

Does Bone Morphogenetic Protein 6 (BMP6) Affect Female Fertility in the Mouse?¹

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ABSTRACT

Bone morphogenetic protein 6 (BMP6) is a transforming growth factor beta superfamily member produced by mammalian oocytes as well as other cell types. Despite well-characterized effects of recombinant BMP6 on granulosa cells *in vitro*, the function of BMP6 *in vivo* has been ill-defined. Therefore, the effects of genetic deletion of the *Bmp6* gene on female mouse fertility were assessed. The mean litter size of *Bmp6*^{-/-} females was reduced by 22% ($P < 0.05$) compared to *Bmp6*^{+/+} controls. Not only did *Bmp6*^{-/-} females naturally ovulate 24% fewer eggs, but competence of *in vitro*-matured oocytes to complete preimplantation development after fertilization *in vitro* was decreased by 50%. No apparent effect of *Bmp6* deletion on either the morphology or the dynamics of follicular development was apparent. Nevertheless, levels of luteinizing hormone (LH)/human chorionic gonadotropin (hCG)-induced transcripts, which encode proteins required for cumulus expansion (HAS2, PTGS2, PTX3, and TNFAIP6), and of epidermal growth factor-like peptides (AREG, BTC, and EREG) were lower in *Bmp6*^{-/-} mice than in controls after administration of a reduced dose of hCG (1 IU) *in vivo*. LH receptor (*Lhcgr*) transcript levels were not significantly lower in *Bmp6*^{-/-} granulosa cells, suggesting that BMP6 is required for processes downstream of LH receptors. To assess whether another oocyte-derived BMP, BMP15, could have BMP6-redundant functions *in vivo*, the fertility of *Bmp15/Bmp6* double mutants was assessed. Fertility was not significantly reduced in double-homozygous mutants compared with that in double-heterozygous controls. Therefore, BMP6 promotes normal fertility in female mice, at least in part, by enabling appropriate responses to LH and normal oocyte quality. Thus, *Bmp6* probably is part of the complex genetic network that determines female fertility.

BMP6, *Bmp6*, early development, fertility, follicles, follicular development, oocyte quality, oocytes, ovary, ovulation rate

INTRODUCTION

Development of mammalian ovarian follicles is coordinated by a complex interaction of multiple signals, including gonadotropins and intrafollicular paracrine/autocrine and juxtacrine signals [1]. Bone morphogenetic proteins (BMPs), members of the transforming growth factor beta superfamily, are produced in ovarian follicles. Attenuating BMP signals in mouse follicular granulosa cells by genetic deletion of its

receptors or signal mediators results in impaired female fertility [2–5]. Therefore, BMP signals play a critical role in coordinating mammalian follicular development [6–8].

The BMP ligand BMP6 is produced by mammalian oocytes as well as by other cell types [9–13]. The effect of recombinant BMP6 on granulosa cell functions has been studied extensively *in vitro*. For example, recombinant BMP6 suppresses follicle-stimulating hormone (FSH)- and/or insulin-like growth factor-induced progesterone production by rat, bovine, and ovine granulosa cells [14–17]. BMP6 decreases FSH receptor (*Fshr*) mRNA levels in rat granulosa cells [14] but enhances *FSHR* mRNA levels in human granulosa cells [12]. Furthermore, BMP6 prevents apoptosis of bovine cumulus cells, which are the granulosa cells associated with oocytes [18]. In addition to these studies *in vitro*, direct ovarian infusion of BMP6 in ewes advances the time of the luteinizing hormone (LH) surge, probably because of a transient increase in serum estradiol (E_2) levels in the BMP6-infused animals [19]. This finding was consistent with a previous report showing that recombinant BMP6 promotes FSH-induced E_2 production by ovine granulosa cells [20]. However, BMP6 has no effect on FSH-induced E_2 production by cultured rat granulosa cells [14]. Therefore, although not conclusive, it appears that BMP6 affects gonadotropin signals in granulosa cells of several mammalian species. Previous studies using *Bmp6*-null (*Bmp6*^{-/-}) mice found a consistent delay in ossification of the developing sternum and did not detect a significant reduction in female fertility [9]; however, it is important to note that the reproductive phenotype of *Bmp6*^{-/-} mice, such as ovarian morphology, granulosa cell development, or sensitivity to exogenous gonadotropin treatments, was not assessed.

To define the reproductive function of BMP6 *in vivo*, the present study attempted to characterize the effects of deletion of the *Bmp6* gene *in vivo* on female mouse fertility and granulosa cell function. Moreover, possible BMP6-redundant functions of the other oocyte-derived BMP, BMP15 [21, 22], on fertility of female mice was assessed using double-mutant (*Bmp6*^{-/-}/*Bmp15*^{-/-}) mice.

MATERIALS AND METHODS

Mice

Mice carrying a *Bmp6*-null (*Bmp6*^{tm1Lex}; hereafter designated as *Bmp6*⁻) allele, in which coding exons 5–7 of *Bmp6* were targeted by homologous recombination, were obtained from Lexicon Genetics, Inc., and were maintained on a C57BL/6J;129S5/SvEvBrd hybrid background in the research colony of the investigators at The Jackson Laboratory. The detailed values for physiological parameters are available online (<http://www.informatics.jax.org/external/ko/lexicon/2523.html>). The *Bmp6*-null allele (*Bmp6*^{tm1Lex}) used in the present study was different from that (*Bmp6*^{tm1Rob}) used in a previous study, in which a part of the *Bmp6* second coding exon, encoding a portion of the pro region, as well as some downstream sequence was targeted [9]. *Bmp6* mutant mice were identified by real-time PCR assay for the exons 6 and 7 and sequences of *Bmp6* gene over its exon 2 sequence (GenBank accession no. NM_007556). The PCR primers used were 5'-GCCAACTACTGTGATGGA GAGTGT-3' and 5'-GTACTCGGGATTCATAAGGTGGA-3' for exons 6 and 7 and 5'-ACCTATCCCAGATTCCTGAGGG-3' and 5'-GCTGATAA GAAAGGTTTGGTTTTTAAAA-3' for exon 2. Mice carrying both *Bmp6*⁻

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and *Bmp15*-null (*Bmp15^{tm1Zuk}*; hereafter designated as *Bmp15^{-/-}*) [23] alleles were produced by mating double-heterozygous (*Bmp6^{+/-}/Bmp15^{+/-}*) females with double-mutant (*Bmp6^{-/-}/Bmp15^{-/-}*) males and were maintained on a C57BL/6J;129S5/S hybrid background. All experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (Institute for Learning and Animal Research).

