

BAX is involved in regulating follicular growth, but is dispensable for follicle atresia in adult mouse ovaries

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Abstract

Mammalian females are endowed with a finite number of primordial follicles at birth or shortly thereafter. Immediately following the formation of the primordial follicle pool, cohorts of these follicles are recruited to begin growth, and this recruitment continues until the primordial follicle population is depleted. Once recruited, a follicle will either grow and ovulate or undergo atresia. Follicle atresia results from the apoptotic death of follicular cells. Members of the BCL-2 family of proteins are important regulators of apoptosis in most cells including in the ovary. Here, we tested the hypothesis that the proapoptotic BAX is an important regulator of follicle survival. We used a variety of histological and biochemical techniques to investigate the impact of *Bax* deletion on follicle growth and death. We observed that the *Bax* deletion results in delayed vaginal opening and altered follicular growth. Young adult *Bax*-deficient ovaries contained increased numbers of primordial follicles and a trend towards reduced numbers of growing follicles. *Bax* deficiency led to a reduction in average litter size, and also a reduction in the number of oocytes ovulated in response to exogenous gonadotropins. In contrast, *Bax* deficiency did not alter follicle atresia. In conclusion, BAX appears to be an important regulator of follicle growth, but is dispensable for follicle atresia in mice.

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Introduction

Immediately following the formation of the primordial follicle population in mammalian ovaries, cohorts of follicles are recruited to begin growth. Follicle recruitment occurs continually throughout the female's reproductive lifespan (Peters 1969; reviewed in Hirshfield 1991, Picton 2001). Though two recent papers (Johnson *et al.* 2004, 2005) have challenged the long-held view that oogenesis is restricted to embryonic life in the majority of mammalian species (Zuckerman 1951), skepticism (Greenfeld & Flaws 2004, Telfer *et al.* 2005), and a recent rebuttal (Eggen *et al.* 2006) refute these challenges. Thus, at present, it is predominately believed that the primordial follicle population is finite and that recruitment from the primordial follicle pool continues only until it has been depleted, at which point infertility ensues and the female is said to have entered reproductive senescence, or menopause in the case of humans (Peters 1969; reviewed in Hirshfield 1991).

Once recruited into the growing population, a follicle has only two fates: ovulation or atresia (follicle death).

Once recruited to initiate growth, the flattened granulosa cells that surround the oocyte in primordial follicles differentiate into cuboidal cells and become mitotically active (reviewed in Hirshfield 1991). Continued proliferation drives the formation of multiple granulosa cell layers and the follicle transitions into first a primary then a secondary or preantral follicle. Preantral follicles also acquire an outer layer of theca cells around their basement membrane that are important for the steroidogenic activity of the antral follicle, the next follicle stage (Hirshfield 1991). Antral follicles are important as they contribute to the ovulatory follicle population and produce steroid hormones, most importantly estradiol (reviewed in Hirshfield 1991).

Follicle growth and survival are regulated by a variety of paracrine and endocrine factors. For example, growth

within and beyond the primary stage is dependent on the actions of the paracrine factor kit ligand (KITL) and the growth differentiation factor (GDF)-9, which are expressed by granulosa cells and the oocyte respectively (Huang *et al.* 1993, Dong *et al.* 1996, Elvin *et al.* 1999; reviewed in Pangas & Matzuk 2004). Conversely, activin A is a negative regulator of preantral follicle growth, promoting these follicles to remain dormant and blocking follicle stimulating hormone (FSH)-induced granulosa cell proliferation (Mizunuma *et al.* 1999). FSH is critical for the transition of follicles to the antral stage (Kumar *et al.* 1997, Balla *et al.* 2003), and for the follicle survival beyond the early antral stage (Hirshfield 1986, 1988, Chun *et al.* 1996). On the other hand, FAS/FASL and tumor necrosis factor α (TNF α) promote follicle atresia (Kaipia *et al.* 1996, Hu *et al.* 2001, Morrison & Marcinkiewicz 2002, Sasson *et al.* 2002, Abdo *et al.* 2003).

Growing follicles that are not rescued by FSH undergo atresia (McGee & Hsueh 2000). Atresia, the fate of more than 99% of follicles (Morita & Tilly 1999), is an apoptotic process (Hughes & Gorospe 1991, Tilly *et al.* 1991), and depending on the follicle stage, it is initiated by oocyte (preantral follicles) or granulosa cell (antral follicle) death (reviewed in Reynaud & Driancourt 2000). Early antral follicles are most sensitive to atresia and require FSH action for survival (Hirshfield 1986, 1988, Chun *et al.* 1996).

Members of the BCL-2 protein family are important regulators of apoptosis in many cell types, and various BCL-2 family members are expressed in growing follicles, including the antiapoptotic BCL-2 and BCL-XL, and the proapoptotic BAX and BAK (reviewed in Hussein 2005). BAX is involved in mediating apoptosis of follicle cells under various conditions. For example, Tilly *et al.* (1995) found that *Bax* expression is reduced following *in vivo* eCG (an FSH analog) treatment in rats, and that follicles cultured in serum-free media in the absence of eCG underwent atresia and exhibited greatly increased *Bax* expression. Further, 4-vinylcyclohexene diepoxide (VCD) and the pesticide methoxychlor (MXC) induce atresia of small preantral and antral follicles respectively, and do so at least in part, through BAX-mediated mechanisms (Springer *et al.* 1996, Hu *et al.* 2001, Borgeest *et al.* 2004, Miller *et al.* 2005).

