REPRODUCTION
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BAX regulates follicular endowment in mice

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Abstract

It is believed that the endowment of primordial follicles in mammalian ovaries is finite. Once follicles are depleted, infertility ensues. Thus, the size of the initial endowment has consequences for fertility and reproductive longevity. Follicular endowment is comprised of various processes that culminate with the incorporation of meiosis-arrested oocytes into primordial follicles. Apoptosis is prominent during follicular endowment, and apoptosis regulatory genes are involved in its regulation. Conflicting data exist with regard to the role of the proapoptotic Bcl-2 associated X protein (BAX) in follicular endowment. Therefore, we investigated the role of BAX during follicular endowment in embryonic and neonatal ovaries. We found that BAX is involved in regulating follicular endowment in mice. Deletion of *Bax* yields increased oocyte numbers in embryonic ovaries and increased follicle numbers in neonatal ovaries when compared with wild-type ovaries. Increased follicular endowment in *Bax*−/− ovaries is not due to enhanced germ cell viability. Further, it is not due to an increased primordial germ cell (PGC) allotment, a delay in the onset of meiosis, or altered proliferative activity of oogonia. Instead, our data suggest that the regulatory activity of BAX in follicular endowment likely occurs during PGC migration, prior to PGC colonization of the gonad.

Reproduction (2007) **133** 865–876

Introduction

Female mammals are endowed with a large complement of primordial follicles within their ovaries at birth or shortly thereafter. Primordial follicles are small dormant structures consisting of an immature oocyte surrounded by a layer of flattened granulosa cells. Throughout the reproductive lifespan of the female, cohorts of these follicles are continuously selected to initiate growth and mature to ovulation. It has long been believed that the primordial follicle population is finite (Zuckerman 1951), and thus once the follicles have been depleted by growth initiation and death, infertility ensues. This long-held notion was recently challenged by the work suggesting that postnatal (PN) oogenesis, and thus the formation of new follicles, occurs in mice (Johnson *et al.* 2004, 2005). These reports have been met with skepticism (Greenfeld & Flaws 2004, Telfer *et al.* 2005) and thus, it is still predominately accepted that oogenesis is restricted to embryonic life and that the primordial follicle population is finite.

The primordial follicle population is formed during embryonic and neonatal life by a series of processes collectively referred to as follicular endowment (reviewed in Hirshfield 1991). Follicular endowment begins with the allocation of a founder population of primordial germ cells (PGCs), which are the embryonic precursors of adult gametes, the male spermatozoa, and female oocyte (reviewed in Starz-Gaiano & Lehmann 2001). PGCs are allocated from precursor cells in the proximal epiblast during gastrulation, with the first PGCs identifiable around embryonic day (E)7.2 in mice, at the base of the allantois (reviewed in Ginsburg *et al.* 1990, Lawson & Hage 1994, Tam & Zhou 1996, De Felici *et al.* 2004).

The founder population of PGC numbers is only about 45 in mice (Lawson & Hage 1994), and migrates through the hindgut to colonize the developing gonad by E11.5 (reviewed in Molyneaux & Wylie 2004). During migration, and after colonization of the gonads, PGCs are proliferative, dividing every 16 h (Tam & Snow 1981, Lawson & Hage 1994) until E13.5, at which point they enter mitotic arrest in males, or meiosis in females (McLaren 1988). The proliferative activity of germ cells expands the germ cell population to 25 000 at E13.5 (Tam & Snow 1981). Coincident with meiotic entrance, germ cell attrition, the apoptotic death of oocytes, begins (reviewed in Reynaud &

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ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-06-0270
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Driancourt 2000). Germ cell death is first observed around E13.5–15.5 in the mouse (Coucouvanis *et al.* 1993, Ratts *et al.* 1995). Attrition leads to a continuous loss of oocytes throughout fetal development and into early neonatal life, resulting in a loss of 65% of germ cells between E13.5 and birth in mice (McClellan *et al.* 2003).

Once PGCs have colonized the gonad midway through gestation, they assume a nonmotile morphology and very closely associate with one another in discrete clusters that resemble *Drosophila* germ cell cysts or nests (GCN; Gomperts *et al.* 1994, Pepling & Spradling 1998, Molyneaux *et al.* 2004). GCN are present in mice by E10.5, and are comprised of a group of interconnected, synchronously dividing germ cells, and surrounded by a layer of somatic cells (Pepling & Spradling 1998, 2001). GCNs begin to break down shortly after birth to facilitate primordial follicle formation (Pepling & Spradling 2001). GCN breakdown appears to occur due to oocyte death within the GCN, which serves to break the connections between oocytes and permits somatic cells to envelop surviving oocytes, thus forming primordial follicles (Pepling & Spradling 2001). In mice, primordial follicle formation is completed by PN day 7 (Pepling & Spradling 2001).

