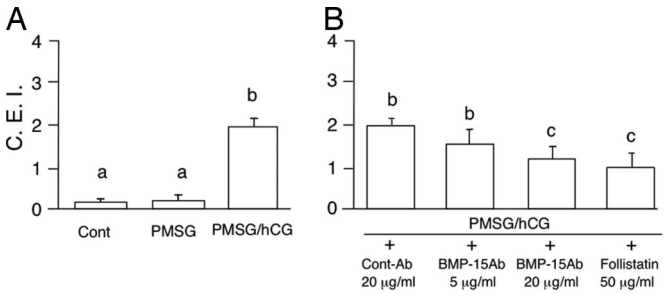

Fig. 3. Effect of anti-mouse BMP-15 Ab on cumulus expansion. Oocytes were collected from immature mice treated with PMSG for 48 h or PMSG followed by hCG for 9 h. After 24 h of culture, conditioned media were collected and used to culture fresh COCs collected from immature mice treated with PMSG for 48 h (*A*). Oocyte conditioned medium collected from mice treated with PMSG/hCG was also cultured with fresh COCs in the presence of IgG fractions purified from the preimmunized (Control-Ab) or anti-mouse BMP-15 (BMP-15 Ab) serum with indicated concentrations, or follistatin-288 (*B*). After 20 h, the degree of cumulus expansion was assessed. Data from three different experiments were combined and represented as the mean ± SEM. Bars with different letters indicate a significant difference at $P < 0.05$.

Discussion

In the current study, we demonstrate that the functional BMP-15 mature protein is barely detectable in the mouse oocytes except for those in the preovulatory follicles after LH/hCG-induced meiotic maturation. This finding is supported by the phenotype of the BMP-15-null mice, in which there are no defects in follicular development and yet decreased ovulation rates are observed due to defects in the ovulation process (7). We have previously reported that, unlike recombinant human BMP-15, the functional mature form of mouse BMP-15 is not produced in an *in vitro* transfection system (9). In contrast to recombinant mouse BMP-15, recombinant mouse GDF-9 produced by *in vitro* cell transfection is readily processed and secreted as its mature GDF-9 protein (15). Similarly, recombinant human GDF-9 (4) and recombinant ovine GDF-9 (16) are also readily processed. Therefore, as opposed to human and ovine BMP-15 and mouse, human, and ovine GDF-9, a defining feature of mouse BMP-15 is an impairment of the proprotein processing. This marked difference in regulation of proprotein processing fits the phenotype of BMP-15-null female mice that are only subfertile (7), whereas GDF-9-null female mice (17), GDF-9 mutant homozygous ewes (3), and BMP-15 mutant homozygous ewes (2, 3) are all infertile because of arrested folliculogenesis at the primary stage. Thus, we hypothesize that the processing of the GDF-9 proprotein into functional mature GDF-9 occurs in the oocytes at least at the primary follicular stage in all these species. In contrast, as demonstrated in the current study, the bioactive mature form of mouse BMP-15 is not present *in vivo* until just before ovulation. Thus, the expression of functional mature BMP-15 is highly controlled in the mouse oocytes in a timely and developmentally regulated manner after the stimulation of the ovulatory sequence by an LH/hCG surge. These findings demonstrate the concept that the availability of bioactive BMP-15 is developmentally regulated with remarkable precision during specific stages after the resumption of meiosis.

In contrast to the dramatic change in the levels of the BMP-15 mature protein during the PMSG/hCG time-course study, the BMP-15 mRNA levels were unchanged. One possible mechanism for the increase in the mature protein could be due to the translational regulation. Indeed, translation of some mRNAs in the