Genetic models have demonstrated the importance of *Bcl-2* in the regulation of follicle atresia (Hsu *et al.* 1996, Morita *et al.* 1999, Flaws *et al.* 2006). *Bax* deletion was said to result in the presence of unusual atretic follicles, in which granulosa cells appeared unable to undergo apoptosis (Knudson *et al.* 1995). Further, Perez *et al.* (1999) demonstrated that atresia of immature follicles is attenuated by *Bax* deletion, leading to a slower depletion of the primordial follicle reserve and a lengthening of female reproductive lifespan. Neither of these reports, however, performed a quantitative assessment of the effect of *Bax* deletion on follicle growth and atresia at all

stages of growth, and its impact on fertility. Such an assessment is warranted for several reasons. First, despite studies showing an involvement of *Bax* in the mediation of atresia by various factors, its physiological role in normal ovarian function is not well established. Second, as *Bax* appears to be involved in regulating oocyte death in immature follicles (Perez *et al.* 1999), the study of its role in regulating antral follicle atresia could highlight similarities or differences in the mechanisms regulating oocyte and granulosa cell death.

The objective of this study, therefore, was to investigate the effect of *Bax* deletion on ovarian function. Through various means, we tested the hypotheses that the enlarged follicle reserve in *Bax*^{-/-} ovaries impacts pubertal onset and estrous cyclicity; that *Bax* is involved in regulating follicle growth; and that *Bax* promotes follicular atresia.

Materials and Methods

Animals and treatment

Wild-type (WT) and *Bax*^{-/-} mice of C57BL/6 background were used in all experiments. Animals were housed in clear plastic cages and maintained on a 12 h light:12 h darkness cycle in a temperature-controlled room (24 ± 1 °C) with 35 ± 4% relative humidity. Mice were provided food and water *ad libitum*. The University of Maryland Institutional Animal Use and Care Committee approved all protocols involving mice.

Screening/genotyping mice

Ear-punch tissue collected from pups was lysed in a 9 μ l water containing proteinase K (4 mg/ml; Ren *et al.* 2001). Tissue was digested for 30 min at room temperature (RT), followed by a 3-min incubation at 100 °C. The lysate was then subjected to PCR using the following primers: common primer GTTGACCAGAGTGGCGTAGG; WT allele-specific primer GAGCTGATCAGAACCATCATG; *Bax*^{-/-} allele-specific primer CCGCTTCCATTGCT-CAGCGG. The PCR conditions were as follows: 35 cycles of 94 °C for 45 s, 59 °C for 1 min 30 s, and 72 °C for 2 min. PCR products were sized by gel electrophoresis. WT mice were identified by the presence of only a 300 bp product, heterozygotes by both a 300 and 506 bp product, and *Bax*^{-/-} mice by only a 506 bp product. Only homozygous WT and *Bax*^{-/-} mice were used in each experiment.

Assessment of pubertal onset and estrous cyclicity

To assess the onset of puberty in juvenile females, mice were observed daily for signs of vaginal opening (Nishimura *et al.* 1989). Estrous cyclicity was monitored by daily vaginal lavage of females in the morning, for a period of at least 1 month beginning around 2 months of

age, and the day of the cycle was determined based on vaginal cytology according to the classification of Pedersen (1970).

Histology

Ovaries were collected on the morning of estrus and fixed in Kahle's solution (4% formalin, 28% ethanol, and 0.34 M glacial acetic acid) for at least 24 h. Following fixation, ovaries were dehydrated and embedded in Paraplast (VWR International, West Chester, PA, USA). Ovaries were serially sectioned at 8 μ m intervals, mounted on glass slides, stained with Weigert's hematoxylin, counterstained with picric acid methyl blue, dehydrated, and mounted in Permaslip (Alban Scientific, Inc., St Louis, MO, USA).

Assessment of follicle development

In every tenth ovarian section, the numbers of primordial, primary, preantral, and healthy antral follicles were counted. Only follicles containing an oocyte with a visible nucleus were counted to avoid double counting, and all counting was done without the knowledge of genotype. Follicles were counted as primordial if they contained an oocyte surrounded by flattened granulosa cells, or a mixture of <7 flattened and cuboidal granulosa cells (Meredith *et al.* 2000). Follicles were counted as primary if they contained an oocyte surrounded by a single layer of seven or more cuboidal granulosa cells. Preantral follicles were those containing an oocyte surrounded by two to four complete layers of granulosa cells. Antral follicles were considered as those that contained five or more complete granulosa cell layers with or without a visible antrum. Antral follicles were considered to be healthy if they had an intact oocyte and <10% pyknotic granulosa cells. Antral follicles were considered to be atretic if their oocyte was degenerating, or there were more than 10% pyknotic granulosa cells. All counts are reported as the raw number counted per ovary without a correction factor applied.