Germ cell death is a prominent feature of follicular endowment and its proper regulation is essential for the endowment of a sufficient follicle population to sustain the fertility of the female. Studies have demonstrated that mutations in apoptosis regulatory genes lead to the dysregulation of follicular endowment, and the presence of a follicle surfeit or deficiency in neonatal ovaries. For example, deletion of the proapoptotic caspase 2 leads to excess follicles in neonatal ovaries (Bergeron *et al.* 1998). Similarly, deletion of acid sphingomyelinase (*Smpd1*), which produces the death-inducing second messenger ceramide through membrane hydrolysis (reviewed in Gulbins 2003), enhances follicular endowment (Morita *et al.* 2000). Deletion of various members of the B-cell lymphoma/leukemia (*Bcl*)-*2* family have been shown to produce similar alterations in follicle numbers in neonates. For example, loss of function of the anti-apoptotic protein BCL-XL leads to follicle deficiency in neonatal ovaries due to decreased oocyte survival in embryonic life (Rucker *et al.* 2000). Similarly, deletion of the anti-apoptotic *Bcl-2* reduces follicular endowment (Ratts *et al.* 1995), whereas overexpression of *Bcl-2* enhances follicular endowment (Flaws *et al.* 2001), presumably due to alterations in oocyte viability (Flaws *et al.* 2006).

In contrast to the effect observed on primordial follicle numbers following the deletion of these anti-apoptotic BCL-2 family members, deletion of the proapoptotic BCL-2 family member *Bax* was not observed to affect follicle numbers in neonatal mice, suggesting that it is not involved in regulating follicular endowment (Perez *et al.* 1999). A lack of effect of *Bax* deletion on oocyte survival and follicle numbers is very surprising given the findings of more recent studies. For example, De Felici *et al.* (1999) showed that BAX is upregulated in ovaries during germ cell attrition and in cultured fetal oocytes undergoing apoptosis. Further, deletion of *Bax* rescues loss of BCL-XL function, restoring follicle numbers to wild-type (WT) levels (Rucker *et al.* 2000). It was also shown that BAX is involved in promoting the death of ectopic germ cells that fail to correctly migrate to the gonads (Stallock *et al.* 2003). These latter studies suggest that BAX is a regulator of germ cell death during follicular endowment. The contradiction between these studies and that of Perez *et al.* (1999) is puzzling, and could suggest that although BAX appears to be involved in regulating oocyte death during follicular endowment, it is not essential. Alternatively, it could be that BAX is involved in regulating follicular endowment, but the data presented by Perez *et al.* (1999) do not allow this interpretation. In the study by Perez *et al.* (1999), follicle numbers at PN4 were compared, and the number of naked oocytes (i.e., those remaining within GCN that have yet to be incorporated into follicles) were not presented. As follicle formation is not completed before PN7 (Pepling & Spradling 2001), PN4 is too early a time point to assess the effect of *Bax* deletion on follicle formation without presenting the total number of oocytes present in the ovaries.

Due to the contradictory data reported in the studies discussed earlier and the equivocal nature of the role of BAX during follicular endowment, we investigated the effect of *Bax* deletion throughout embryonic and neonatal ovarian development. Specifically, we tested the hypothesis that BAX negatively regulates follicular endowment by promoting oocyte death during germ cell attrition and GCN breakdown.

Materials and Methods

Animals and treatment

WT and *Bax*−/− mice in a C57BL/6 background were used in all experiments. For experiments involving embryos, timed matings were established and the morning of the observation of a vaginal plug was designated as E0.5. The day of birth was considered to be PN0. 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 9 μl 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:

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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 and identified by gel electrophoresis. Only homozygous WT and *Bax*−/− mice were used in each experiment.

Identification of PGCs

Whole embryos were collected at E8.5 and were genotyped using yolk sac tissue. Embryos were fixed in 4% paraformaldehyde for 2 h at RT, after which they were rinsed thrice in PBS and incubated in 70% ethanol for at least 1 h at RT. After rinsing in PBS, alkaline phosphatase activity within embryos was detected by exposing them to a staining solution containing 0.5 mg/ml Fast Red TR (Sigma), 10 μg/ml α-napthyl phosphate (Sigma), 10% $MgCl_2$, and 4.5% borax in water, for 7–10 min. Staining was stopped by rinsing embryos in PBS, and they were then stored in 70% glycerol in PBS. Embryos were mounted on glass slides and PGCs (identified by dark red staining) were counted, and compared between WT and *Bax*−/− embryos.