Fertility Assessment

To assess reproductive performance, 6-wk-old mutant or control female mice were mated with age-matched, wild-type (*Bmp6^{+/+}*) B6SJLF1 male mice. The number of pups per litter was recorded for a 6-mo period.

Histological Assessment of Ovaries

Ovaries of 18-day-old or 6-mo-old mutant and littermate control female mice were fixed in Bouin solution, embedded in paraffin, serially sectioned (thickness, 5 μ m), and stained with hematoxylin-eosin. The numbers of follicles at each developmental stage in ovaries of 18-day-old mice were estimated by counting follicles in every third section where the germinal vesicle of the oocyte was observed [24]. The criteria used for classifying developmental stages of follicles were as follows: primordial follicle, oocyte surrounded by a single squamous layer of follicular somatic cells; primary follicle, single layer of cuboidal granulosa cells; early or late secondary follicles, more than one or two layers, respectively, of granulosa cells and no antrum; early antral follicle, small antrum is in the process of forming; midantral follicle, a single antral space separating the mural granulosa cells from the cumulus cells surrounding the oocyte; and atretic follicle, contains a degenerated oocyte, an oocyte that has resumed meiosis, or cumulus cells dissociated from oocyte, and pyknotic granulosa cells are common.

Assessment of Natural and Gonadotropin-Induced Ovulation Rate

To assess the number of oocytes ovulated naturally, 2-mo-old *Bmp6^{+/+}* or *Bmp6^{-/-}* female mice were mated to vasectomized males. The female mice were assessed for a vaginal plug every morning. Oviductal oocytes were counted when a plug was found. To assess the ovulatory response to exogenous gonadotropin, 20-day-old female mice were injected with 5 IU of equine chorionic gonadotropin (eCG) and, 48 h later, were given 1 or 5 IU of human chorionic gonadotropin (hCG). Oviductal oocytes were counted 14 to 16 h after hCG injection.

Hormone Analyses

Levels of FSH, LH, E₂, and testosterone in serum of 20-day-old female mice were measured by the Ligand Assay and Analysis Core Laboratory (University of Virginia, Charlottesville, VA). The serum was prepared by centrifugation and stored at -80°C until assayed for hormonal levels. The limits of detection for each assay were 1.3 ng/ml, 0.04 ng/ml, 0.081 ng/ml, and 23 pg/ml for the FSH, LH, testosterone, and E₂ assay, respectively. Samples that exhibited values below the limits of detection were assigned the threshold value.

Reverse-Transcription Real-Time PCR

To assess steady-state levels of transcripts, reverse-transcription real-time PCR was conducted as reported previously [25]. The PCR primers used were 5'-CTCTCAGAATGATGTCTTGGAGGTAAT-3' and 5'-GATGTACAGCA GATTGTTAGCCTTTTC-3' for *Fshr* (GenBank accession no. NM_013523), 5'-GGAGAACAATTCGCCCTTTCTT-3' and 5'-GATGGACTCCACAC AAACCTTCCA-3' for *Cyp19a1* (NM_007810), 5'-CAAGAACATCCAGGCC AACATT-3' and 5'-ACCTTCAAGTTGTGTGCCATCTC-3' for *Cyp11a1* (NM_019779), and 5'-AAGCCTTGGGCATACTCAAC-3' and 5'-CCCAC ATCTGGCACCATT-3' for *Star* (NM_011485). The other PCR primers used were reported previously [25–28]. The results were normalized to a housekeeping gene, ribosomal protein L19 (*Rpl19*), by the 2^{- Δ ACT} method [29] and then presented as the levels relative to a control group.

Induction and Evaluation of Cumulus Expansion In Vitro

Bicarbonate-buffered Minimum Essential Medium α (Life Technologies, Inc.) with Earle salts, supplemented with 75 μ g/ml of penicillin G, 50 μ g/ml of streptomycin sulfate, 0.23 mM pyruvate, and 3 mg/ml of bovine serum albumin (Sigma-Aldrich Co.), was used as a basic culture medium. Cumulus cell-oocyte complexes (COCs) were isolated from 22- to 24-day-old *Bmp6^{+/+}* or *Bmp6^{-/-}* female mice that had been primed with 5 IU of eCG 44 to 48 h earlier [30]. To

TABLE 1. Fertility analysis of *Bmp6* mutant female mice.^a

| Genotype | No. of females | Average litter size | Litters/mo. |
|---------------------------|----------------|---------------------|-----------------|
| <i>Bmp6^{+/+}</i> | 6 | 8.24 \pm 0.47 | 1.29 \pm 0.03 |
| <i>Bmp6^{+/-}</i> | 6 | 7.04 \pm 0.72 | 1.36 \pm 0.03 |
| <i>Bmp6^{-/-}</i> | 6 | 6.43 \pm 0.55* | 1.14 \pm 0.08 |

^a Wild-type (*Bmp6^{+/+}*), *Bmp6* heterozygous (*Bmp6^{+/-}*), and homozygous mutant (*Bmp6^{-/-}*) female mice were bred with *Bmp6^{+/+}* males and litter size and litters per month were recorded for 6 months.