Assessment of apoptosis using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays

Ovaries were collected and fixed in Kahle's solution, dehydrated, embedded in Paraplast, serially sectioned at 5–8 μ m intervals, and mounted on glass slides. Sections were subjected to TUNEL analysis using the ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon International, Temecula, CA, USA), according to the manufacturer's instructions. Sections were counterstained with crystal violet-free methyl green (FD Neuro Technologies, Inc., Baltimore, MD, USA), and then mounted in Permaslip. Apoptotic cells were classified as those staining dark brown by the assay.

Assessment of apoptosis using 3'-end labeling

Ovaries were collected from females aged 3 months on the morning of estrus and frozen at -70°C until use. Ovaries from two females per genotype were pooled and DNA was isolated using the DNeasy kit (Qiagen). In each reaction, 0.5 μ g DNA was labeled with [α - ^{32}P]ddATP (GE Healthcare, formerly Amersham) using the Terminal Transferase Reaction kit (Roche Diagnostic Corporation). Labeled DNA was precipitated and separated on a 2% agarose gel. Individual lanes were excised from the gel after it was dried, and were subjected to scintillation counting.

In vitro assessment of atresia

Follicles were isolated from immature WT and *Bax* $-/-$ females following the procedure of Flaws *et al.* (1995). Females were dosed with 5 IU PMSG, and antral follicles were isolated in unsupplemented Eagle's Minimum Essential Media (MEM; GIBCO) 2 days later. Follicles were either snap-frozen immediately upon collection (time 0, no incubation; $n=10$ follicles per vial), or were cultured for 24 h at 37°C in MEM supplemented with 0.1% BSA (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate, after which they were snap-frozen ($n=10$ follicles per vial). Isolated DNA, 0.25–0.5 μ g, was then subjected to 3'-end labeling reactions as above.

Estradiol assays

Blood was collected on the morning of estrus from WT and *Bax* $-/-$ mice and subjected to enzyme-linked immunosorbent assay (ELISA) for the measurement of estradiol levels. ELISA kits and reagents were obtained from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA). The assay was run according to the manufacturer's instructions. All samples were run in duplicate. The minimum detection limit, as stated in the instructions of the kit, was 7 pg/ml. The average intra- and interassay coefficients of variation were 4.2 and 8.2% respectively.

FSH assay

Blood was collected from WT and *Bax* $-/-$ mice on the morning of estrus and FSH assays were carried out by RIA using reagents from the National Hormone and Pituitary Distribution Program. Rat FSH hormone antigen, rat FSH antiserum, and mouse FSH RP were provided by the National Institute of Diabetes and Digestive and Kidney Diseases. Iodination reagents (IODO-BEADS 28665, 28666) were purchased from Pierce (Rockford, IL, USA). A standard curve was prepared and cold standards and samples (100 μ l) were added to labeled tubes along with primary antibody (FSH at 1:1400) and iodinated FSH. Samples were shaken and

stored at 4 °C overnight. On day 2, secondary antibody was added (1:10 dilution) along with 2% normal rabbit serum (Sigma-Aldrich) and incubated at RT for 5 min. The tubes were centrifuged for 15 min at 3000 r.p.m., supernatant was decanted, and pellets were counted in a gamma counter for 1 min each. All samples were run in duplicate. Sensitivity for the FSH assay was 200 pg/ml. The average intra- and interassay coefficients of variation were 6.7 and 2.7% respectively.

Statistical analysis

Follicle numbers between WT and *Bax*^{-/-} ovaries at 3 months of age were compared using Student's *t*-test, as were the mean FSH and estradiol levels, age at puberty, and the percent of time spent in estrus. Follicle numbers between WT and *Bax*^{-/-} ovaries at 13 months of age were compared using Mann-Whitney tests due to the smaller number of animals and the distribution of the data. The 3'-end labeling data for *Bax*^{-/-} ovaries were normalized to WT values and the data were compared using a one-way *t*-test. Litter sizes were compared by one-way ANOVA. *P* values <0.05 were considered to be statistically significant.

Results

We first assessed the effect of *Bax* deletion on the age at pubertal onset and estrous cyclicity. As shown in Fig. 1, vaginal opening was significantly delayed in *Bax*^{-/-} females (postnatal day (PN) 36.6 ± 0.9) when compared with WT females (PN 32.7 ± 0.8; *n* = 9 WT females; *n* = 8 *Bax*^{-/-} females; *P* < 0.01). Shortly after the females reached puberty, we monitored estrous cyclicity for 1–1.5 months on a daily basis. As shown in Fig. 1, estrous cyclicity, based on the percentage of days that females