Follicle counts

To assess naked oocyte and follicle numbers at E13.5, E15.5, PN4, and PN7, ovaries were collected and fixed in Kahle's solution (4% formalin, 28% ethanol, and 0.34 M glacial acetic acid) for at least 24 h. Females were distinguished from males based on the absence of the testes-specific coelomic vessel, which forms by E12.5 (Brennan *et al.* 2002). Following fixation, ovaries were dehydrated and embedded in Paraplast (VWR International, West Chester, PA, USA). Ovaries were serially sectioned at 8 μm intervals, mounted on glass slides, stained with Weigert's hematoxylin–picric acid methyl blue, and mounted with Permaslip (Alban Scientific Inc, St Louis, MO, USA). In every fifth section in embryonic ovaries or tenth section in neonatal ovaries, the numbers of naked oocytes, primordial, primary, and preantral follicles were counted. Only follicles containing a visible nucleus were counted to avoid double counting and all counting was done without the knowledge of genotype. Oocytes were counted as naked if they were present in clusters of at least two oocytes with an absence of intervening somatic cells. Follicles were counted as primordial if they contained an oocyte surrounded by flattened granulosa cells, or a mixture of less than seven flattened and cuboidal granulosa cells. 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. Follicle counts are reported as the raw number counted per ovary without a correction factor applied.

Whole-mount immunohistochemistry (wmIHC)

Ovaries were collected on the morning of E12.5, PN4, and PN7 and washed twice briefly in PT (0.1% Triton X-100 in PBS) and once for at least 30 min. Ovaries were then incubated in PT+5% BSA or heat-inactivated horse serum (hiHS), for 30–60 min, before being incubated with primary antibody diluted in PT with or without 5% BSA overnight at 4 °C (anti-phosphohistone H3 (Ser28); 1:100; Upstate Cell Signaling Solutions, Charlottesville, VA, USA) to identify mitotic cells (Gurley *et al.* 1975, Atchison *et al.* 2003), and anti-mouse CD31 platelet endothelial cell adhesion molecule (PECAM-1; 1:50; BD Biosciences Pharmingen, San Jose, CA, USA), and anti-STAT3 (C20; 1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) to identify germ cells). Ovaries were then washed in PT+1% BSA, or hiHS, for 30 min, and treated with RNase A for 30 min. DNA was labeled with propidium iodide or TOTO-3 (Invitrogen Molecular Probes) for 20 min, and the ovaries were washed in PT+1% BSA, or hiHS, for 30 min. Fluorescein isothiocyanate (FITC) conjugated secondary antibody (Invitrogen Molecular Probes) was diluted in the ratio of 1:250 in PT+5% BSA, or hiHS, and incubated with the ovaries for 2 h at RT. Ovaries were then washed thrice in PT+1% BSA, or hiHS, for 30 min, then briefly with PBS before being mounted in either Vectashield (Vector Laboratories, Burlingame CA) or Prolong Antifade Kit (Invitrogen Molecular Probes) with Secure-seal imaging spacers (Sigma). Confocal analysis was performed by a Zeiss LSM410 laser-scanning microscope. Images were taken near the surface of the ovary, and then 20 μm into the ovary at 63×. At least four images were recorded for each ovary. A phosphohistone H3-labeling index was determined by dividing the number of labeled germ cells by the total number of germ cells. GCN breakdown was assessed at PN4 and PN7 as described in Pepling & Spradling (2001). Briefly, the number of single oocytes (i.e., those separated from the GCN and incorporated into follicles) relative to the number of oocytes in nests was determined using confocal stacks from 12 to 20 different ovaries. Anti-STAT3 was used as a marker for germ cells in these assays, because it specifically labels the cytoplasm of germ cells and appears continuous when cells are present as nests (Murphy *et al.* 2005).

Assessment of BrdU incorporation

To assess 5-bromo-2-deoxyuridine (BrdU) incorporation, we used the technique of Schmahl *et al.* (2000) with minor modifications. Pregnant mice received a single

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i.p. injection of BrdU (50 mg/kg body weight) on the morning of E12.5, and embryos were collected 2 h later. Ovaries were isolated from embryos and fixed in 5.3% formaldehyde in PBS overnight at 4 °C, after which they were washed thrice in PBS. Ovaries were rinsed in 0.6% Triton X-100 in PBS and then in reaction buffer (0.1 M Tris (pH 7.5), 50 mM NaCl, and 10 mM $MgCl_2$) thrice for 3 min, and then incubated for 1 h at 37 °C in 50 U/ml DNase I (Roche Diagnostic Corporation). Ovaries were then rinsed in 0.6% Triton X-100 in PBS and incubated with 5% BSA in PT for 1 h at RT. Ovaries were incubated with primary antibodies overnight at 4 °C (anti-BrdU (1:20; BD Biosciences), anti-VASA (1:500), a germ cell marker that was a gift from Toshiaki Noce). The second day followed as described earlier in the wmIHC section.