* Significant difference from *Bmp6^{+/+}* value ($P < 0.05$).

induce cumulus expansion, the COCs were cultured in the basic culture medium supplemented with 5% serum and 10 ng/ml of epidermal growth factor (EGF; BD Biosciences). Some of the COCs were taken for analysis of transcript levels after 3 h of EGF treatment. Cumulus expansion index [31] was assessed after 14 h of culture.

Assessment of Developmental Competence of the Oocytes

To assess the preimplantation developmental competence of oocytes, *Bmp6^{+/+}* and *Bmp6^{-/-}* oocytes were matured and fertilized with *Bmp6^{+/+}* sperm in vitro. Subsequent preimplantation development up to the blastocyst stage was assessed as reported previously [32].

Statistical Analysis

All experiments were repeated at least three times, and results are presented as the mean \pm SEM. The Tukey-Kramer honestly significant differences test was used to compare multiple groups, and a *t*-test was used for paired comparison using the computer software JMP (SAS Institute, Inc.). A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Fertility of *Bmp6^{-/-}* Mutant Female Mice

Wild-type (*Bmp6^{+/+}*), heterozygous (*Bmp6^{+/-}*), or *Bmp6^{-/-}* female mice were mated with *Bmp6^{+/+}* males, and the numbers of pups and litters per month were recorded. Although no significant difference was found in frequency of litters, the mean litter size of *Bmp6^{-/-}* female mice (6.43 \pm 0.55 pups/litter) was reduced significantly ($P < 0.05$), by approximately 22%, compared to that of *Bmp6^{+/+}* mice (8.24 \pm 0.47 pups/litter) (Table 1). Therefore, *Bmp6^{-/-}* female mice are subfertile.

Morphology of the *Bmp6^{-/-}* Ovary

The ovaries of 18-day-old and 6-month-old *Bmp6^{-/-}* mice displayed normal histology when compared with those of *Bmp6^{+/+}* mice (Fig. 1A). No significant differences were found in numbers of follicles at each developmental stage between 18-day-old *Bmp6^{+/+}* and *Bmp6^{-/-}* ovaries (Fig. 1B). Therefore, development of ovarian follicles in *Bmp6* mutant mice appears to be morphologically normal.

Reduced Response of the *Bmp6^{-/-}* Ovary to LH/hCG

To resolve the etiology of reduced fertility in *Bmp6^{-/-}* female mice, the number of naturally ovulated oocytes in vivo was compared between adult *Bmp6^{+/+}* and *Bmp6^{-/-}* mice without exogenous gonadotropin treatment. The number of oocytes ovulated by *Bmp6^{-/-}* mice (6.30 \pm 0.47 oocytes) was significantly lower than that ovulated by wild-type mice (8.33 \pm 0.61 oocytes) ($P < 0.05$) (Fig. 2A).

The effect of exogenous gonadotropin treatment on ovulation rate was examined using immature mice. The eCG-primed *Bmp6^{+/+}*, *Bmp6^{+/-}*, or *Bmp6^{-/-}* mice were injected with hCG, which mimics the LH surge, and the number of ovulated oocytes was determined after 14 h (Fig. 2B). *Bmp6^{-/-}* mice treated with 5

