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Loss of the Proapoptotic BH3-Only Protein BCL-2 Modifying Factor Prolongs the Fertile Life Span in Female Mice[1]

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ABSTRACT

The duration of the female fertile life span is influenced by the number of oocytes stored in the ovary as primordial follicles. Cell death, both during ovarian development in the embryo and in the postnatal ovary, plays a critical role in determining how many primordial follicles are established and maintained within the ovary. However, the roles of individual apoptotic regulators in mediating cell death within the ovary have not yet been characterized. In this study, gene targeted mice were used to investigate the role of BCL-2-modifying factor (BMF), a proapoptotic protein belonging to the BH3-only subgroup of the BCL-2 family, in determining the number of primordial follicles maintained in the adult ovary and the length of the fertile life span. Stereological analysis of ovaries showed that *Bmf*$^{-/-}$ mice had significantly more primordial follicles than wild-type (WT) control animals at Postnatal Days 100, 200, 300, and 400 but not at Day 20. No differences were observed between WT and *Bmf*$^{-/-}$ mice in the number of ova shed following ovulatory stimulation with exogenous gonadotropins. *Bmf*$^{-/-}$ females were fertile and produced the same number pups/litter as WT females, but *Bmf*$^{-/-}$ females produced litters more frequently and consequently more offspring than WT females over a 6-mo period. Furthermore, the fertile life span of *Bmf*$^{-/-}$ females was significantly extended compared to WT females. Our findings support an important role for BMF in determining the number of primordial follicles maintained in the ovary throughout adult reproductive life and thus indicate that the length of female fertility may be extended by increasing the number of primordial follicles maintained within the ovary through inhibition of BMF.

apoptosis, BCL-2, BMF, fertility, follicles, ovary, primordial follicles

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INTRODUCTION

Females are endowed with a fixed number of primordial follicles at birth, and this stockpile is the source of all oocytes for future ovulations [1–3]. Throughout life, the pool of primordial follicles is gradually depleted either as a result of death or through their natural progression into growing follicles. The majority of follicles that recommence folliculogenesis undergo atresia, which is characterized by apoptosis of the supporting granulosa cells followed by oocyte degeneration [4, 5], with only a small number of oocytes surviving to be ovulated [4, 6–10]. Over time, the ovarian reserve becomes so diminished that females become infertile. Surprisingly, little is known about the mechanisms that regulate the size of the ovarian primordial follicle reserve and the duration of the fertile life span. However, some studies suggest that apoptosis, both during ovarian development and in the postnatal ovary, plays a critical role in determining how many primordial follicles are established and maintained within the ovary [11–16].

Recent progress toward elucidating the mechanisms of apoptosis in the ovary has highlighted the role of the intrinsic (also known as the ''mitochondrial,'' ''BCL-2-regulated,'' or ''stress'') apoptotic pathway [17]. This pathway is controlled by members of the BCL-2 family of proteins, which comprises both antiapoptotic and proapoptotic proteins that function coordinately to control caspase-executed cellular destruction. The proapoptotic members of the BCL-2 family fall into two categories: multi-BH-domain proapoptotic BAX-like molecules (BAX, BAK, and BOK) and the BH3-only proteins (BAD, BID, BIK, BIM, BMF, HRK, NOXA, and PUMA), which contain a single BH3 (BCL-2 homology 3) domain. BH3-only proteins are activated transcriptionally and posttranscriptionally by developmental cues and diverse cytotoxic stimuli. BH3-only proteins initiate apoptosis by binding and neutralizing the antiapoptotic BCL-2 family members, including BCL2, BCL-XL, BCL-W, MCL-1, and A1 [18, 19]. This interaction prevents inhibition of ''primed'' BAX/BAK at the mitochondrial membrane, which results in cytochrome c release, formation of the apoptosome, caspase activation, and cellular destruction [18]. Some BH3-only proteins (BIM, tBID, and PUMA) contribute to apoptosis initiation also by direct activation of BAX/BAK.

Several studies in mice have suggested that prosurvival BCL-2 and BCL-X, as well as proapoptotic BAX and BOK, play critical roles in the regulation of germ cell loss during ovarian development and in determining the number of primordial follicles initially endowed and subsequently maintained in the adult ovary [4, 20, 21]. Additionally, both BID and BIM have been implicated in granulosa cell apoptosis [22].

However, very little is known about the role of another BH3-only protein, BMF, in apoptosis within the ovary. BMF has previously been shown to regulate B-cell homeostasis and lymphocyte apoptosis [10], mammary gland morphogenesis [11], and anoikis, a specialized type of apoptosis elicited by loss of cell adhesion of mammary epithelial cells [10] and intestinal epithelial cells [12]. In the testis, BMF is proposed to regulate the apoptosis of germ cells in response to their detachment from Sertoli cells [23]. In the current study using genetically modified mice, we investigated the role of BMF in mediating apoptosis within the ovary, with a particular focus on the number of primordial follicles maintained in the adult ovary and relationship between primordial follicle number and the duration of fertility.

MATERIALS AND METHODS

Mice

Bmf$^{-/-}$ mice on a C57BL/6 background have been described previously [24]. These animals and control C57BL/6 mice were maintained on a 12L:12D cycle in a temperature-controlled high-barrier facility (Monash University ARL) with free access to mouse chow and water. All animal procedures and experiments were performed in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals and approved by the Monash University School of Biomedical Sciences Animal Ethics Committee.