3′-End labeling

3′-End labeling analysis of apoptosis within ovaries at E15.5 was performed by combining at least 20 ovaries (ten embryos) from each genotype. Ovaries were collected and frozen at −70 °C until use. Ovarian DNA was isolated using the DNeasy kit (Qiagen). In each reaction, 0.1–1 μg 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 drying and were subjected to scintillation counting. At least three separate labeling experiments were performed for each genotype from the same grouped DNA.

Transferase dUTP nick end labeling (TUNEL) assay

Ovaries were collected and fixed in Kahle's solution, dehydrated, embedded in Paraplast, serially sectioned at 8 μm intervals, and mounted on glass slides. Sections were subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (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. For comparison of numbers of apoptotic oocytes between genotypes in neonatal ovaries, eight serial sections per ovary were subjected to TUNEL, and the number of labeled oocytes was counted in every second section. Only one ovary per animal was subjected to TUNEL analysis, with at least three ovaries per genotype. The number of apoptotic oocytes was determined without knowledge of genotype.

Immunohistochemistry

Embryonic ovaries were collected and fixed in 4% paraformaldehyde for 2 h at RT, after which they were rinsed in PBS, dehydrated, embedded in Paraplast, serially sectioned at 8 μm intervals, and mounted on glass slides. Sections were deparaffinized and rehydrated to water. Antigen retrieval was performed in boiling citrate buffer (10 mM) for 5 min. Slides were washed thrice in PBS, incubated in a 3% H_2O_2 solution in PBS for 10 min and washed thrice in PBS. Sections were blocked with 5% hiHS for 1 h, after which endogenous biotin sites were blocked using an avidin–biotin blocking kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. Slides were washed thrice in PBS and then incubated with primary antibody diluted in the ratio of 1:100 in PBS for 48 h at 4 °C. Anti-SCP3 (synaptonemal complex protein 3) and anti-DMC1 were generous gifts of Dr Chris Ottolenghi at the National Institute on Aging. After incubation with primary antibodies, slides were washed thrice in PBS, and then incubated with appropriate biotin-conjugated secondary antibodies diluted in the ratio of 1:250 in PBS for 1 h at RT. Slides were washed thrice in PBS, and then incubated with the Vectastain Elite ABC reagent (Vector Laboratories) for 1 h at RT. Slides were washed thrice in Tris buffer (pH 7.2), and then incubated in a 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) solution in Tris buffer for 5–10 min at RT. Slides were then washed thrice in Tris buffer, thrice in PBS, counterstained with picric acid–methyl blue, and dehydrated and mounted in Permaslip.

Statistical analysis

PGC, oocyte, and follicle numbers between WT and *Bax*−/− ovaries were compared using Student's *t*-test. GCN breakdown and proliferation data were compared using the Mann–Whitney test. Data for *Bax*−/− ovaries from 3′-end labeling were normalized to WT values and the data were compared using a one-way *t*-test. *P* values <0.05 were considered to be statistically significant.

Results

Effect of Bax *deletion on follicular endowment*

Morphological assessment of oocyte and follicle numbers demonstrated that deletion of *Bax* increases follicular endowment in mouse ovaries. At all time points between E15.5 and PN7, there were significantly more oocytes and follicles in *Bax*−/− when compared with WT ovaries (Fig. 1). At E15.5, 4675.2±216.6 oocytes were counted in *Bax*−/− ovaries compared with 3650.8±385.7 oocytes in WT ovaries ($n=4$ WT ovaries, $n=5$ *Bax*−/− ovaries; $P<0.05$; Fig. 1A).

Figure 1B shows the results of follicle counts in PN4 ovaries. There were significantly more naked oocytes