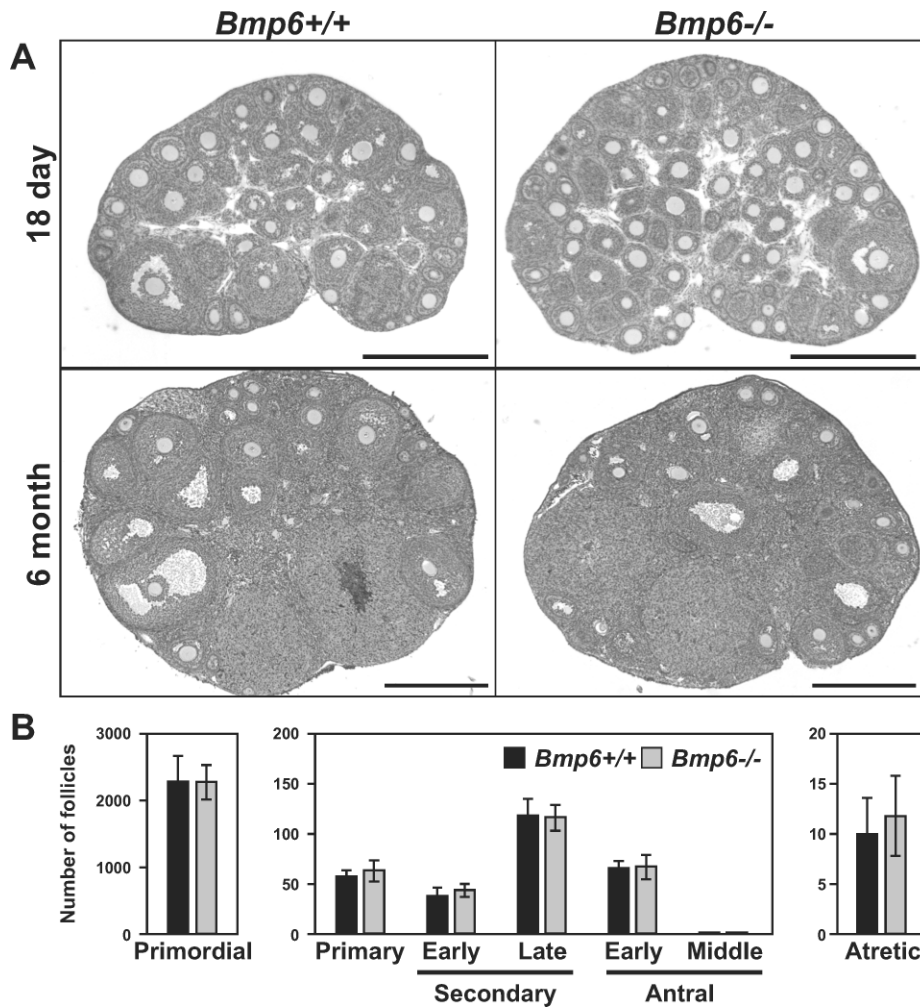


FIG. 1. Morphology of *Bmp6* mutant ovaries. **A**) Representative photographs of ovaries of 18-day-old (**top**) and 6-mo-old (**bottom**) *Bmp6*^{+/+} and *Bmp6*^{-/-} mice are shown. Bar = 500 μ m. **B**) Follicular count of unstimulated 18-day-old *Bmp6*^{+/+} (black bars) and *Bmp6*^{-/-} (gray bars) mice. Follicle counts were conducted for primordial follicles and the other developmental stages of follicles using three or nine ovaries of each genotype, respectively.

IU of hCG ovulated oocytes at numbers comparable to those in *Bmp6*^{+/+} and *Bmp6*^{+/-} mice. However, when *Bmp6*^{-/-} mice were treated with a lower dose of hCG (1 IU), *Bmp6*^{-/-} mice ovulated significantly fewer oocytes, whereas littermate *Bmp6*^{+/+} and *Bmp6*^{+/-} mutant mice ovulated the same numbers of oocytes as at the 5 IU dose (54.2 ± 5.1 , 41.3 ± 5.4 , and 10.8 ± 5.0 oocytes for *Bmp6*^{+/+}, *Bmp6*^{+/-}, and *Bmp6*^{-/-}, respectively; $P < 0.05$) (Fig. 2B). These results suggest that *Bmp6*^{-/-} female mice are subfertile, at least in part because of reduced ovulatory response to LH/hCG.

Serum levels of FSH, LH, and testosterone in 20-day-old female mice without exogenous gonadotropin treatment were examined (Fig. 2C). Serum E₂ levels were below the limit of detection in both *Bmp6*^{+/+} and *Bmp6*^{-/-} mice (not shown). Whereas the serum FSH and testosterone levels were comparable between *Bmp6*^{+/+} and *Bmp6*^{-/-} mice, the LH level trended to be higher in *Bmp6*^{-/-} mice compared to *Bmp6*^{+/+} mice, but this difference was not statistically different ($P = 0.13$).

Cumulus Expansion

The LH signal to the COC is indirect and mediated by EGF-like growth factors encoded by *Areg*, *Btc*, and *Ereg* transcripts that are expressed by mural granulosa cells [33]. The EGF-like growth factor signal in cumulus cells is amplified by a positive-feedback mechanism, which involves expression of the EGF-like growth factors by the cumulus cells [34]. The EGF-like growth factor signal induces cumulus expansion, which

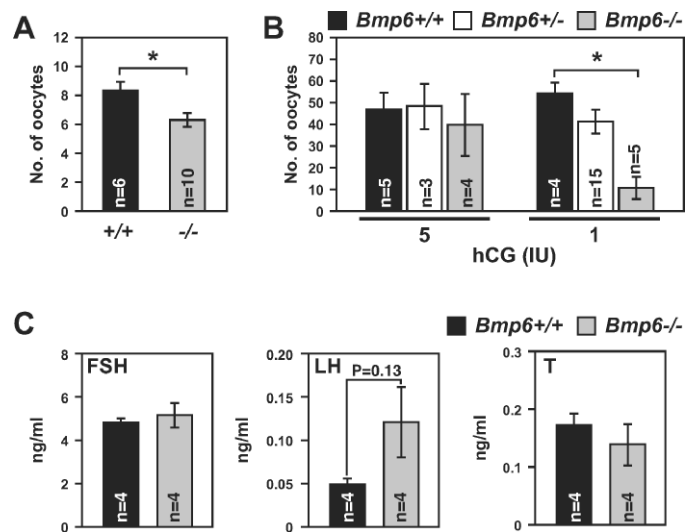
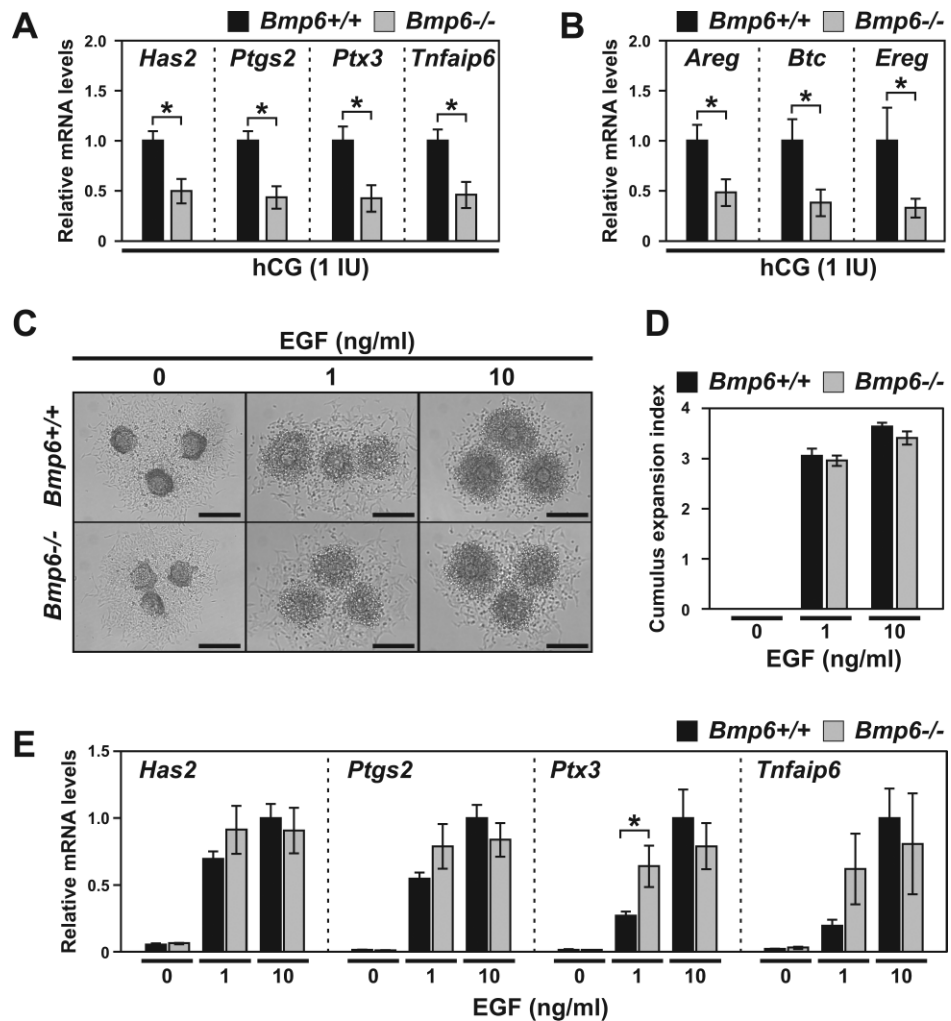