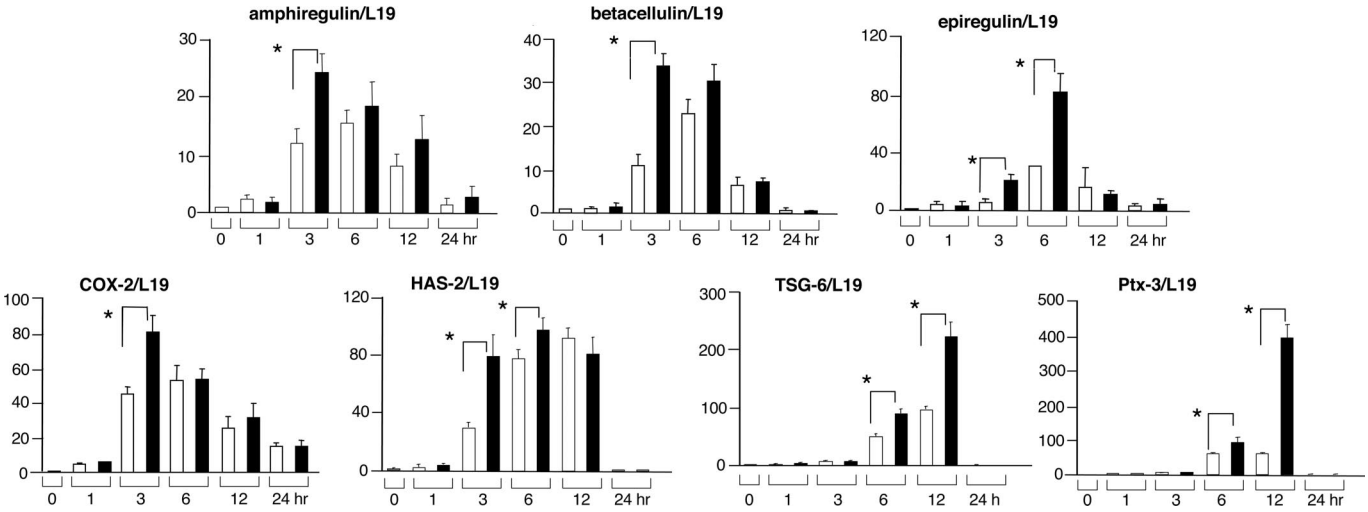

Fig. 4. Effect of BMP-15 on the steady-state mRNA levels of genes regulating cumulus expansion. COCs obtained from PMSG-treated mice were cultured with or without BMP-15 (300 ng/ml) for different time intervals (0–24 h). Total RNA was then extracted from the COCs and subjected to real-time PCR to determine the mRNA levels of the indicated genes. Data are normalized to L19 mRNA levels and shown as relative ratio to the mRNA level at time 0 (without culture). Filled and open bars represent the relative mRNA levels obtained from the culture in the presence or absence of BMP-15, respectively. Data from three different experiments were combined and represented as the mean ± SEM. *, a significant difference at $P < 0.05$ between the indicated pairs.

oocytes is activated by cytoplasmic polyadenylation through a specific cis-element called the cytoplasmic polyadenylation element (CPE) that is usually located <100 nt upstream of the polyadenylation recognition signal, AAUAAA, in the 3′ noncoding region (18). However, the 3′ noncoding region of mouse BMP-15 does not contain the CPE (GenBank accession no. NM009757), suggesting that BMP-15 mRNA translation is not regulated by cytoplasmic polyadenylation. Furthermore, we were unable to detect the BMP-15 proprotein at any time point in any of the samples, although the processed mature form was present. Therefore, it is possible that, unless the mouse BMP-15 proprotein is processed into the stable mature form, it is targeted to degradation shortly after translation. The labile nature of the proprotein compared with the mature form has been demonstrated by a pulse-chase analysis of metabolically radiolabeled BMP-7 (19). Other BMPs such as BMP-3, -4, -6, and -7 were also detected only as mature proteins in various tissue extracts (20–22), suggesting that their proproteins are immediately processed or degraded after translation.

What does BMP-15 mature protein do just before ovulation in mice? Given that the BMP-15-null mice exhibit defects in the processes of ovulation, fertilization, and early embryonic development (7, 23), and our present finding that a dynamic change in the level of mouse BMP-15 mature protein occurs after the LH/hCG-induced onset of the ovulation process, we have investigated the role of the BMP-15 mature protein in controlling key cellular activities involved in ovulation and postovulation events. Specifically, we first asked whether BMP-15 has the ability to induce cumulus expansion, the process critical for normal ovulation, fertilization, and early embryonic development. It is known that oocytectomized cumulus shells require both FSH and fully grown oocytes for their expansion (12, 24). Among various molecules evaluated for their ability to induce cumulus expansion, TGF-β and GDF-9 have been shown to induce cumulus expansion *in vitro* in the presence of FSH, although there is a currently some debate as to whether FSH is required for the effect of GDF-9 (possibly because of variation in the degree of sample purity) (25). In the present study, we have found that a purified preparation of recombinant human BMP-15 can stimulate the expansion of cumulus cells of the mouse COC in the absence of FSH.