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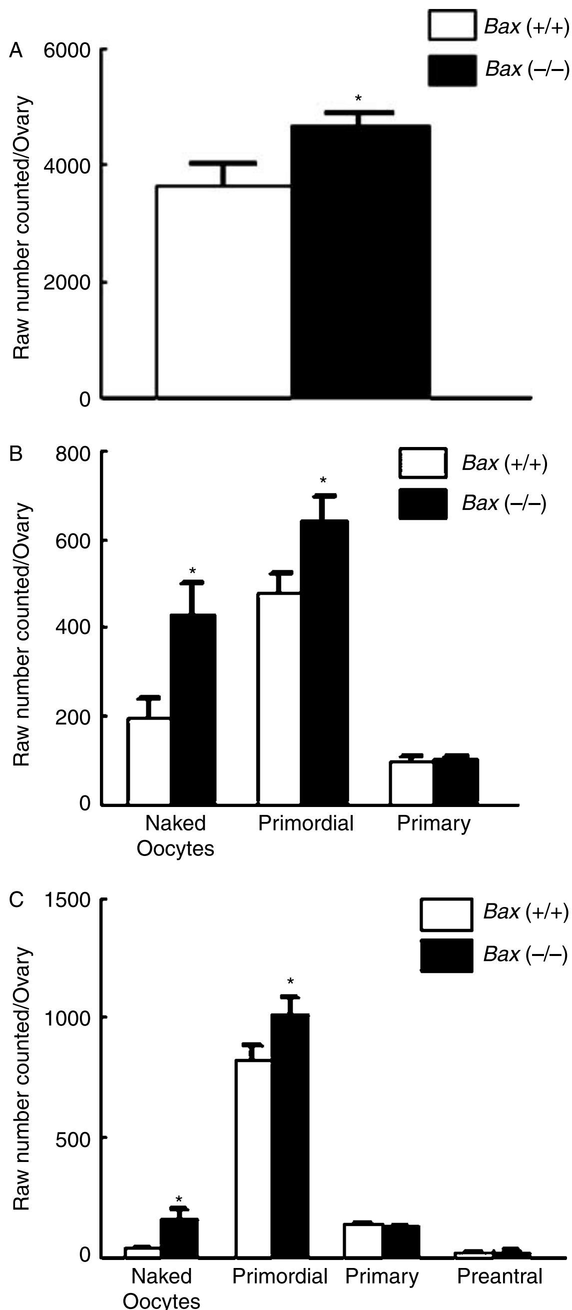

Figure 1 Effect of *Bax* deletion on follicular endowment as assessed by oocyte and follicle counts in WT and *Bax−/−* ovaries. (A) E15.5 ($n=4$ WT ovaries, $n=5$ *Bax−/−* ovaries; $*P<0.05$). (B) PN4 ($n=7$ WT ovaries, $n=7$ *Bax−/−* ovaries; $*P<0.05$). (C) PN7 ($n=6$ WT ovaries, $n=8$ *Bax−/−* ovaries; $*P<0.05$). Bars represent the mean±S.E.M. of the raw number counted per ovary.

counted in *Bax−/−* ovaries (431.1±69.5) when compared with WT ovaries (192.6±43.63; $n=7$ WT ovaries, $n=7$ *Bax−/−* ovaries; $P<0.05$). Similarly, there were significantly more primordial follicles counted in *Bax−/−* ovaries (642.1±56.53) when compared with WT ovaries (477.6±43.04; $P<0.05$). No differences were seen in primary follicle numbers ($P=0.73$).

Figure 1C shows naked oocyte and follicle numbers in ovaries from PN7 mice. Again, the number of naked oocytes counted in *Bax−/−* ovaries was significantly greater compared with WT ovaries. There were 161.3±37.7 naked oocytes counted in *Bax−/−* ovaries compared with 35.0±4.0 in WT ovaries ($n=6$ WT ovaries, $n=8$ *Bax−/−* ovaries; $P<0.05$). Similarly, there were significantly more primordial follicles counted in *Bax−/−* ovaries (1019.4±61.1) at PN7 when compared with WT ovaries (827.2±60.0; $P<0.05$). No differences were seen in the number of primary or preantral follicle numbers between WT and *Bax−/−* ovaries ($P=0.39$).

Effect of Bax *deletion on GCN breakdown*

Figure 2A and B shows the dramatic differences in the morphology of *Bax−/−* and WT ovaries at PN4, in terms of naked oocytes within GCN. In the WT ovary (Fig. 2A), GCNs persisted but appeared small. In contrast, the *Bax−/−* ovary (Fig. 2B) contained large GCNs throughout its cortex. Based on these morphological differences, together with the fact that we observed significantly more naked oocytes in *Bax−/−* when compared with WT ovaries at both PN4 and PN7, we investigated whether *Bax* deletion impacted GCN breakdown. Using confocal analysis, we observed that GCN breakdown appeared to be delayed in the absence of *Bax*, such that 38.9±3.1% of oocytes in WT ovaries at PN4 had been incorporated into follicles, while only 16.9±2.5% had been incorporated into follicles in *Bax−/−* ovaries ($n=12$ WT ovaries, $n=20$ *Bax−/−* ovaries; $P<0.001$; Fig. 2C). Similarly, at PN7, a larger, but not statistically significant, percentage of oocytes in WT ovaries (55.9±5.0%) had been incorporated into follicles when compared with *Bax−/−* ovaries (45.6±2.4%; $n=18$ WT ovaries, $n=20$ *Bax−/−* ovaries; $P=0.14$; Fig. 2C). Representative confocal images of WT and *Bax−/−* ovaries at PN4 are shown in Fig. 2D and E respectively.