FIG. 2. Ovulation rates and serum hormone levels in *Bmp6* mutant mice. **A** and **B**) Numbers of oocytes ovulated in vivo in adult *Bmp6*^{+/+} (black bars) and *Bmp6*^{-/-} (gray bars) mice (**A**) and gonadotropin-induced ovulation in immature *Bmp6*^{+/+} (black bars), *Bmp6*^{+/-} (open bars), and *Bmp6*^{-/-} (gray bars) mice (**B**) were examined. **C**) Serum FSH, LH, and testosterone (T) levels in immature *Bmp6*^{+/+} (black bars) and *Bmp6*^{-/-} (gray bars) mice without exogenous gonadotropin treatment were examined. Serum levels of E₂ were below the limit of detection in both *Bmp6*^{+/+} and *Bmp6*^{-/-} mice (not shown). * $P < 0.05$.

FIG. 3. Steady-state levels of LH-induced transcripts in hCG-treated *Bmp6* mutant mice. **A** and **B** The eCG-primed immature *Bmp6*^{+/+} (black bars) or *Bmp6*^{-/-} (gray bars) mice were injected with 1 IU of hCG, and levels of transcripts encoding cumulus expansion-related proteins (**A**) and EGF-like growth factors (**B**) in COCs at 3 h post-hCG injection were examined. **C–E** COCs isolated from eCG-primed immature *Bmp6*^{+/+} (black bars) or *Bmp6*^{-/-} (gray bars) mice were treated with 1 or 10 ng/ml of EGF in vitro. Cumulus expansion index (**D**) and levels of transcripts (**E**) were assessed after 14 and 3 h of EGF treatment, respectively. Bar = 200 μ m. **P* < 0.05.



requires expression of proteins encoded by *Has2*, *Ptgs2*, *Ptx3*, and *Tnfaip6* transcripts by cumulus cells [27, 35–40].

To test whether the response to the LH signal is lower in cumulus cells of *Bmp6* mutant mice, eCG-primed *Bmp6*^{+/+} or *Bmp6*^{-/-} mice were injected with 1 IU of hCG, and after 3 h, the steady-state levels of these transcripts were determined in COCs in vivo. The levels of these LH-induced transcripts were all significantly down-regulated in *Bmp6*^{-/-} COCs compared with those in *Bmp6*^{+/+} COCs (*P* < 0.05) (Fig. 3, A and B). The levels of *Btc* and *Ereg* transcripts in *Bmp6*^{-/-} mural granulosa cells 3 h after treatment with hCG in vivo tended to be lower than those in *Bmp6*^{+/+} mural granulosa cells; however, the differences were not statistically significant because of a high variation among the experiments (data not shown).

Although the above results suggest that *Bmp6*^{-/-} follicles are less responsive to LH/hCG, it was possible that *Bmp6*^{-/-} COCs express lower levels of these transcripts because of an impaired development of cumulus cells or oocytes. To address this, COCs of eCG-primed *Bmp6*^{+/+} or *Bmp6*^{-/-} mice without hCG treatment were treated with EGF to induce cumulus expansion in vitro. The degree of EGF-induced cumulus expansion by *Bmp6*^{-/-} COCs in vitro was comparable to that of *Bmp6*^{+/+} COCs (Fig. 3, C and D). In addition, the expression levels of the cumulus expansion-related transcripts by *Bmp6*^{-/-} COCs were comparable with, or even greater than, those detected in *Bmp6*^{+/+} COCs when they were treated with EGF in vitro (Fig. 3D). Therefore, *Bmp6* mutant COCs can express comparable levels of transcripts required for cumulus expansion

in vitro; however, they do not express full levels of these transcripts in vivo in response to the lower dose of hCG (1 IU). These results strongly support the idea that follicular cells of *Bmp6* mutant mice are less responsive to LH/hCG and that reduced numbers of ovulated eggs in the mutant oocytes may be caused, at least in part, by defects in cumulus expansion.

Quality of *Bmp6*^{-/-} Oocytes

Developmental competence of *Bmp6*^{-/-} oocytes to complete preimplantation development was assessed in vitro (Fig. 4). The percentages of oocytes that resumed meiosis (germinal vesicle breakdown), extruded the first polar body, and developed to the 2-cell stage were comparable for *Bmp6*^{+/+} and *Bmp6*^{-/-} oocytes fertilized with *Bmp6*^{+/+} sperm in vitro. However, the ratio of 2-cell embryos that developed to the blastocyst stage was significantly lower in *Bmp6*^{-/-} oocytes than in *Bmp6*^{+/+} oocytes. Therefore, in addition to the reduced LH response in *Bmp6*^{-/-} mice, lower oocyte quality may contribute to the subfertility of *Bmp6*^{-/-} female mice.