Tissue Collection and Assessment of Follicle Numbers

Ovaries from wild-type (WT) and *Bmf*$^{-/-}$ mice (Day 20, 100, 200, 300, 400, and 545, n = 6/age/genotype) harvested for stereological examination were fixed in Bouin solution and processed into hydroxyethyl methacrylate resin (Technovit 7100; Kulzer and Co.). Ovaries were serially sectioned at 20 μm with a Leica RM2165 microtome (Leica Microsystems Nussloch GmbH) and stained with periodic acid-Schiff and counterstained with hematoxylin. Stereological data were obtained as previously described in detail [25, 26] using a 100× oil immersion objective on an Olympus BX50 microscope equipped with an Autoscan stage (Autoscan Systems Pty Ltd) in conjunction with the CASTGRID stereological system (CAST 2002; Olympus). Briefly, every third section from a random start was evaluated, and an estimation of follicle number in the ovary was determined by multiplying raw count of oocytes sampled (Q^-) by all three sampling fractions ($1/f_1$, $1/f_2$, and $1/f_3$) [25]. This is achieved through sampling of three parameters: sections, depth, and field.

Assessment of Atretic Follicle Numbers

The number of atretic follicles was determined by direct counting of consecutive 20-μm glycomethacrylate sections encompassing whole cross sections of the ovary using the fractionator/physical dissector method as previously described [25]. Primordial, primary, and secondary follicles were considered to be atretic if the oocyte was degenerating as indicated by an irregular plasma membrane or was eosinophillic or if granulosa cells were fragmented or had pyknotic condensed nuclei. Antral follicles were considered atretic if more than 10% of their granulosa cells were apoptotic or if they contained a degenerating oocyte.

Ovulation Induction

WT and *Bmf*$^{-/-}$ mice aged 20, 150, and 300 days (n = 6/genotype) were superovulated with an intraperitoneal (i.p.) injection of 5 IU eCG (5 IU/ml; Folligon; Intervet) in 0.1 ml saline (0.9% NaCl; British Drug House), followed 48 h later with an i.p. injection of 5 IU hCG (5 IU/ml; Chorulon; Intervet) in 0.1 ml saline (0.9% NaCl; Pfizer). Mice were killed by cervical dislocation 12–15 h after hCG injection. Both oviducts were excised and placed in 15-mm culture dishes (Becton Dickinson) containing 2–4 ml prewarmed PBS and opened with forceps to release the cumulus-oocyte complexes (COCs), which were visualized using a stereoscopic microscope (Leica Wild M8). The COCs were treated with hyaluronidase (2 mg/ml) in PBS for 30 sec to dissociate the cumulus cells and obtain denuded oocytes for assessment of meiotic maturation, and the number of oocytes present in the oviducts of each mouse was recorded.

Assessment of Fertility

At 6-wk of age, WT and *Bmf*$^{-/-}$ females were housed as individual breeding pairs with 6-wk-old WT (C57BL/6) males (n = 6 pairs of mice/genotype). Litter size, frequency, and general health of pups were monitored until breeding females reached 18 mo of age. Males were replaced at 12 mo of age to ensure fertility.

Immunohistochemistry

Tissue sections (5 μm) were deparaffinized and rehydrated prior to commencing. Antigen retrieval was performed by microwaving slides for 10 min in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by immersion in 10% hydrogen peroxide in MilliQ water for 30 min, after which the slides were washed three times in 0.1 M Tris, 150 mM NaCl, and 0.1% v/v Tween buffer (TNT). The sections were incubated in a blocking solution, 10% normal rabbit serum in room temperature for 30 min, and subsequently incubated with rat antibody to BMF (17A9; ENZO Life Sciences) [24] (1:50) overnight at 4°C. After washing the slides three times in TNT, sections were incubated with rabbit anti-rat IgG HRP (Dako; 1:400) for 30 min, followed by staining using a Vectastain ABC kit (Vector Laboratories). BMF-positive cells were visualized by the addition of 0.05% DAB chromagen (Dako) for ~1 min. The reaction was stopped by placing the slides into distilled H_2O. The sections were counterstained using neat Harris hematoxylin, dehydrated through a graded series of alcohols, and mounted under glass coverslips with DPX (Sigma). Ovarian sections from *Bmf*$^{-/-}$ mice were used as negative controls and to confirm antibody specificity. Cleaved caspase-3 (R&D Systems; AF-835; 1:1000) staining was performed on sections adjacent to those used for BMF immunostaining so that BMF expression could be correlated with follicular atresia.

TUNEL Staining

Apoptotic cells in ovarian sections were detected using terminal deoxynucleotidyl transferase (TdT)-mediated TUNEL, using the ApopTag Peroxidase in situ apoptosis detection kit (Chemicon International). Ovarian sections, mounted on superfrost slides, were deparaffinized and rehydrated prior to commencing. The slides were washed (5 min) in PBS (1.0 mM, pH 7.4) and then transferred into equilibration buffer. After 10 min at room temperature in a humidified chamber, working-strength TdT enzyme was added while negative control sections received PBS. Slides were placed in humidified chamber at 37°C for 1 h. The slides were then washed with stop/wash buffer at room temperature for 10 min. After three (5-min) washes in PBS, antidigoxigenin conjugate was applied to all sections and incubated for 30 min at room temperature in a humidified chamber. This was followed by four (5-min) PBS washes. Apoptotic cells were visualized by the addition of 0.05% DAB chromagen (Sigma) for ~1 min. The reaction was stopped by placing the slides into distilled H_2O. The sections were lightly counterstained using neat Harris hematoxylin, dehydrated through a graded series of alcohols, and finally mounted under glass coverslips with DPX (Sigma). TUNEL staining was performed on three to four randomly selected slides. On each slide, one section was used for TUNEL analysis, while the other acted as a negative control (no addition of TdT).

Statistical Analysis

Data are presented as mean ± SEM, and statistical analysis of follicle numbers was performed using GraphPad Prism software (GraphPad Software, Inc.). Data were normally distributed and analyzed by one-way analysis of variance, and the significance was determined by the Newman-Keuls post hoc multiple comparison test. Differences were considered significant when $P <$ 0.05.