Effect of Bax *deletion on oocyte death*

As GCN breakdown is thought to occur by oocyte death (Pepling & Spradling 2001), we hypothesized that *Bax* deletion reduces oocyte death in neonatal ovaries resulting in delayed GCN breakdown. To test this hypothesis, we performed TUNEL assays on neonatal *Bax−/−* and WT ovaries (Fig. 3). In contrast to our hypothesis, more apoptotic oocytes were observed in *Bax−/−* ovaries compared with WT ovaries at both PN1 and PN4. At PN1, *Bax−/−* ovaries contained 32.9±7.5 TUNEL-positive oocytes per section compared 14.1±3.1 TUNEL-positive oocytes per section in WT ovaries ($n=6$ WT ovaries, $n=4$ *Bax−/−* ovaries; $P<0.05$; Fig. 3A). Similarly, at PN4, *Bax−/−* ovaries contained 21.13±3.17 TUNEL-positive oocytes per section when compared with 7.5±1.76 per

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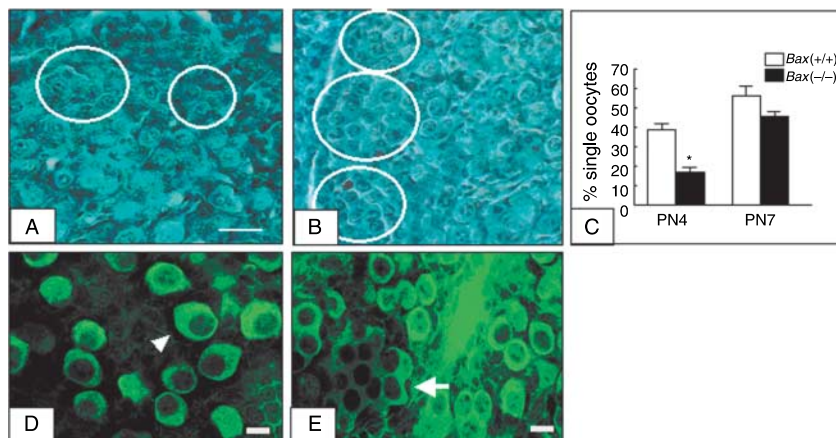

Figure 2 Germ cell nest breakdown. Representative photomicrographs at PN4 for (A) WT and (B) *Bax*−/− ovaries. GCN are encircled in white. Magnification, 40×. Scale bar, 50 µm. (C) The percentage of single oocytes in WT and *Bax*−/− ovaries at PN4 (n=12 WT ovaries, n=20 *Bax*−/− ovaries; *P<0.001) and PN7 (n=18 WT ovaries, n=20 *Bax*−/− ovaries; P=0.14). Bars represent mean ± S.E.M. Representative confocal image of a (D) WT and (E) *Bax*−/− ovary at PN4 labeled with anti-Stat3 used to calculate the percentage of single oocytes shown in C. The arrow points to an intact germ cell nest. The arrowhead points to a single oocyte. Magnification, 63×. Scale bar, 20 µm.

section in WT ovaries (n=3 WT ovaries, n=4 *Bax*−/− ovaries; P<0.05; Fig. 3B). Representative TUNEL sections are shown in Fig. 3C and D.

These findings suggest that differences in GCN breakdown and follicle numbers in neonatal *Bax*−/− when compared with WT ovaries are not due to enhanced oocyte survival during follicle formation. Therefore, we next tested the hypothesis that increased follicle numbers in *Bax*−/− mice are due to enhanced oocyte survival during germ cell attrition, by performing 3′-end labeling reactions at E15.5. As shown in Fig. 4A, more apoptosis was observed in *Bax*−/− ovaries when compared with WT ovaries. Scintillation counts of radio-labeled DNA revealed that *Bax*−/− ovaries contained 232.4±9.6%, the labeling seen in WT ovaries (P<0.001; Fig. 4B). At E18.5, there was no difference in the amount of labeling (*Bax*−/− 134.02+28.4% of WT; P=0.35; Fig. 4C). TUNEL analysis showed that the primary cell type undergoing apoptosis at E15.5 is oocytes (Fig. 4D), suggesting that like in neonatal ovaries *Bax* deficiency does not enhance oocyte survival.