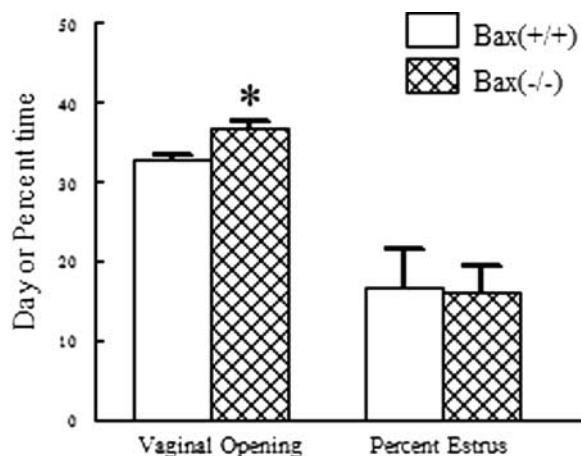


Figure 1 Effect of *Bax* deletion on the onset of puberty and estrous cyclicity. (A) Age at vaginal opening in WT and *Bax*^{-/-} females; and (B) percentage of time spent in estrus in females aged 2–3 months. Bars represent mean ± s.e.m. **P* < 0.01.

exhibited an estrous smear, did not differ between *Bax*^{-/-} (16.1 ± 3.4%) and WT females (16.8 ± 4.7%; *n* = 6 WT females; *n* = 8 *Bax*^{-/-} females; *P* = 0.9).

We next investigated the effect of *Bax* deletion on folliculogenesis. To do this, we performed morphometric analysis of follicle numbers at 3 and 13 months of age and compared them between *Bax*^{-/-} and WT animals. Fig. 2A shows healthy follicle numbers for *Bax*^{-/-} and WT animals at 3 months of age. *Bax*^{-/-} ovaries contained significantly more primordial follicles (353.2 ± 42.4) when compared with WT ovaries (256.2 ± 19.3; *n* = 10 WT ovaries; *n* = 9 *Bax*^{-/-} ovaries; *P* < 0.05). The number of primary follicles was not different in *Bax*^{-/-} ovaries (207.0 ± 26.2) when compared with WT ovaries (168.6 ± 17.3; *P* = 0.23). There was a trend towards decreased numbers of larger growing follicles *Bax*^{-/-} ovaries. The number of preantral follicles in *Bax*^{-/-} (67.0 ± 6.3) was lower than in WT ovaries (85.7 ± 9.5; *P* = 0.13), as was the number of antral follicles (WT = 32.0 ± 3.8, *Bax*^{-/-} = 23.4 ± 2.5; *P* = 0.08), but neither was statistically significant. Interestingly, *Bax*^{-/-} ovaries contained both inclusion cysts (Fig. 3A), as well as large hemorrhagic cysts (Fig. 3B). While occasional inclusion cysts were observed in WT ovaries, we did not observe hemorrhagic cysts.

Follicle numbers at 13 months of age are shown in Fig. 2B. *Bax*^{-/-} ovaries contained significantly more primordial follicles (80.7 ± 19.9) when compared with WT ovaries (21.8 ± 4.8; *n* = 4 WT ovaries; *n* = 6 *Bax*^{-/-} ovaries; *P* < 0.05). There were also significantly more primary follicles in *Bax*^{-/-} (89.3 ± 10.4) when compared with WT (48.5 ± 11.2) ovaries (*P* < 0.05), as well as more preantral follicles in *Bax*^{-/-} (34.5 ± 7.5) when compared with WT (19.3 ± 3.3) ovaries (*P* < 0.05). In contrast, there was no statistically significant difference in the number of antral follicles in *Bax*^{-/-} (16.7 ± 2.2) when compared with WT (10.0 ± 1.9) ovaries (*P* = 0.11).

As there was a strong statistical trend towards fewer antral follicles in *Bax*^{-/-} when compared with WT ovaries at 3 months of age, we retrospectively examined litter records to see if the average litter size was smaller in *Bax*^{-/-} when compared with WT females. We

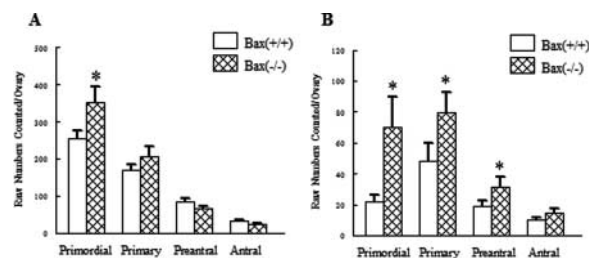


Figure 2 Effect of *Bax* deletion on folliculogenesis. Healthy follicle numbers at (A) 3 months of age; and (B) 13 months of age. Bars represent mean ± s.e.m. **P* < 0.05.

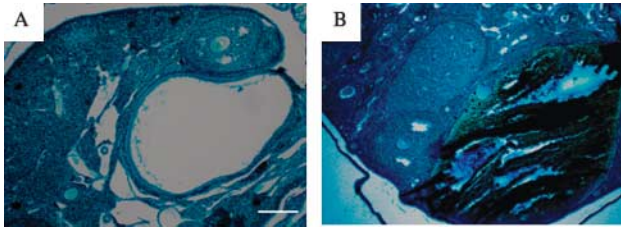


Figure 3 Examples of cystic structures in *Bax*^{-/-} ovaries. (A) Inclusion cyst; (B) hemorrhagic cyst. Magnification $\times 10$, scale bar = 150 μm .

compared breedings between WT males and WT females, *Bax*^{+/-} males and *Bax*^{+/-} females, and *Bax*^{+/-} males and *Bax*^{-/-} females (Fig. 4). When compared with WT breedings (10.68 ± 0.5 pups/l; $n = 59$), average litter size was decreased in both *Bax*^{+/-} breedings (7.95 ± 0.41) and *Bax*^{-/-} breedings (8.96 ± 0.25 pups/l; $n = 138$; $P < 0.005$).