RESULTS

Primordial Follicle Numbers Are Elevated in Adult Bmf$^{-/-}$ Mice Compared to WT Controls

Follicles were enumerated in ovaries from WT and *Bmf*$^{-/-}$ prepubertal and adult mice to determine if BMF-mediated apoptosis influences the number of follicles maintained in the ovary during reproductive life. Ovaries from prepubertal (Day 20) WT and *Bmf*$^{-/-}$ mice contained a similar number of primordial follicles (Fig. 1A). Primordial follicle numbers then fell significantly in both WT and *Bmf*$^{-/-}$ ovaries between Day

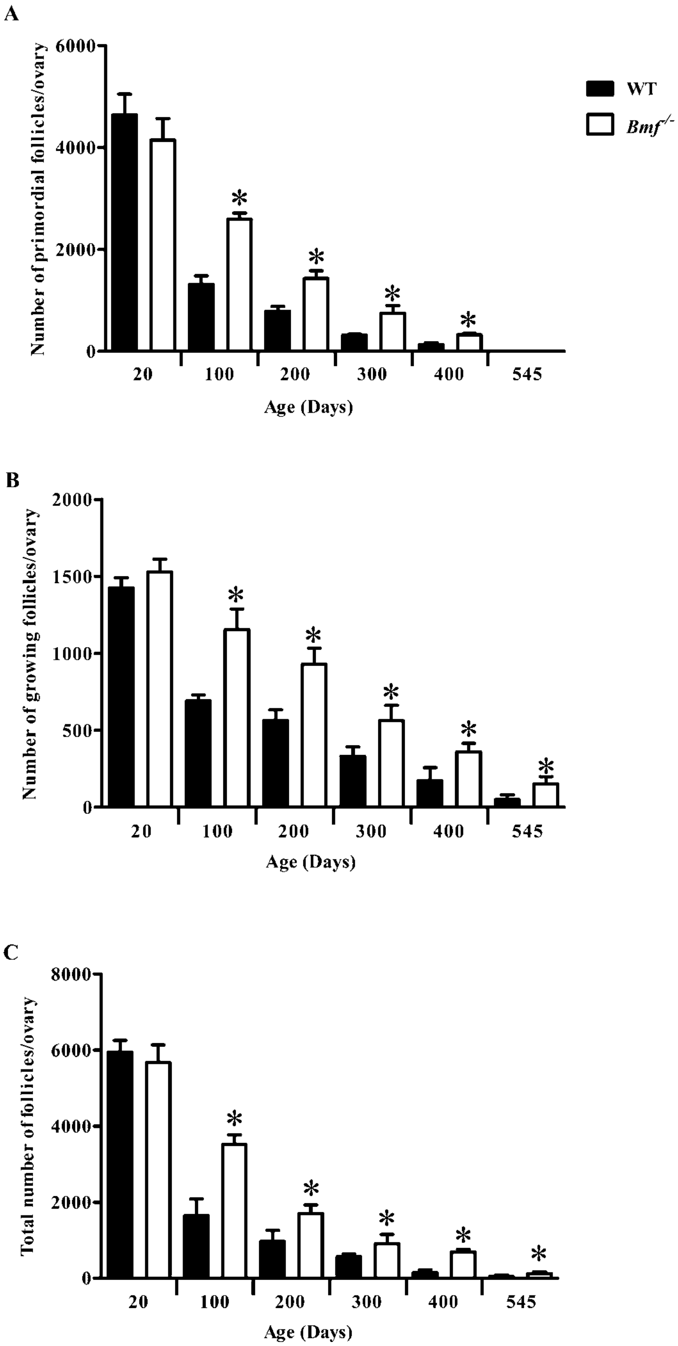

FIG. 1. BMF deletion results in increased follicle numbers during adult reproductive life. Primordial (**A**), growing (**B**), and total (**C**) follicles were enumerated in ovaries from WT and *Bmf*$^{-/-}$ mice from Postnatal Days 20 to 545. Data are presented as mean ± SEM, n = 6 mice/group, *$P < 0.05$ when compared to same-age WT value.

20 and Day 100. However, considerably fewer primordial follicles were lost during this period in *Bmf*$^{-/-}$ compared to WT mice. The number of primordial follicles was significantly increased in ovaries from *Bmf*$^{-/-}$ mice compared to those from WT controls at Day 100 (WT 1318.3 ± 164.2 vs. *Bmf*$^{-/-}$ 2591.8 ± 120.7 follicles/ovary, $P < 0.001$). Primordial follicle numbers remained elevated in *Bmf*$^{-/-}$ compared to WT ovaries on Days 200 (WT 791.0 ± 89.5 vs. *Bmf*$^{-/-}$ 1431.8 ± 137.6 follicles/ovary, $P < 0.05$), 300 (WT 320.0 ± 19.5 vs. *Bmf*$^{-/-}$ 748.0 ± 133.0 follicles/ovary, $P < 0.05$), and 400 (WT 135.0

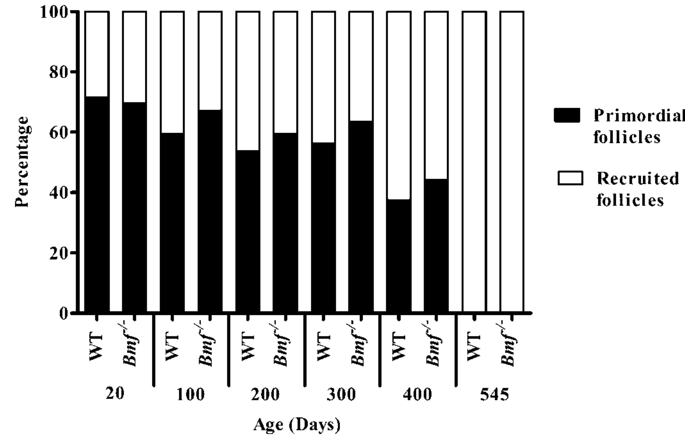

FIG. 2. Proportion of primordial and growing follicles in *Bmf*$^{-/-}$ and WT ovaries. The percentages of total follicles in either the primordial or the growing pool in ovaries from WT and *Bmf*$^{-/-}$ mice were compared across the fertile life span. n = 6 mice/group.