Transcript Levels in *Bmp6* Mutant Follicles

Levels of transcripts encoding receptors for EGF (*Egfr*), FSH (*Fshr*), or LH (*Lhcgr*), as well as those of glycolytic enzymes (*Pfkfb* and *Ldha*) [30] and steroidogenic proteins (*Cyp11a1*, *Cyp19a1*, and *Star*), were compared in cumulus and mural granulosa cells of eCG-primed *Bmp6*^{+/+} and *Bmp6*^{-/-} mice.

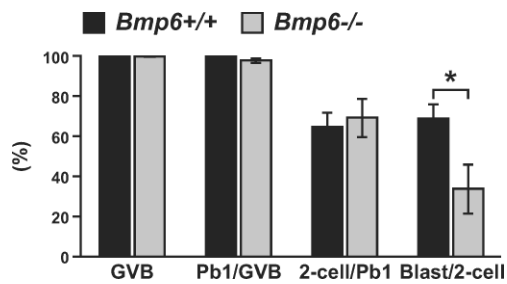


FIG. 4. Developmental competence of *Bmp6* mutant oocytes. Oocytes of *Bmp6*^{+/+} (black bars) and *Bmp6*^{-/-} (gray bars) mice were matured and fertilized in vitro, and the preimplantation embryonic development was assessed (n = 6). Blast, blastocyst-stage embryo; GVB, oocytes that underwent germinal vesicle breakdown; Pb1, oocytes extruded the first polar body; 2-cell, 2-cell embryo. **P* < 0.05.

Levels of transcripts encoding oocyte-produced growth factors (*Bmp15*, *Gdf9*, *Fgf8*, and *Tgfb2*) were examined as well.

As expected, *Bmp6* transcripts were not detected in *Bmp6*^{-/-} oocytes (Fig. 5A). Transcript levels of the oocyte-produced growth factors other than *Bmp6* mRNA were not significantly different in *Bmp6*^{+/+} and *Bmp6*^{-/-} oocytes, suggesting that deficiency in *Bmp6* did not affect production of these other oocyte factors (Fig. 5A). Although the levels of *Lhcgr* and *Cyp19a1* transcripts in mural granulosa cells and of *Pfkip* and *Ldha* in cumulus cells of *Bmp6*^{-/-} mice tended to be lower than those in *Bmp6*^{+/+} mice, the differences were not statistically significant (Fig. 5, B and C). Overall, levels of transcripts examined were not significantly different between *Bmp6*^{+/+} and *Bmp6*^{-/-} granulosa cells.

Bmp6/Bmp15 Double-Mutant Mice

Although significant species differences exist in functions of, and response to, BMP ligands [16, 41, 42], BMP6 and the other oocyte-derived BMP, BMP15 [21, 22], appear to have several redundant roles in regulating granulosa cell functions. They can act through the same BMP receptor [6], and their intercellular signals are mediated by the MAD homolog 1/5/9 (SMAD1/5/9; formerly known as SMAD1/5/8) pathway in granulosa cells [15, 17, 43, 44]. Both recombinant BMP6 and BMP15 suppress apoptosis in bovine granulosa cells [18] and increase mRNA levels of inhibitor of DNA binding 1 (ID1) in ovine granulosa cells [45]. Therefore, a possible redundant function of BMP6 and BMP15 was assessed using mutant mice homozygous for both *Bmp6* and *Bmp15* (*Bmp6*^{-/-}/*Bmp15*^{-/-}).

The mean litter size and number of litters per month of *Bmp6*^{-/-}/*Bmp15*^{-/-} female mice tended to be lower than those of double heterozygous (*Bmp6*^{+/-}/*Bmp15*^{+/-}) littermates; however, these differences were not statistically significant (Fig. 6, A and B). The histology of 6-mo-old *Bmp6*^{-/-}/*Bmp15*^{-/-} ovaries was grossly normal when compared with *Bmp6*^{+/-}/*Bmp15*^{+/-} littermates (n = 3) (Fig. 6C). Formation of ovarian granulosa cell tumors was reported in several mutant-mouse models in which follicular BMP signal had been attenuated [3–5]. For example, all of *Smad1/Smad5* double-homozygous mutant female mice exhibit evidence of tumors after 3 mo of age [4]. We examined more than 10 female *Bmp6*^{-/-}/*Bmp15*^{-/-} mice older than 6 mo but did not observe granulosa cell tumors.

DISCUSSION

Despite extensive studies conducted using isolated granulosa cells in vitro, evidence for a role of BMP6 in female fertility in vivo has been lacking. The present study shows that female mice