Reduced litter sizes were not due to increased pup mortality prior to weaning due to an inability of *Bax*^{-/-} females to nurse. *Bax*^{-/-} females were able to nurse, as seen from the presence of milk in the stomach of neonatal pups, and the fact that the average weight of pups in litters from WT (12.4 ± 0.95 g; $n = 3$ litters) and *Bax*^{-/-} (12.3 ± 1.09 g; $n = 5$ litters; $P = 0.95$) dams were not different.

We next investigated the impact of *Bax* deletion on follicle atresia. As shown in Fig. 5A, we observed that there were similar numbers of atretic antral follicles in *Bax*^{-/-} (26.2 ± 2.2) when compared with WT (23.8 ± 2.2) ovaries at 3 months of age ($P = 0.44$). Since there was a trend towards fewer healthy antral follicles in *Bax*^{-/-} ovaries, the percentage of atretic antral follicles was significantly greater in *Bax*^{-/-} ($53.1 \pm 3.4\%$) when compared with WT mice ($42.4 \pm 3.3\%$; $P < 0.05$; Fig. 5B). TUNEL analysis confirmed that pyknotic cells used to morphologically classify a follicle as atretic were in fact apoptotic cells (Fig. 5C and D).

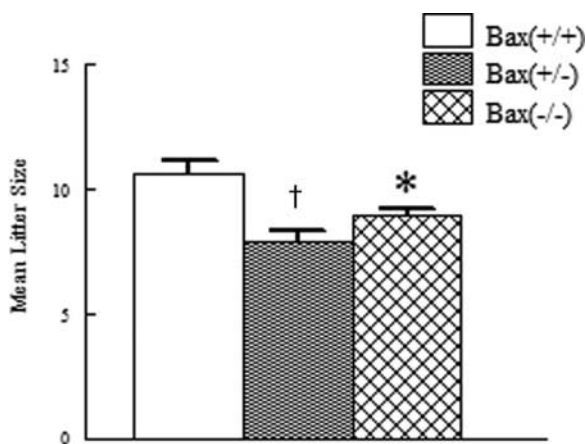


Figure 4 Effect of *Bax* deletion on average litter sizes for WT, *Bax*^{+/-}, and *Bax*^{-/-} females. Bars represent mean \pm S.E.M. * $P < 0.005$ and † $P < 0.001$.

We next performed 3'-end labeling of DNA isolated from whole ovaries. There was no difference in the amount of labeling in *Bax*^{-/-} when compared with WT ovaries (*Bax*^{-/-} $130.8 \pm 27.6\%$ of WT; $P = 0.35$; Fig. 6A and B). Similar to what we observed from 3'-end labeling experiments using whole ovaries, *Bax*^{-/-} follicles at time 0 h had similar labeling as WT follicles (*Bax*^{-/-} $117.8 \pm 12.9\%$ of WT; $P = 0.3$; Fig. 6C and D). In contrast, *Bax*^{-/-} follicles collected following culture for 24 h in serum-free media had significantly less labeling when compared with WT follicles (*Bax*^{-/-} $74.3 \pm 4.7\%$ of WT; Fig. 6C and D; $P < 0.05$).

We next tested whether the strong statistical trend in the reduction of antral follicle numbers was due to decreased FSH production or responsiveness. FSH levels did not differ between *Bax*^{-/-} and WT females at PN35 and 3 months of age (Fig. 7A). Next, we performed superovulation experiments to investigate the responsiveness of *Bax*^{-/-} ovaries to exogenous gonadotropins (Fig. 7B). In response to gonadotropins, *Bax*^{-/-} females ovulated significantly fewer oocytes (31.14 ± 5.7) when compared with WT ovaries (54.5 ± 6.1 ; $n = 8$ WT females, $n = 7$ *Bax*^{-/-} females; $P < 0.05$).

Finally, we tested whether the strong statistical trend in the reduction of growing follicles was due to altered estradiol production. To do this we measured serum estradiol levels. As shown in Fig. 7C, estradiol levels at 3 months of age were significantly higher in *Bax*^{-/-} (97.95 ± 7.2 pg/ml) when compared with WT females (69.9 ± 6.1 pg/ml; $n = 4$ WT females, $n = 4$ *Bax*^{-/-} females; $P < 0.05$). At 13 months of age, however, estradiol levels were equivalent between *Bax*^{-/-} and WT females ($P = 0.6$).