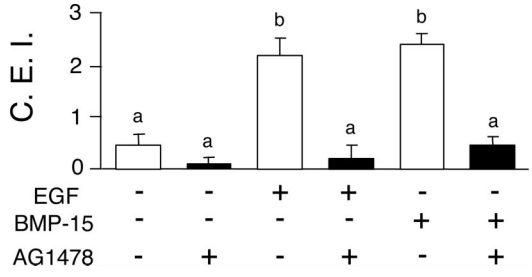

Fig. 5. Effect of AG1478 on EGF- or BMP-15-induced cumulus expansion. Mouse COCs were preincubated with 10 μM AG1478, a selective inhibitor of EGF-R, or DMSO (control) for 30 min before culturing with or without BMP-15 (300 ng/ml) or EGF (50 ng/ml) for 20 h. The degree of cumulus expansion was then assessed and expressed in terms of C.E.I. Data from three different experiments were combined and represented as the mean ± SEM. Bars with different letters indicate a significant difference at $P < 0.05$.

To address the physiological relevance of this finding, we used the conditioned media collected from the culture of oocytes obtained from mice treated with PMSG for 48 h or PMSG followed by hCG for 9 h. Interestingly, the oocyte conditioned medium from PMSG/hCG-treated mice exhibited a marked increase in the degree of cumulus expansion in the absence of FSH, whereas that from mice treated with PMSG alone had no effect. Currently, the accepted mechanism for the cumulus expansion process is that fully grown oocytes secrete the cumulus expansion enabling-factors (CEEFs) that promote cumulus expansion by virtue of FSH (12). However, our present data indicate that a factor from oocytes of LH/hCG-primed mice does not require FSH in the culture media for its ability to expand cumulus cells. Moreover, the activity of this factor was attenuated by BMP-15 antibody, establishing the physiological relevance of endogenous BMP-15 in regulating cumulus expansion in mice.

Su *et al.* (23) demonstrated that the FSH-induced degree of cumulus expansion in the COCs from BMP-15-null mice is not statistically different from that of heterozygous BMP-15 mutant mice. Moreover, Gui and Joyce (26) used an RNA interference approach to investigate the role of BMP-15 and GDF-9 in promoting cumulus expansion. In their study, oocytes from PMSG-treated mice were microinjected with long double-stranded RNA to knockdown the expression of GDF-9 or BMP-15. Interestingly, cumulus expansion was severely inhibited in cumulus shells cocultured with GDF-9 knockdown oocytes, whereas knockdown of BMP-15 had

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little effect on cumulus expansion (26). These results suggest that BMP-15 is not required for cumulus expansion. However, our present data indicate that recombinant human BMP-15 as well as endogenous mouse BMP-15 (from hCG-primed oocytes) have the ability to induce cumulus expansion *in vitro*. The plausible explanation for this discrepancy is that the COCs used by Su *et al*. (23) and the oocytes used by Gui and Joyce (26) were obtained from mice treated with PMSG alone for 44–48 h, and we found that the production of the functional mature BMP-15 requires the sequential treatment with PMSG for 48 h and hCG for 9 h or PMSG treatment alone for at least 60 h (Fig. 1). Accordingly, it is not surprising that oocytes from mice treated with PMSG alone for 44–48 h had no effect.

The process of cumulus expansion involves secretion of a hyaluronan-rich matrix by the cumulus cells and expression of a number of proteins required for matrix formation and retention (27). It has long been known that the mid-cycle LH surge triggers cumulus cell expansion; however, this effect of LH must be indirect because the LH receptor is expressed in theca and mural granulosa cells and virtually absent in cumulus cells and oocytes of the mouse preovulatory follicles (28). A major breakthrough in this issue occurred with the discovery by Park *et al*. (14) that the LH surge induces the expression of EGF-like growth factors (amphiregulin, betacellulin, and epiregulin) in mural granulosa cells by virtue of the LH receptor signaling (14). These factors, in turn, stimulate the expression of downstream genes necessary for normal cumulus expansion, such as COX-2, HAS-2, and TSG-6 in the cumulus cells (14, 27). Additionally, *in situ* hybridization and PCR analysis have shown that these EGF-like growth factors are also expressed in mouse cumulus cells during ovulation (14, 29, 30). Therefore, we hypothesized that oocyte-derived BMP-15 may regulate the gene expression of amphiregulin, betacellulin, and epiregulin in the mouse cumulus cells, leading to the induction of cumulus expansion. Our present data do, in fact, support this hypothesis; indeed, BMP-15 induced the expression of these genes in cumulus cells within 3 h, which is comparable with the reported effect of LH in mural granulosa cells (14).