± 27.0 vs. *Bmf*$^{-/-}$ 326.3 ± 21.9 follicles/ovary, $P < 0.05$; Fig. 1A). Primordial follicles could no longer be detected in either *Bmf*$^{-/-}$ or WT ovaries on Day 545 (Fig. 1A). The growing follicles were also enumerated in WT and *Bmf*$^{-/-}$ ovaries. Increased numbers of growing follicles were observed in *Bmf*$^{-/-}$ compared to WT ovaries between 100 and 545 days of age (Fig. 1B).

The Proportion of Follicles Recruited in the Growing Follicle Pool Is Similar in Ovaries from WT and Bmf$^{-/-}$ *Mice*

An increased number of primordial follicles stored in the ovary in adult life may arise if the numbers or proportions of follicles recruited into the growing follicle pool are reduced. To investigate this possibility, the percentages of total follicles in the primordial or growing pool were compared between ovaries from WT and *Bmf*$^{-/-}$ mice (Fig. 2). Although *Bmf*$^{-/-}$ mice had abnormally increased numbers of follicles in their ovaries (Fig. 1C), the proportions of follicles in the primordial and the actively growing follicular pool were comparable between WT and *Bmf*$^{-/-}$ mice (Fig. 2). Moreover, in both WT and *Bmf*$^{-/-}$ ovaries, an age-related increase in the proportions of follicles in the growing follicle pool were observed; in both genotypes of mice, approximately 30% to 40% of total follicles were growing at Day 100, compared to 60% at Day 400, with all remaining follicles having commenced folliculogenesis at Day 545.

BMF Is Expressed in the Granulosa Cells of Some Atretic Follicles

BMF expression was analyzed by immunohistochemistry in ovaries from adult WT females (Fig. 3). BMF was not detected in the oocytes or granulosa cells from primordial or primary follicles at any age. BMF was also not detected in healthy secondary and antral follicles. However, BMF immunoreactivity was found in the granulosa cells of some large atretic secondary and antral follicles. These follicles were confirmed to be atretic by immunostaining for activated caspase-3 (Fig. 3).

Follicular Atresia

Follicular morphology assessment and the TUNEL assay were used to determine whether BMF is essential for granulosa