Effect of Bax *deletion on PGC allocation*

As enhanced follicular endowment due to *Bax* deletion is not due to decreased oocyte death, we tested several further hypotheses to explain it. First, we tested whether *Bax* deletion increases the size of the initial founding population of PGCs. Figure 5A and B shows representative photos of PGCs in WT and *Bax*−/− embryos respectively. As shown in Fig. 5C, *Bax* deletion did not increase the size of the initial PGC allocation (WT=65.3+3.6, *Bax*−/−=56.3±4.2 PGCs; n=11 WT embryos, n=12 *Bax*−/− embryos; P=0.13).

Effect of Bax *deletion on the onset of meiosis*

We next tested the hypothesis that *Bax* deletion delays the onset of meiosis by examining the expression of two markers of meiosis. We first performed PCR analysis of *Scp3* gene expression at E13.5–15.5. As shown in Fig. 6A, the *Scp3* gene was appropriately expressed in *Bax*−/− when compared with WT ovaries. Next, we examined whether SCP3 and DMC1 protein were appropriately expressed in *Bax*−/− when compared with WT ovaries. As shown in Fig. 6C–F, SCP3 and DMC1 are appropriately expressed in *Bax*−/− when compared with WT ovaries.

Effect of Bax *deletion on oogonia proliferation*

Finally, we tested the hypothesis that *Bax* deletion increases germ cell proliferation. For this hypothesis to have validity, there would have to be differences in germ

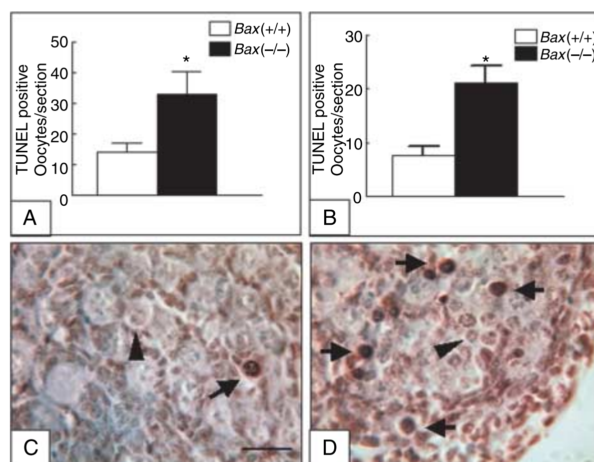

Figure 3 Effect of *Bax* deletion on oocyte death in neonatal ovaries as assessed by TUNEL analysis. (A) Number of TUNEL-positive oocytes per section in ovaries at PN1 (n=6 WT ovaries, n=4 *Bax*−/− ovaries; *P<0.05). (B) Number of TUNEL-positive oocytes per section in ovaries at PN4 (n=3 WT ovaries, n=4 *Bax*−/− ovaries; *P<0.05). Bars represent mean ± S.E.M. Representative photomicrographs of PN1 ovaries subjected to TUNEL analysis from (C) WT pups and (D) *Bax*−/− pups. Arrows point to TUNEL-positive oocytes, arrowheads point to healthy oocytes. Magnification, 63×. Scale bar, 30 µm.

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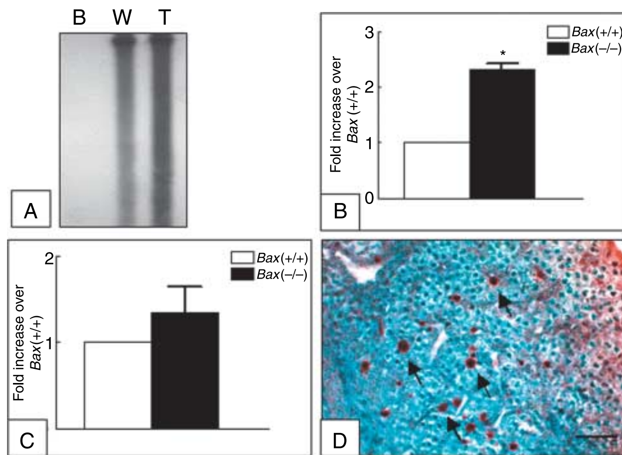

Figure 4 Effect of *Bax* deletion on germ cell attrition as assessed by 3′-end labeling reactions of DNA isolated from WT and *Bax*−/− ovaries. (A) Representative gel from a reaction using DNA isolated from ovaries at E15.5 (B, blank; W, WT; and T, *Bax*−/−). Fold increase of *Bax*−/− labeling over WT at (B) E15.5 and (C) E18.5 (DNA from at least 20 grouped ovaries per genotype were used in each reaction). Bars represent mean ± S.E.M. *$P<0.001$. (D) TUNEL assay of ovaries from E15.5 demonstrated that oocytes were the primary cell type undergoing apoptosis. Arrows point to TUNEL positive oocytes. Magnification, 40×. Scale bar, 50 μm.

cell number between *Bax*−/− and WT ovaries at the completion of the proliferative period. This is so, as at E13.5, there were significantly more germ cells in *Bax*−/− (1413.8 ± 61.6) when compared with WT (1122.8 ± 117.9) ovaries ($n=4$ WT ovaries, $n=8$ *Bax*−/− ovaries; $P<0.05$; Fig. 7).