Discussion

This study demonstrates the importance of *Bax* in the regulation of female reproductive physiology. We observed that the age at vaginal opening was delayed in *Bax*^{-/-} when compared with WT females. The age at vaginal opening is an indicator of the onset of puberty, and thus, these data suggest that puberty is delayed following *Bax* deletion. It is possible though that *Bax* deficiency impacts the physical process of vaginal opening itself. FSH levels at PN35, around the time of pubertal onset, are equivalent between WT and *Bax*^{-/-} females, suggesting that rather than affecting hormone production, *Bax* deletion interferes with apoptosis in the vaginal mucosa. Vaginal opening is an apoptotic event stimulated by estradiol and regulated by BCL-2 family members (Rodriguez *et al.* 1997) and thus, delayed vaginal opening in *Bax*^{-/-} females is likely due to an absence of *Bax* in the vaginal mucosa, and defective apoptosis of these cells.

As BAX is a proapoptotic protein that is expressed in the ovary, and has been shown to be involved in regulating immature follicle atresia and the mediation

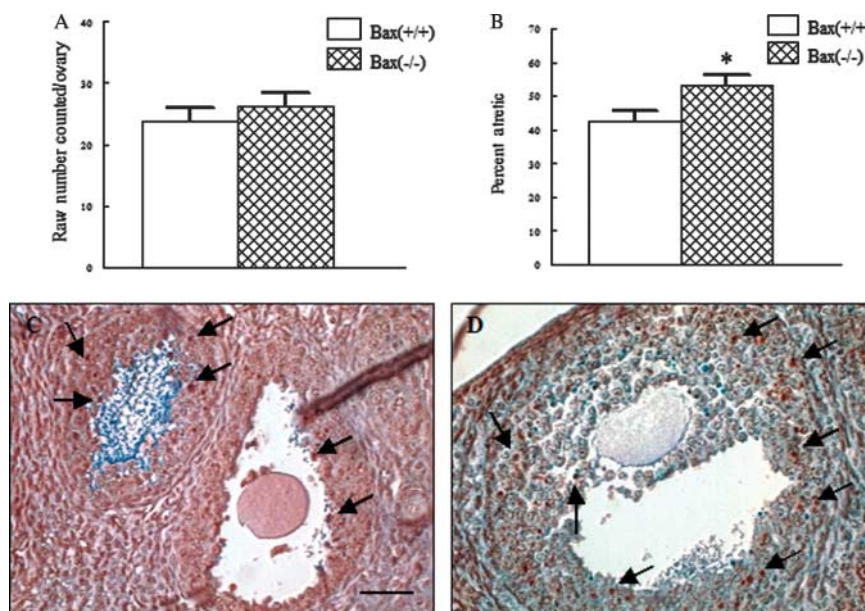


Figure 5 Effect of *Bax* deletion on antral follicle atresia *in vivo* at 3 months of age. (A) Mean number of atretic antral follicles; (B) percentage of antral follicles that are atretic. Bars represent mean \pm S.E.M. * $P < 0.05$. TUNEL assays on (C) WT and (D) *Bax*^{-/-} ovaries. Arrows point to darkly stained, TUNEL-positive cells. Magnification $\times 40$, scale bar = 50 μ m.

of follicle atresia induced by a variety of toxicants (Tilly *et al.* 1995, Springer *et al.* 1996, Perez *et al.* 1999, Hu *et al.* 2001, Matikainen *et al.* 2001, Borgeest *et al.* 2004, Miller *et al.* 2005), we hypothesized that *Bax* deletion would enhance follicle survival and lead to the presence of increased numbers of healthy follicles. This was not the case, and instead, we observed a defect in folliculogenesis following *Bax* deletion. We observed increased primordial follicle numbers in young adult *Bax*^{-/-} when compared with WT mice, corroborating the results of Perez *et al.* (1999). Further, we observed that, while primary follicle numbers were not different, there was a statistical trend towards reduced numbers of preantral ($P = 0.13$) and antral ($P = 0.08$) follicles in *Bax*^{-/-} when compared with WT ovaries on the morning of estrus. The early antral follicles present on the morning of estrus represent the pool from which ovulatory follicles will be selected. Thus, our data showing reduced numbers of these follicles in *Bax*^{-/-} when compared with WT ovaries, suggest that fewer antral follicles are available to be selected to grow to the ovulatory stage in *Bax*^{-/-} when compared with WT ovaries. This is supported by the fact that significantly fewer oocytes were ovulated in *Bax*^{-/-} when compared with WT females in response to exogenous gonadotropins. Exogenous gonadotropin treatment recruits additional large secondary follicles into the ovulatory pool, and its effects are dependent on the number of these follicles when it is administered (Hirshfield 1991). Since *Bax*^{-/-} females ovulated fewer follicles in response to gonadotropin treatment, it suggests that fewer follicles were available for cyclic recruitment (i.e., the recruitment during each cycle of

antral follicles from the growing pool of secondary follicles (McGee & Hsueh 2000)).

The biological significance of *Bax* deletion is further supported by the fact that litter sizes were reduced in *Bax*^{-/-} females when compared with WT females.