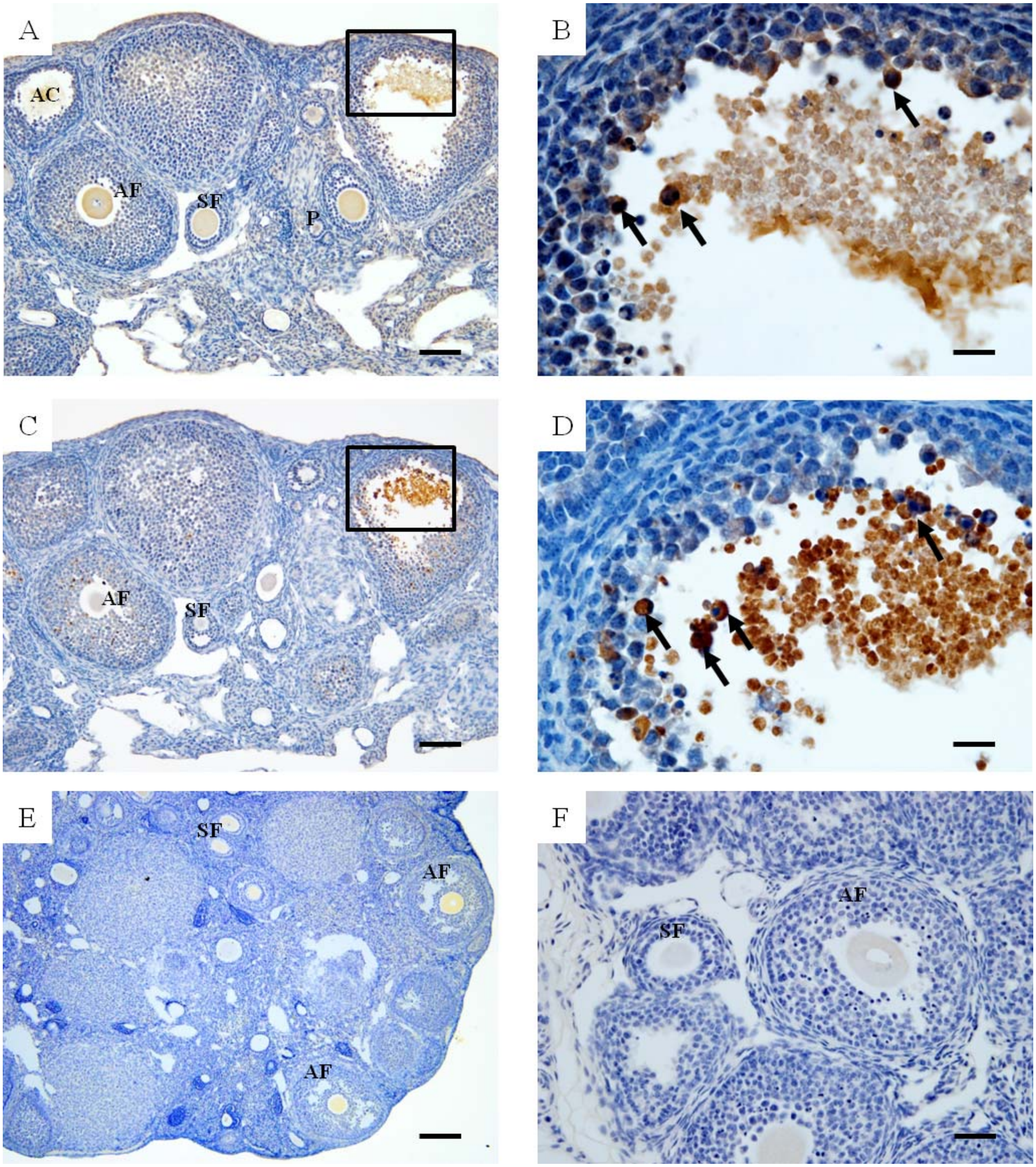

FIG. 3. Expression of BMF in ovaries from adult WT mice. BMF expression was assessed by immunohistochemistry in WT adult ovaries (**A**, **B**) and correlated with expression of activated caspase-3, a marker of apoptotic cells (**C**, **D**). Representative negative control showing absence of staining in a WT ovary when the primary antibody was omitted (**E**). Very light brown staining of some oocytes in **E** (and also evident in **A**) reflects low levels of nonspecific background staining. Representative negative control showing absence of staining in apoptotic granulosa cells in a *Bmf*$^{-/-}$ ovary (**F**). P, primary follicle; SF, secondary follicle; AF, antral follicles; AC, antral cavity. Arrows indicate positive brown staining in granulosa cells for BMF (**B**) and caspase-3 (**D**). Sections were counterstained with hematoxylin (blue nuclear staining). Bar = 200 μm (**A**, **C**, **E**), 20 μm (**B**, **D**), and 50 μm (**E**, **F**).

cell apoptosis and follicular atresia. Primordial and primary follicles with apoptotic oocytes or granulosa cells were not detected in females of either genotype. Fragmented, pyknotic, and TUNEL-positive granulosa cells were, however, detected in large secondary and antral follicles in ovaries from both WT and *Bmf*$^{-/-}$ mice (Fig. 4A). Interestingly, at Day 20, the numbers of atretic follicles were significantly decreased in ovaries from *Bmf*$^{-/-}$ mice compared to those from WT females (Fig. 4B). However, at Day 100 and beyond, the numbers of atretic follicles were found to be comparable between in *Bmf*$^{-/-}$

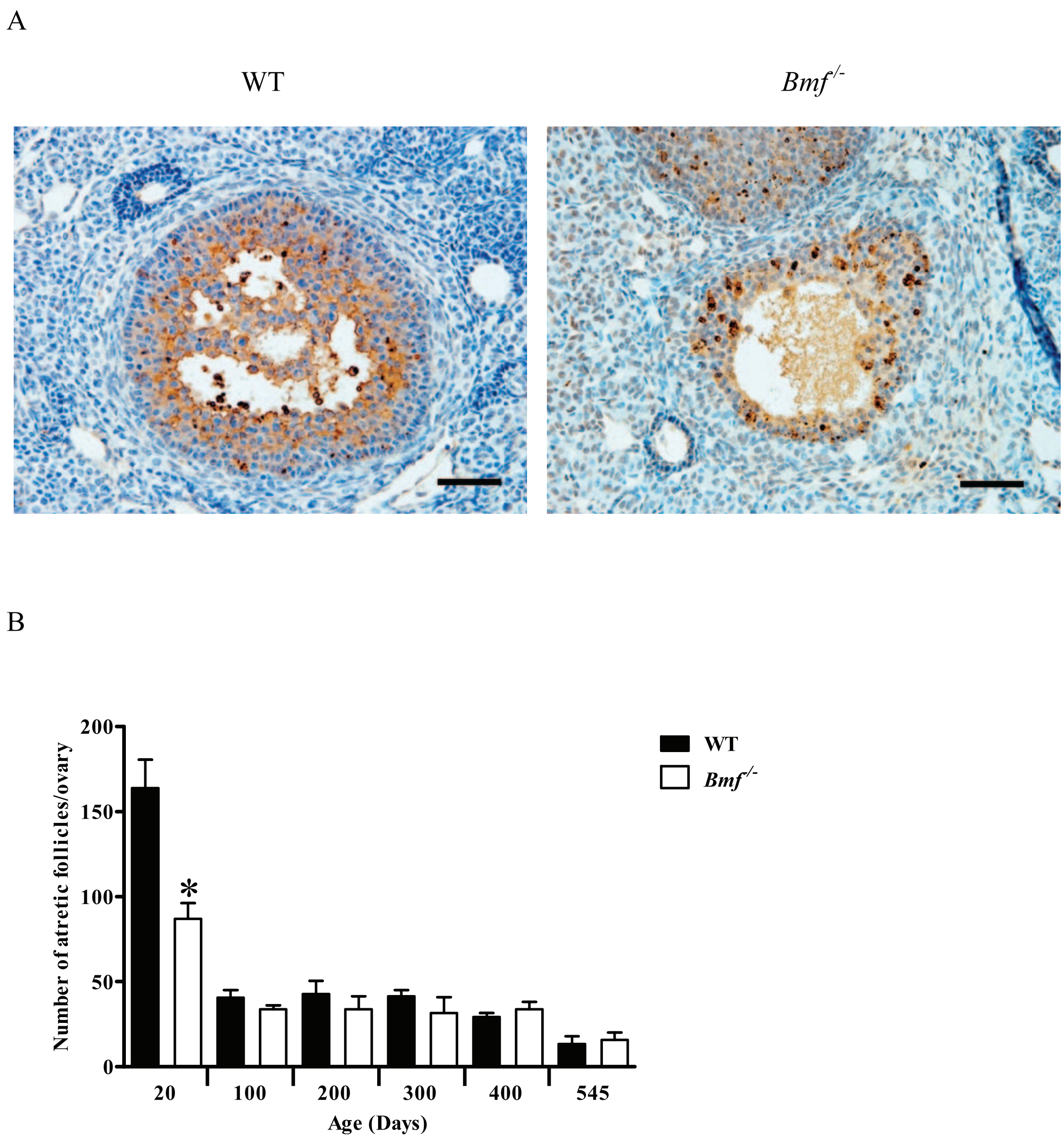

FIG. 4. Follicular atresia in ovaries from WT and *Bmf*$^{-/-}$ mice. **A**) Follicular morphology and TUNEL staining (brown) were used to examine apoptosis in ovaries from WT and *Bmf*$^{-/-}$ mice. Bar = 50 μm. Sections were counterstained with hematoxylin (blue nuclear staining). **B**) Determination of the number of atretic follicles present in ovaries from WT and *Bmf*$^{-/-}$ mice from Postnatal Days 20 to 545. Data are presented as mean ± SEM, n = 6 mice/group, $^{*}P <$ 0.005 when compared to values from age-matched WT females.

and WT mice (Fig. 4B). In addition, a greater number of atretic follicles were observed in both WT and *Bmf*$^{-/-}$ ovaries at Day 20 compared to Days 100–545 (Fig. 4B).