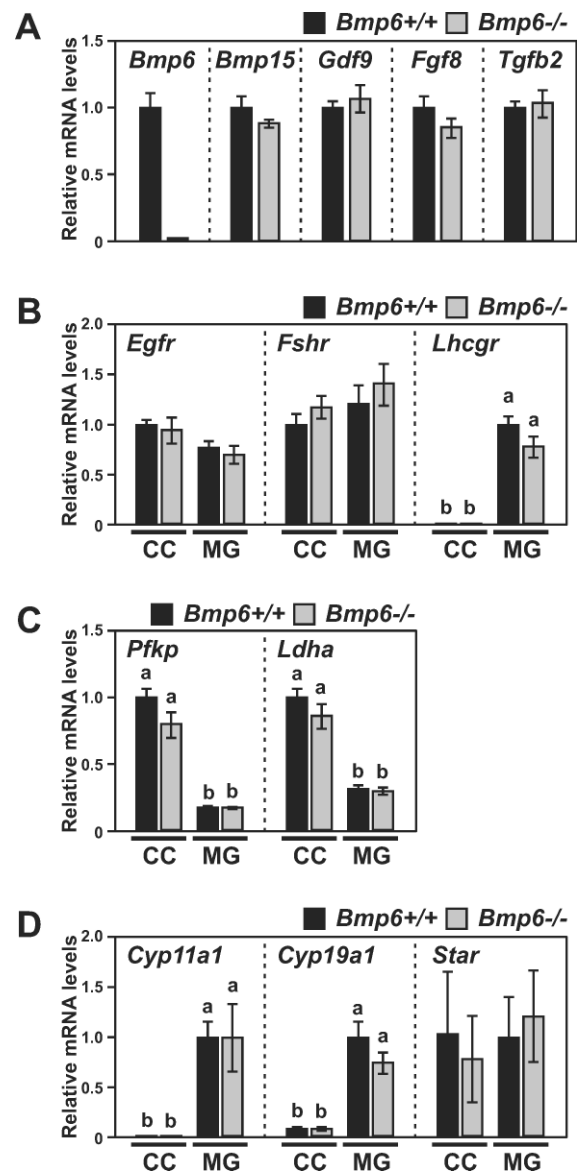
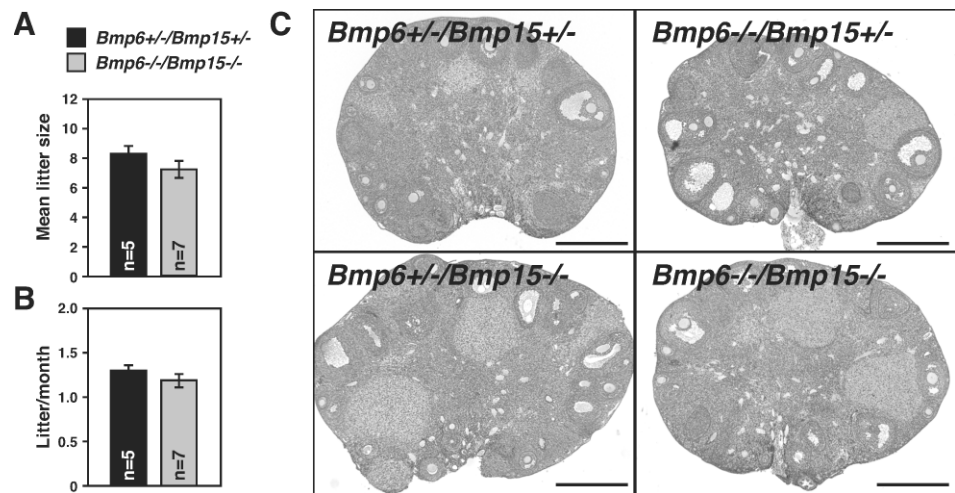


FIG. 5. Transcript levels in oocytes (A) or granulosa cells (B–D) of eCG-primed *Bmp6* mutant mice. Levels of transcripts encoding growth factors in oocytes (n = 5; A) as well as receptors (n = 6 for *Fshr* and *Egfr* and 11 for *Lhcgr*; B), glycolytic enzymes (n = 6; C), and steroidogenic proteins (n = 8; D) in granulosa cells of *Bmp6*^{+/+} (black bars) and *Bmp6*^{-/-} (gray bars) mice were assessed. Values indicated by different letters (a and b) are significantly different (*P* < 0.05). CC, cumulus cells; MG, mural granulosa cells.

deficient in BMP6, at least in the strain of mice used for the present study, exhibit a 22% reduction in litter size. *Bmp6*^{-/-} ovaries are less responsive to LH/hCG, and cumulus cells of *Bmp6*^{-/-} mice express decreased levels of the LH-induced transcripts after treatment with lower dose of hCG in vivo. However, transcript levels for LH receptor are not significantly lower in *Bmp6*^{-/-} ovaries, suggesting that BMP6 is required for processes downstream of LH receptor expression. In addition, *Bmp6*^{-/-} oocytes are less competent to complete preimplantation development in vitro when fertilized with wild-type sperm. Therefore, BMP6 is required for normal fertility of female mice by enabling appropriate responses to LH and promoting normal oocyte quality. The underlying mechanisms by which BMP6 affects the actions of the ovulatory surge of LH remain to be

FIG. 6. Ovarian morphology and fertility analysis of female mice deficient in both *Bmp6* and *Bmp15*. **A** and **B**) Double-heterozygous (*Bmp6*^{+/-}/*Bmp15*^{+/-}, black bars) and double-homozygous (*Bmp6*^{-/-}/*Bmp15*^{-/-}, gray bars) mutant female mice were bred with *Bmp6*^{+/+}/*Bmp15*^{+/+} male, and litter size (**A**) and number of litters per month (**B**) were recorded for 6 mo. **C**) Representative photographs of ovaries of 6-mo-old mutant mice are shown. Bar = 500 μ m.



investigated. Mouse oocytes express *Bmp6* [9], but other cell types also express this ligand [10–13]. Therefore, effects of *Bmp6* deletion on fertility, responses to LH/hCG, or oocyte quality could result from systemic effects rather than from the absence of oocyte-derived BMP6.

In vitro studies using recombinant ligands showed that BMP6 can affect FSH signaling in granulosa cells either positively or negatively, depending on mammalian species. BMP6 suppresses *Fshr* mRNA expression and FSH-induced progesterone production in diethylstilbestrol-primed rat granulosa cells without affecting E₂ production or *Cyp19a1* transcript levels [14, 17]. In contrast, BMP6 promotes FSH-induced E₂ production by ovine granulosa cells [20]. Furthermore, BMP6 enhances *FSHR* mRNA levels in human granulosa cells [12]. In the present study, expression levels of well-known FSH-regulated transcripts in granulosa cells, such as *Lhcgr*, *Cyp19a1*, and *Fshr* transcripts, were not significantly changed in *Bmp6*^{-/-} granulosa cells. BMP6 belongs to the 60A subfamily of the BMP ligands, which also includes BMP5, BMP7, BMP8A, and BMP8B [46, 47]. In rodent ovaries, BMP5 and BMP7 are produced by granulosa cells and theca cells, respectively [6, 48], and regulate FSH signaling in granulosa cells in vitro [48, 49]. Therefore, these BMPs may compensate for the absence of BMP6 with regard to regulating FSH signaling in vivo.