COX-2 and HAS-2 control the synthesis of prostaglandins and hyaluronan, respectively. Because the mRNA expression of these genes is induced by EGF-like growth factors (14), we further examined whether BMP-15 regulates the mRNA expression of COX-2 and HAS-2 and found that BMP-15 does induce their mRNA expression. BMP-15 also induced the mRNA expression of TSG-6 and Ptx-3, which are hyaluronan-binding proteins that stabilize the hyaluronan structural backbone in the expanded cumulus matrix (27). It is notable that, based on the present results from the time-course experiments, the mRNA expression of TSG-6 and Ptx-3 peaks after 12 h of BMP-15 stimulation, whereas EGF-like growth factors peak after 3–6 h of stimulation. Because TSG-6 is a downstream target of the EGF-like growth factors (14), the delayed up-regulation of TSG-6 and Ptx-3 expression is compatible with a mediatory role for the EGF-like growth factors in the BMP-15-signaling pathway. Furthermore, the fact that a selective inhibitor of EGF-R tyrosine kinase totally abolishes the ability of BMP-15 to stimulate cumulus expansion indicates the crucial role of the cascaded expression of these genes in cumulus cells in response to the oocyte factor, BMP-15.

There are no observable defects in folliculogenesis in mice with a targeted deletion of *Bmp15*; nevertheless, these mice do exhibit defects in the ovulation process as well as in the quality of fertilizable oocytes, resulting in reduced litter size (7). Interestingly, there is a remarkable similarity in the phenotype of the BMP-15-null mice and the mice lacking the genes responsible for cumulus expansion (7, 31–33). Mice null for COX-2 have impaired ovulation associated with defective cumulus expansion (34). TSG-6-null mice are unable to assemble a hyaluronan-rich extracellular matrix, which leads to a failure of cumulus expansion (33). The defects in the integrity of the cumulus–oocyte complex were also demonstrated in the Ptx-3-null mice (31). It is known that properly expanded cumulus cells surrounding the oocyte contribute not only to the ovulation process but also to fertilization (27). Thus, these phenotypes are reminiscent of BMP-15-null mice (7). Taken together, the defect in the ovulation process, as well as the reduced fertilization rate observed in BMP-15-null mice, is likely due to the lack of the stimulatory activity of BMP-15, which affects multiple parameters, including expression of amphiregulin, betacellulin, epiregulin, COX-2, HAS-2, TSG-6, and Ptx-3.

Mice null for activin receptor-like kinase 6 (ALK-6, or BMP type IB receptor), the type I receptor for BMP-15 (35), are viable but infertile, primarily because of defects in cumulus expansion (36). ALK-6 is expressed in oocytes, cumulus and mural granulosa cells of antral follicles in the mouse (36). In contrast to the wild-type mice, the COCs recovered from oviducts of the ALK-6-null mice are more aggregated and retained fewer cumulus cells. Given that these oocytes are fertilizable *in vitro*, the authors concluded that fragile cumulus–oocyte complexes in the oviducts are the primary cause of infertility in ALK-6-null mice, which is a characteristic phenotype also observed in BMP-15-null mice (7). In contrast to BMP-15, the type I receptor for GDF-9 is ALK-5 (37). GDF-9 was demonstrated to be an inducer of cumulus expansion *in vitro* (15, 26, 38). However, this ability of GDF-9 seems to be insufficient *in vivo* to form the stably expanded cumulus cells surrounding the oocyte, which is essential for normal fertilization because the intact GDF-9/ALK-5 signaling system retained in the ALK-6-null mice failed to compensate for the loss-of-function of ALK-6. Likewise, the observed defects in cumulus expansion in BMP-15-null mice also support this notion. Moreover, the ALK-6-null mice exhibited no obvious abnormality in developing follicles, a phenotype similar to BMP-15-null mice.

Collectively, we propose that the EGF-like growth factors are produced not only by mural granulosa cells induced by LH (14), but also by the cumulus cells induced by BMP-15, and together promote cumulus expansion. After ovulation, COCs are separated from the mural granulosa cells, and further downstream molecules (COX-2, HAS-2, TSG-6, and Ptx-3) that are induced by EGF-like growth factors in cumulus cells in response to BMP-15 facilitate successful fertilization by forming a stably expanded cumulus matrix. Given that the level of functional mature BMP-15 is dramatically increased just before ovulation *in vivo*, the physiological role of BMP-15 in the mouse may be restricted to promoting cumulus matrix expansion and stabilization during and after ovulation. Therefore, we speculate that the species-specific differences in the defects caused by mutations in the *Bmp15* gene between monoovulatory ewes and women and polyovulatory mice may be attributed to the timing of processing of the BMP-15 proprotein into the functional mature BMP-15, which is available beginning at the early stages of folliculogenesis in monoovulatory mammals but not until the time of ovulation in polyovulatory mammals, a factor that could be involved in determining ovulation quota.