Ovulation Rates Are Not Affected by Loss of BMF and Increased Follicle Number

WT and *Bmf*$^{-/-}$ mice were stimulated with exogenous hormones to determine whether an increase in follicle number resulted in an increase in the number of oocytes ovulated. Oocytes were collected from immature mice (20 days), young adults (150 days), and older adults (300 days). No difference was found in the number of oocytes ovulated by WT and *Bmf*$^{-/-}$ females at any age (Fig. 5).

Fertility Is Abnormally Prolonged in Bmf$^{-/-}$ *Mice*

The overall fertility and duration of the fertile life span of *Bmf*$^{-/-}$ mice were compared to those of WT females to determine if an increased number of primordial follicles maintained within the ovary in adult life can confer enhanced and prolonged reproductive capacity. *Bmf*$^{-/-}$ females were fertile and produced similar numbers of pups/litter as WT females when mated with WT (C57BL/6) males (Table 1). However, *Bmf*$^{-/-}$ females produced more litters than WT females over a 6-mo period (Table 1). Furthermore, the fertile life span of *Bmf*$^{-/-}$ females was extended when compared to WT females (Table 1); the average age at the end of fertility was 295 days for WT versus 360 days for *Bmf*$^{-/-}$ females (Table 1). Notably, one *Bmf*$^{-/-}$ female was pregnant when the study was terminated at 545 days. The combined effect of increased litter frequency and extended duration of fertility

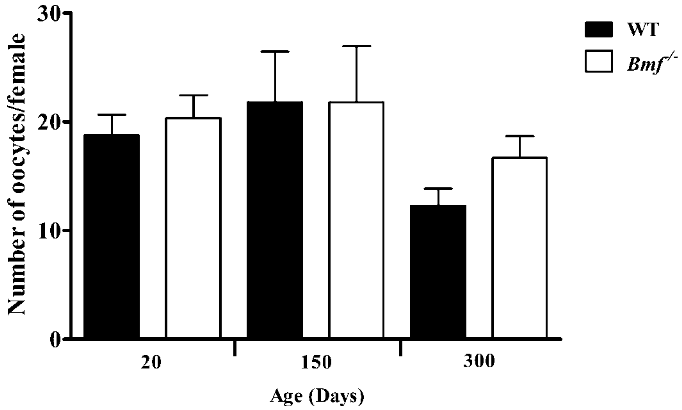

FIG. 5. The ovulatory response to exogenous gonadotropin stimulation in WT and *Bmf*$^{-/-}$ mice. The numbers of oocytes ovulated by prepubertal, young, and aged WT and *Bmf*$^{-/-}$ mice following stimulation with eCG and hCG were determined. Data are presented as mean ± SEM, n = 6 mice/group with no significant differences.

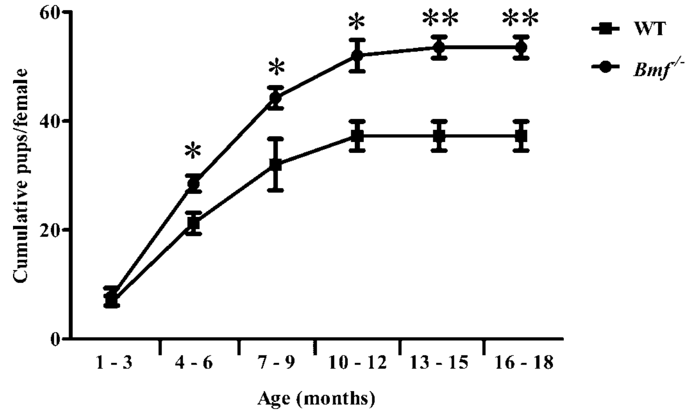

FIG. 6. Fertility of female *Bmf*$^{-/-}$ mice compared to WT controls. Reproductive capacity of mice, expressed as the cumulative number of pups weaned per WT and *Bmf*$^{-/-}$ females over a period of 18 mo. Data are presented as mean ± SEM, n = 6 mice/group, *$P < 0.05$ and **$P < 0.005$ when compared to same-age WT value.

resulted in *Bmf*$^{-/-}$ females producing significantly more pups than WT females over the course of the study (WT 37.3 ± 2.7 vs. *Bmf*$^{-/-}$ 53.5 ± 1.9 pups/female, $P < 0.005$; Fig. 6).

Ovarian morphology and follicular endowment were assessed at the end of the fertility trial, when females were 545 days of age. The primordial follicle pool was exhausted in both WT and *Bmf*$^{-/-}$ ovaries, but primary follicles were still present in low numbers (WT 50.4 ± 33.5; *Bmf*$^{-/-}$ 128.0 ± 51.3 follicles/ovary, $P = 0.24$). Notably, even at this advanced age, *Bmf*$^{-/-}$ females contained significantly more secondary and antral follicles than their WT counterparts (Fig. 7A). In particular, large antral follicles were still evident in ovaries from *Bmf*$^{-/-}$ females but were not present in those from WT controls (Fig. 7B).