In the present study, we observed a tendency for increased basal LH level in the serum of *Bmp6*^{-/-} female mice. The E₂ levels in the serum of both wild-type and *Bmp6*^{-/-} mice were below the limit of detection and, therefore, could not be compared. The apparently higher basal LH level in *Bmp6*^{-/-} mice probably results from the relatively poor response of granulosa cells to LH, because mice with reduced LH sensitivity, such as *Lhcgr* knockout female mice, also exhibit increased serum LH levels [50, 51]. Alternatively, it is possible that although differences in the kinetics of follicular development were not detected between 18-day-old wild-type and *Bmp6* mutant mice, the progression of follicular development in 20-day-old mice may vary between these genotypes, and it may result in different feedback effects on the pituitary secretion of LH. These possibilities remain to be elucidated. It is unlikely that these levels can be attributed to the direct effect of loss of BMP6 in the pituitary, because BMP6 has no effects on secretion of LH by rat and ovine pituitary cells [52, 53].

In addition to BMP6, BMP15 is produced by mammalian oocytes [21, 22]. *Bmp15*-null (*Bmp15*^{-/-}) female mice are subfertile because of defective granulosa cell functions and reduced developmental competence of oocytes [23, 54].

Cumulus cells of *Bmp15*^{-/-} mice exhibit reduced levels of transcripts encoding enzymes for glycolysis and cholesterol biosynthesis before the LH surge [26, 55, 56]. Furthermore, recombinant human BMP15 promotes cumulus expansion in vitro [57]. Therefore, BMP15 plays a critical role in regulating granulosa cell functions. Surprisingly, in the present study, we did not observe significant reduction in fertility of double-mutant (*Bmp6*^{-/-}/*Bmp15*^{-/-}) female mice compared with that of double-heterozygous (*Bmp6*^{+/-}/*Bmp15*^{+/-}) mice, despite the fact that the double-mutant mice are deficient in BMP15. In addition to the redundant functions of BMP15 and BMP6, some nonredundant functions of these BMPs are apparent [14, 58, 59]. BMP15 promotes follicular development in mice [60], but BMP6 appears to suppress the FSH signaling in vitro, which would be required for normal follicular development in vivo. Nevertheless, we did not observe any morphological differences in follicular development and FSH signaling in immature *Bmp6*^{-/-} mice. One possible explanation for the absence of significant effects of *Bmp15* deletion on fertility of the double-mutant mice is that deletion of the *Bmp6* gene may rescue some of the defects caused by BMP15 deficiency in the follicles. This possibility remains to be tested.

Female fertility in various species is affected by BMP signaling in different ways. Ewes carrying homozygous mutation in *BMP15* are infertile because of a block in folliculogenesis at the primary stage [61, 62]. Likewise, immunization against BMP15 in ewe and cattle results in impaired follicular development and ovulation rate [63, 64]. Ewes carrying a mutation in a gene encoding one of the BMP receptors, *BMPR1B* (also known as *ALK6*), exhibit increased fertility [65–68]. On the other hand, mice deficient in *Bmp15* [23, 54], *Bmp6* [9] and present study), and both *Bmp6* and *Bmp15* (present study) exhibit relatively modest defects in female fertility compared with those in other mammalian species. However, prevention of BMP signaling in granulosa cells by conditional deletion of their downstream signaling molecules, *SMAD1/5/9*, results in development of metastatic granulosa cell tumors, and the female mutant mice become infertile [4, 5]. Likewise, female mice deficient in *Bmpr1b* exhibit defective cumulus expansion and are infertile [2]. In addition, conditional deletion of the other BMP receptor, *BMPR1A*, in granulosa cells produces a severe reduction in female fertility, and female mice deficient in both *Bmpr1a* and *Bmpr1b* suffer from ovarian granulosa cell tumors [3]. Therefore, BMP signaling is, indeed, critical for ovarian follicular development and female fertility in mice. Oocyte-derived BMP ligands and the other BMP ligands produced by follicular somatic cells may constitute a total and complementary

BMP signaling system in follicles, and this entire BMP signaling system is probably essential for normal fertility in female mice.

Multiple processes, including follicular development, ovulation, fertilization, and subsequent preimplantation development, determine female fertility. Each process is affected by multiple factors, including genetic variability as well as environmental influences. For example, previous studies have identified several quantitative trait loci (QTL) that determine hormone-induced ovulation rate or litter size in mice [69–73]. Therefore, ovulation and litter size are quantitative processes controlled by a complex interaction among multiple genes. The *Bmp6* locus was not identified by the previous QTL linkage analyses as a candidate locus that determines ovulation rate or litter size [69–73]; however, the present study has clearly shown that a deletion in the *Bmp6* gene does affect ovulation rate and litter size in mice. Therefore, *Bmp6* is probably part of the complex genetic network that determines female fertility. Although a 22% decrease in fertility, whether caused by *Bmp6* deletion or any other genetic or environmental factor, may seem mild in the context of a laboratory model, this difference could determine the success or failure of a species or population in the wild and have significant agricultural economic impact.

In summary, the present study has shown that BMP6 is required for normal fertility in female mice, at least in part, by enabling appropriate responses to LH and promoting normal oocyte quality *in vivo*. However, in addition to BMP15, other BMP ligands produced in ovarian follicles, such as BMP5 and BMP7, may compensate for the absence of BMP6 and prevent more drastic consequences to ovarian function and fertility in female mice.

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