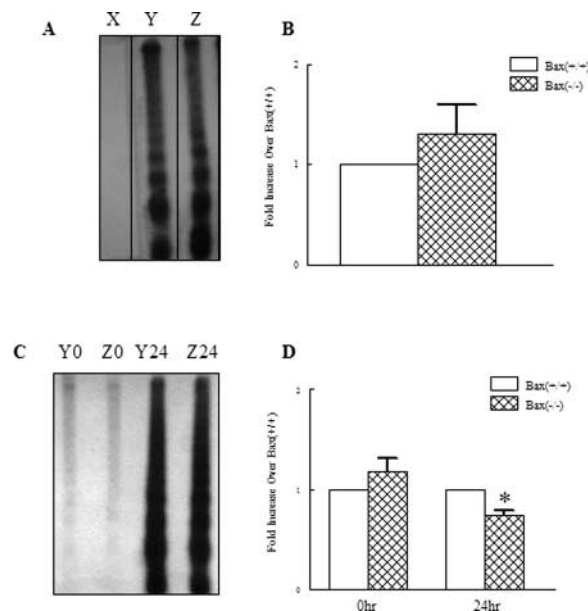


Figure 6 Effect of *Bax* deletion on atresia. 3'-End labeling of DNA isolated from whole ovaries at 3 months (A and B) or from follicles isolated from females at PN28 and cultured for 0 or 24 h in serum-free media (C and D). (A) Representative gel (X, blank; Y, WT; and Z, *Bax*^{-/-}); (B) fold increase of labeling of *Bax*^{-/-} DNA over WT DNA; (C) representative gel (Y0 = WT 0 h; Z0 = *Bax*^{-/-} 0 h; Y24 = WT 24 h; and Z24 = *Bax*^{-/-} 24 h); and (D) fold increase of labeling of *Bax*^{-/-} DNA over WT DNA. Bars represent mean \pm S.E.M. * $P < 0.05$.

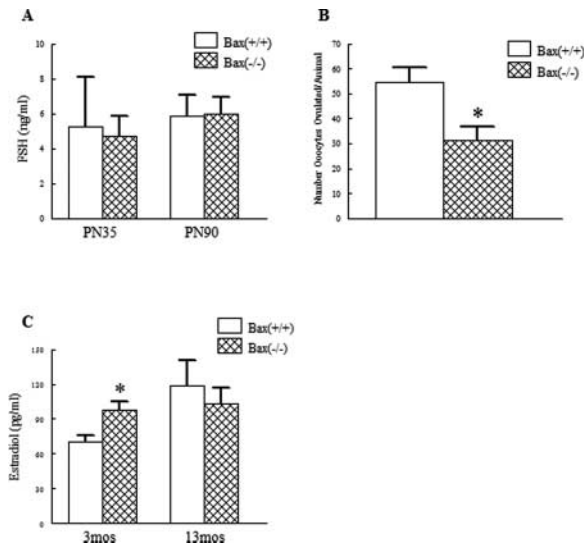


Figure 7 Effect of *Bax* deletion on hormone production and responsiveness to FSH. (A) Serum FSH levels at PN35 and PN90; (B) number of oocytes ovulated in response to exogenous gonadotropins; and (C) serum estradiol levels at 3 and 13 months. Bars represent mean \pm S.E.M. **P* < 0.05.

While it is possible that decreased litter sizes were due to the fact that we used *Bax*^{+/-} males in our breedings, we feel that differences in litter size were due to a female defect. Despite the fact that *Bax*^{-/-} males are infertile due to an inability of spermatozoa to enter meiosis (Knudson *et al.* 1995), and heterozygous breedings in this study produced smaller litter sizes when compared with WT, Russell *et al.* (2002) demonstrated that *Bax*^{+/-} males are fertile and phenotypically identical to WT males. Therefore, we think that decreased litter sizes are due to a female cause, and we believe that this caused reduced antral follicle numbers in *Bax*^{-/-} ovaries. Future studies should examine *Bax*^{-/-} ovaries at other times during the estrous cycle to determine if the number of preovulatory follicles is reduced when compared with WT.

The reduced number of healthy antral follicles in *Bax*^{-/-} ovaries is not due to altered atresia. This suggests that, in some way, *Bax* deficiency perturbs the process of cyclic recruitment, such that in its absence the proper number of antral follicles growing to ovulation is not maintained. The feedback loop of estradiol and inhibin on FSH production normally acts to tightly regulate the number of antral follicles (Hirshfield 1991). Even though there were fewer preantral follicles in *Bax*^{-/-} ovaries, we would have expected that this feedback loop would have acted to rescue an increased percentage of these follicles to maintain ovulatory capacity. This is seen in models of chemical reduction of follicular reserve size, in which there is a great reduction in the number of immature follicles, but no difference in the number of mature healthy follicles (Hirshfield 1994).

It is unclear how *Bax* deletion might affect cyclic recruitment. Our data suggest that it is not due to decreased FSH output. Further, it does not appear to be due to reduced estradiol production. This was surprising given that there are fewer antral follicles, and antral follicles produce estradiol. A similar finding, however, has been seen by others. For example, Anzalone *et al.* (2001) observed that middle-aged unilateral ovariectomized rats produced similar amounts of estradiol as young intact rats, despite the fact that they had significantly fewer growing follicles. Reduced numbers of growing follicles in *Bax*^{-/-} ovaries could be due to defective follicle growth, potentially due to reduced granulosa cell proliferation. Recent data have demonstrated that BCL-2 family members regulate cellular proliferation in Bonnefoy-Berard *et al.* (2004), and Knudson *et al.* (2001) showed that overexpression of *Bax* enhances proliferation of thymocytes. Thus, it is possible that in the absence of *Bax*, granulosa cell proliferation is reduced, and this could be due to a defect downstream of estradiol.