DISCUSSION

Females are born with a fixed number of oocytes stored within the ovary as primordial follicles [1–3]. It is from this initial pool of primordial follicles that all oocytes for ovulation are drawn. However, most growing follicles are not ovulated but instead undergo atresia [4, 6–10]. Thus, cell death plays a critical role in determining both the number of primordial follicles maintained within the ovary and the rate of follicular loss throughout postnatal life [1, 27]. Despite the significance of cell death in influencing the number of available oocytes and follicles and thus the length of the fertile life span, the genes that regulate germ cell death during ovarian development and follicular atresia during reproductive life have not been fully characterized. In this study, we identified the proapoptotic BH3-only BCL-2 family member BMF as a key regulator of primordial follicle number in the ovaries of adult mice. We showed that deletion of *Bmf* in mice results in increased numbers of primordial follicles being retained in the ovary between Days 20 and 100, which resulted in a sustained elevation in primordial follicle numbers until Day 400. Furthermore, we demonstrated that increasing the number of primordial follicles maintained during adulthood can prolong fertility.

Numerous studies in mice and women have shown that premature depletion of the primordial follicle reserve leads to early loss of fertility (mice and women) and early menopause (women) [12, 14, 28–31]. However, studies investigating the impact of increasing the size of the ovarian reserve on the duration of the fertile period are rare and have produced conflicting results. One study demonstrated that neonatal administration of exogenous activin A significantly increased the number of primordial follicles formed initially but that follicle numbers were comparable to those in vehicle-treated animals by Postnatal Day 19 [1]. Thus, excess follicles were not maintained, and the fertile life span was unlikely to be prolonged in this setting. Furthermore, young activin A-treated animals ovulated a higher number of oocytes, but the oocytes were of poor quality compared to normal oocytes from untreated WT animals [1]. Similarly, the ovaries of neonatal mice overexpressing the prosurvival protein BCL-2 have significantly more primordial follicles than WT mice, but the numbers of follicles returned to WT levels by Postnatal Day 60 [32]. Therefore, an increase in the number of follicles initially endowed within the ovary does not guarantee that elevated primordial follicle numbers will be maintained during reproductive life. To account for these observations, Bristol-Gould [1] proposed an ‘‘ovarian quorum sensing mechanism’’ that acts around the time of puberty to eliminate excess follicles from the ovary in order to normalize follicle numbers and ensure that only high-quality oocytes are retained in the adult primordial follicle pool [1].

In contrast to these two studies, we have shown that compared to WT mice, BMF-deficient females have increased numbers of follicles within their ovaries and that this is

TABLE 1. Fertility of *Bmf*$^{-/-}$ compared to WT female mice.[a]

	Over 6-mo period			Over fertile life span		
Genotype	No. of pups/litter	No. of litters	No. of pups weaned	No. of litters	No. of pups weaned	Age at last litter
WT (n = 6 pairs)	7.5 ± 0.5	3.5 ± 0.3	26 ± 4.0	4.7 ± 0.6	37.3 ± 2.7	295 ± 40.1
Bmf$^{-/-}$ (n = 6 pairs)	8 ± 0.7	5.3 ± 0.5*	36 ± 2.2*	6.8 ± 0.8*	53.5 ± 1.9**	360 ± 30.1*

[a] Data are presented as mean ± SEM; *$P < 0.05$ and **$P < 0.005$ when compared to WT.