Materials and Methods

PMSG, EGF, and AG1478 were purchased from Calbiochem, and hCG was from Sigma Aldrich. Ovine FSH (NIDDK-oFSH-S20) was provided by A. F. Parlow (National Hormone and Pituitary Program, Torrance, CA). Recombinant human BMP-15 and follistatin-288 were prepared as described (39, 40). Female C57BL/6 mice were from Charles River Laboratories.

Analysis of BMP-15 Protein and mRNA Expression in Mouse Oocytes. All animal protocols were approved by the University of California, San Diego Institutional Animal Care and Use Committee. The level of the endogenous BMP-15 during the ovulation process in the mouse was studied by using (*i*) fully differentiated mature germinal vesicle (GV) intact oocytes from preovulatory follicles from 24-day-old mice that had been primed with 5 units of PMSG for 48 h (41) and (*ii*) meiotically active oocytes at the GV break down

(GVBD), MI and MII stages of meiosis from mice treated with 5 units of PMSG for 48 h followed by the treatment with 5 units of hCG for 2, 5, and 9 h, respectively. Additionally, oocytes were obtained from mice treated with PMSG alone for 48, 56, 60, or 63 h.

The denuded oocytes were prepared as described (42). Oocyte lysates were prepared by suspending the oocytes in lysis buffer [Tissue-PE LB containing 5 mM DTT, 5 mM EDTA, and 10 μl/ml Protease Arrest (Genotech, St. Louis, MO)]. Six micrograms of each lysate was subjected to Western immunoblotting by using the anti-mouse BMP-15 antibody (1:25,000), which was prepared in house following the same procedure as described earlier (39), except that mouse BMP-15 mature protein produced in *Escherichia coli* was used as an antigen. Western blotting analysis was carried out as described (9). To determine the specificity of the BMP-15 antibody, it was incubated with an excess of antigen before use. The mRNA expression analysis was carried out by quantitative PCR using 100 oocytes in each group.

Collection and Culture of COCs. COCs were obtained by using a 28-gauge needle from preovulatory follicles of 24-day-old female mice that had been primed with PMSG (5 units) for 48 h. Ten COCs were cultured in 15 μl of Eagle's MEM in the presence of 5% FBS, and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) was also added to prevent GVBD. According to a previous report, IBMX (0.1 mM) has no effect on cumulus expansion (12). The complexes were overlaid with mineral oil in Petri dishes and cultured with or without FSH or BMP-15 for 20 h at 37°C.

Culture of COCs with Oocyte Conditioned Medium. Oocytes from PMSG-primed mice or PMSG followed by hCG (5 units) for 9 h were cultured in MEM containing 5% FBS at a concentration of 4 oocytes per μl. The conditioned media were then tested for their effect on cumulus expansion as described above by using COCs collected from immature mice treated with PMSG for 48 h. The experiments to examine the effect of BMP-15 antibody were conducted by using commercially available antibody (H-83; Santa Cruz Biotechnology) because it exhibited better blocking efficiency than the antibody produced in house. This antibody has no cross-reactivity with GDF-9 (Fig. 6).

Assessment of Cumulus Expansion. After culturing the COCs for 20 h, the degree of cumulus expansion was assessed according to a subjective scoring system, a scale of 0 (no expansion) to +4 (maximal expansion) (24, 43). Data are expressed in terms of cumulus expansion index, which is defined as the average expansion value for a particular group.

RNA Extraction and Real-Time PCR. Total RNA preparation, reverse transcription, and real-time PCR analysis were performed as described (44). Primer sequences and real-time PCR conditions are described in Table 1, which is published as supporting information on the PNAS web site. Each sample was analyzed in triplicate. The relative expression level of each gene was normalized to L19 mRNA levels.

Statistical Analysis. All experiments were performed at least three times, and data were combined. Multiple comparisons were performed by using one-way ANOVA with post hoc comparisons employing Fisher's test. The unpaired *t* test was used to compare results between control and treated samples in the quantitative PCR study. Statistical significance was considered to be $P < 0.05$.

Note Added in Proof. After we submitted this article, Shimada *et al*. (45) reported the expression and function of EGF-like growth factors in cumulus cells during the ovulatory process.

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