Similar to the results of Perez *et al.* (1999), we observed a prolonged maintenance of an enlarged follicular reserve in *Bax*^{-/-} ovaries. Further, we also observed significantly more immature growing follicles, but similar number of antral follicles in *Bax*^{-/-} when compared with WT ovaries late in life. The reversal in follicle number differences between *Bax*^{-/-} and WT ovaries at 13 months when compared with 3 months, i.e. there were more growing follicles in *Bax*^{-/-} when compared with WT ovaries at 13 months and fewer at 3 months, is likely due to the fact that the follicular reserve in WT mice is nearly exhausted and follicle output is diminishing, and not due to accelerated growth in *Bax*^{-/-} ovaries.

The most surprising observation in this study was that *Bax* deletion did not affect the amount of antral follicle atresia *in vivo*. Unaltered follicle atresia following *Bax* deletion is surprising based on a study by Knudson *et al.* (1995), in which it was stated that granulosa cell apoptosis is reduced following *Bax* deletion. However, this was based on the observation of abnormal follicle structures in *Bax*^{-/-} ovaries without biochemical assessment of granulosa cell apoptosis. We demonstrated here via morphological and biochemical means that *Bax*-deficient granulosa cells are fully competent to undergo apoptosis. This was evidenced by the fact that there were similar numbers of atretic antral follicles in *Bax*^{-/-} when compared with WT ovaries, and that the total amount of apoptosis occurring in *Bax*^{-/-} when compared with WT ovaries was similar. Further, TUNEL analysis demonstrated the presence of apoptotic granulosa cells in *Bax*^{-/-} ovaries. These data suggest that *in vivo* there is a redundant factor(s) that promotes follicle atresia in the absence of BAX. A likely candidate is BAK, which has been shown in a variety of cell types to have a redundant function with BAX. In some cells, the

deletion of both factors is required to affect apoptosis (Lindsten *et al.* 2000, Wei *et al.* 2001, Zong *et al.* 2001, Degenhardt *et al.* 2002). Therefore, it is possible that BAK is able to function in the absence of BAX to promote follicle atresia.

Interestingly, if there is redundancy between BAX and BAK, it does not extend to all means of induction of atresia. This is based on the observation that there were different consequences of *Bax* deletion on follicle atresia *in vivo* and *in vitro*. When follicles were cultured in serum-free media for 24 h, *Bax*^{-/-} follicles underwent less apoptosis than WT follicles. Serum-free culture mimics a growth factor withdrawal situation. A protective effect of *Bax* deletion on oocyte viability following growth factor withdrawal has been demonstrated (De Felici *et al.* 1999, Stallock *et al.* 2003), suggesting that granulosa cells are similarly less susceptible to death following growth factor withdrawal in the absence of *Bax*.

That *Bax* deficiency is protective to follicles *in vitro*, but not *in vivo*, suggests that an extra-follicular atretogenic factor(s) may exist that acts via a BAX-independent pathway to promote follicle atresia. A major cause of atresia is FSH withdrawal, which leads to a reduction in the intra-follicular expression of survival proteins, such as XIAP and FLIP, and an increase in the intra-follicular expression of death factors, such as FAS/FASL (Hirshfield 1986, 1988, Chun *et al.* 1996; reviewed in Jiang *et al.* 2003). Since *Bax* deletion is protective to isolated follicles, but not to intact follicles, extra-follicular factors may also be involved in promoting atresia. It is also possible that the array of factors whose expression changes following FSH withdrawal is different in isolated versus intact follicles, such that *in vivo*, factors are involved that act via a BAX-independent pathway to promote atresia.

That *Bax* deletion does not protect antral follicles from atresia *in vivo*, contrasts Perez *et al.* (1999) who observed that *Bax* deletion attenuates small follicle atresia. As atresia of immature follicles is initiated by oocyte death and antral follicle atresia is initiated by granulosa cell death (reviewed in Reynaud & Driancourt 2000), our data, together with those of Perez *et al.* (1999), suggest that different mechanisms act to regulate apoptosis in oocytes and granulosa cells. Thus, as atresia of small follicles is reduced by *Bax* deletion (Perez *et al.* 1999), it appears that oocyte death proceeds via a BAX-dependent pathway, whereas granulosa cells die via a BAX-independent pathway.

In summary, we have shown that *Bax* is important for normal follicle development, but that under physiological conditions, it is dispensable for antral follicle atresia. Our data support the idea that follicle atresia is an active process *in vivo*, and that it may involve extra- as well as intra-follicular death-inducing factors. Future studies using the *Bax*-deficient model will be useful in determining whether

extra-follicular factors are important for stimulating atresia, and for their identification.

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