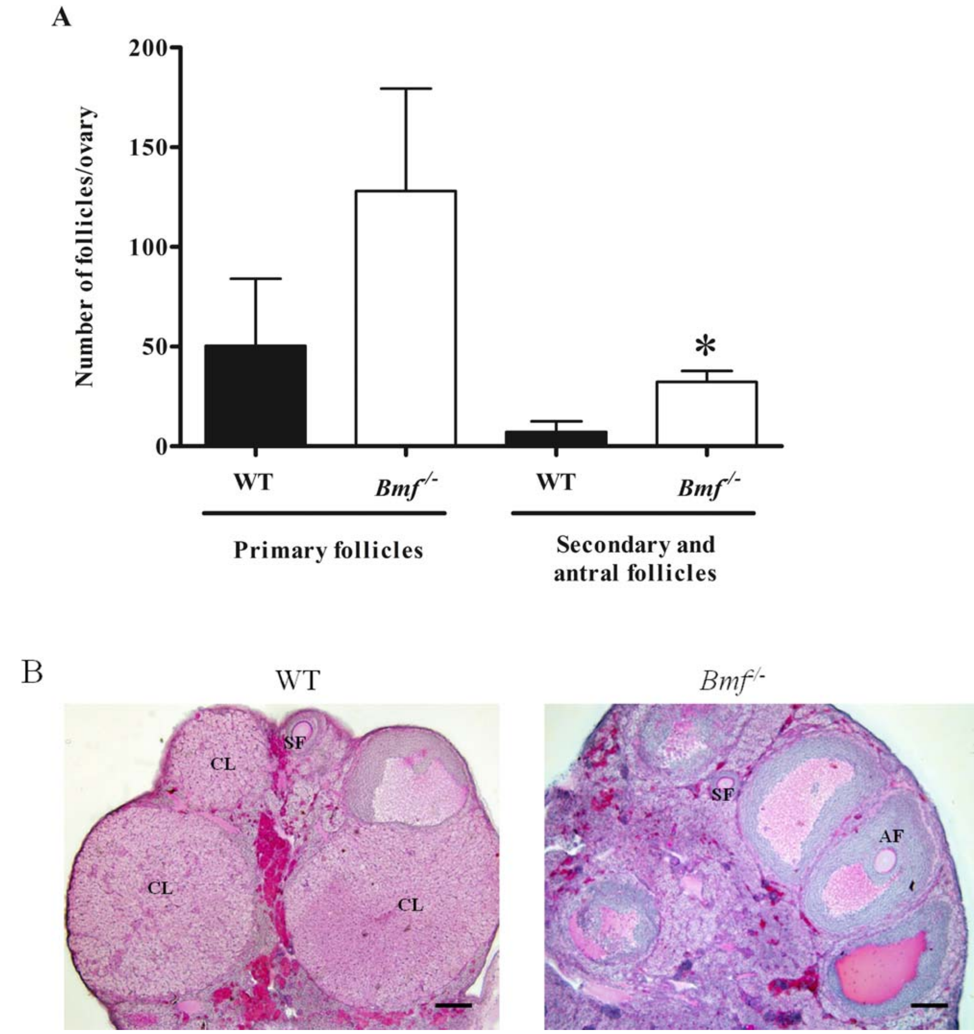

FIG. 7. Morphology and follicular endowment of ovaries from WT and $Bmf^{-/-}$ mice at 545 days of age. **A**) The numbers of primary, secondary, and antral follicles in ovaries from WT and $Bmf^{-/-}$ females at 545 days of age were determined. n = 6 mice/group, $*P < 0.05$ when compared to WT females. **B**) Representative image of $Bmf^{-/-}$ ovaries containing large antral follicles, which were not present in WT ovaries. Sections were PAS stained. SF, secondary follicle; AF, antral follicles; CL, corpora lutea. Bar = 200 μm.

sustained throughout adult life and even into advanced age (from Day 100 until Day 545). Remarkably, increased primordial follicle numbers correlated with an extended duration of fertility with no apparent negative impact on the health of the offspring or the mother. Our findings are consistent with one previous study by Perez et al. [33] demonstrating that mice deficient in the proapoptotic protein BAX also have an excess supply of primordial follicles in adulthood that is maintained into advanced chronological age, though fertility was not extended. Notably, it was found that prolonged ovarian function in female BAX-deficient mice was associated with an improvement in many age-related health problems, including improved bone density and reduced muscle loss [33]. In contrast, a second study by Ke et al. [21] found that the number of primordial follicles in ovaries from $Bax^{-/-}$ mice was comparable to that in WT females at 1 yr of age and that increased primordial follicle number was observed only in ovaries from female mice lacking both BOK and BAX ($Bok^{-/-}Bax^{-/-}$). The difference in findings between these two studies may be attributed to the use of significantly different counting methodologies; Perez et al. reported follicle numbers per ovary, whereas Ke et al. reported follicle density in the form of primordial follicle numbers per standard unit of area.

After establishment of the initial ovarian reserve, the number of primordial follicles maintained in the ovary throughout reproductive life is influenced by 1) the rate of primordial follicle death and 2) the rate of recruitment into the growing follicle pool [27, 34, 35]. In this study, we showed that although ovaries from $Bmf^{-/-}$ mice contained more primordial and growing follicles than those from WT females, the proportions of total follicles that were growing were comparable to those detected in WT ovaries. Thus, a reduction in the number of follicles or the rate of recruitment to commence follicular development could not explain the sustained increase in primordial follicle numbers detected in $Bmf^{-/-}$ females. Additionally, these results indicate that a fixed proportion rather than a fixed number of follicles are recruited into the growing follicle pool and that this proportion is age dependent.

We investigated the hypothesis that loss of BMF protected oocytes and/or granulosa cells from apoptosis and thus follicles from atresia in both prepubertal and adult ovaries. The number of dying or dead primordial and primary follicles detected either morphologically or through caspase-3 immunoreactivity or TUNEL assay was negligible in adult ovaries of both WT and $Bmf^{-/-}$ females. We did not detect BMF in oocytes or granulosa cells at these stages of follicular development. These observations are consistent with previous work demonstrating that it is difficult to detect atretic primordial follicles in normal healthy ovaries, even during periods of significant primordial follicle loss [27]. BMF protein was observed in apoptotic granulosa cells of some large secondary and antral follicles. However, similar numbers of atretic follicles were observed in WT and $Bmf^{-/-}$ ovaries from Days 100 to 545, indicating that BMF is dispensable for follicle atresia in adult ovaries. In contrast, follicle atresia was abnormally reduced in ovaries from Day 20 mice $Bmf^{-/-}$ mice, suggesting that BMF may be required for follicular atresia prior to adulthood.

We have shown that BMF acts in the ovary to mediate primordial follicle loss between Postnatal Days 20 and 100 (i.e.. the period of time when primordial follicle numbers are first seen to be abnormally elevated in $Bmf^{-/-}$ ovaries). Interestingly, the prepubertal/pubertal period has previously been identified as a time of primordial follicle loss in both mice and humans [1, 36, 37]. Bristol-Gould et al. [1] and Tingen et al. [27] reported that primordial follicle numbers in mice fell by approximately 50% between Postnatal Days 19 and 45 before stabilizing in the adult ovary, and it was proposed that this loss was due primarily to the death of primordial follicles rather than to recruitment into the growing follicle pool. Despite the loss of thousands of primordial follicles from the ovary during this time, few active caspase-3 or cleaved PARP1-positive primordial follicles were detected, nor were atretic primordial follicles identified in large numbers morphologically. Thus, the mechanisms underlying prepubertal/pubertal primordial follicle loss have remained elusive. Our study shows that BMF may be a key factor in directly or indirectly mediating primordial follicle loss at this time. Thus, our future work will determine which cell types within the ovary are targeted for death by BMF between Days 20 and 100. From these future studies, we will establish if BMF acts directly on primordial follicles (either oocytes or granulosa cells) or if BMF acts on growing follicles or even stromal components in the ovary to indirectly mediate primordial follicle depletion.

Characterizing the mechanisms of primordial follicle loss during postnatal life is important for understanding the regulation of primordial follicle number and its impact on the fertile life span. We have identified BMF as one such regulator in mice; when BMF is deleted in mice, a greater number of primordial follicles are retained in the ovary, resulting in prolonged fertility. This study improves our understanding of the regulation of primordial follicle number and demonstrates that the duration of the fertile life span may be prolonged by blocking key members of the apoptotic pathway.

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