Next, to test whether oogonia were more proliferative in *Bax*−/− when compared with WT ovaries, we compared the percentage of oogonia that had incorporated BrdU, as well as the percentage of oogonia that labeled with phosphohistone H3 at E12.5. As shown in Fig. 8A, there was no difference in BrdU incorporation between *Bax*−/− ovaries (50.58 ± 3.1%) when compared with WT ovaries (50.73 ± 3.2%; $n=11$ WT ovaries, $n=6$ *Bax*−/− ovaries; $P=0.96$). Similarly, as shown in Fig. 8B, there was no difference in the percentage of oogonia that labeled with phosphohistone H3 (WT = 1.03 ± 0.26%, *Bax*−/− = 1.49 ± 0.32%; $n=10$ WT ovaries, $n=12$ *Bax*−/− ovaries; $P=0.28$). Representative confocal images are shown in Fig. 8C–E.

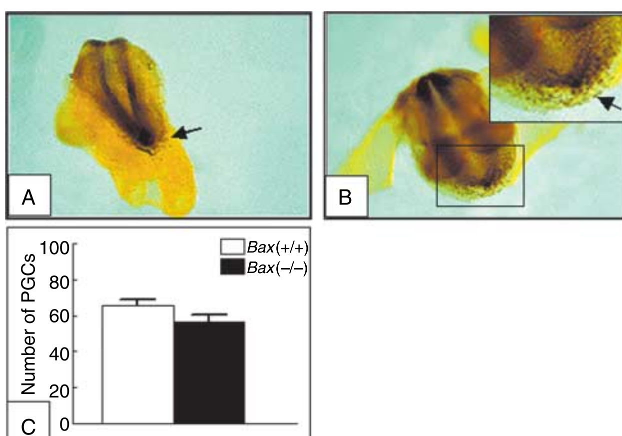

Figure 5 Effect of *Bax* deletion on germ cell allocation as assessed by primordial germ cell numbers at E8.5. Representative embryos for (A) WT and (B) *Bax*−/−. Region of the embryo containing PGCs is highlighted by the arrow. The inset in B is an enlargement of the area within the square with PGCs being the small red-staining cells highlighted by the arrow. (C) Mean number of PGCs per embryo ($n=11$ WT embryos, $n=12$ *Bax*−/− embryos; $P=0.13$). Bars represent the mean ± S.E.M.

Discussion

Perez *et al.* (1999) reported that *Bax* deletion does not affect follicular endowment. We sought to revisit the question of *Bax*'s involvement in follicular endowment in part, because Perez *et al.* (1999) made their assessment of the effect of *Bax* deletion on follicle numbers at PN4, too early a time point as follicle formation is not completed before PN7 (Pepling & Spradling 2001). Additionally, work subsequent to Perez *et al.* (1999) is highly suggestive of a role for *Bax* in regulating follicular endowment. Specifically, studies have shown that it is upregulated during germ cell attrition, promotes the death of ectopic PGCs, and corrects the BCL-XL loss of function phenotype, equilibrating oocyte death to WT levels (De Felici *et al.* 1999, Rucker *et al.* 2000, Stallock *et al.* 2003). Thus, we undertook these studies due to the equivocal role for BAX in regulating follicular endowment. In this report, we go beyond these earlier studies and clearly demonstrate that BAX is involved in follicular endowment. This was demonstrated by the fact that there is a surfeit of oocytes and follicles in *Bax*−/− ovaries when compared with WT ovaries at various times before and near the completion of primordial follicle formation.

Our findings contradict those of Perez *et al.* (1999) with regard to the effect of *Bax* deletion on follicle numbers at PN4, in which we observed an increased number of primordial follicles in *Bax*−/− ovaries when compared with WT ovaries at this time point, whereas they did not observe any difference in follicle numbers between *Bax*−/− and WT mice at this time point. The reason for this discrepancy is not clear. One possibility is the way in which primordial follicles were classified. We classified a follicle as primordial even if it had a mixture of squamous and cuboidal granulosa cells, despite the fact these follicles have traditionally been regarded as transitioning into primary follicles. We classified these follicles as primordial based on a study by Meredith *et al.* (2000) in which they demonstrated that these 'transitional' follicles are actually primordial follicles. Discrepancy between our study and Perez *et al.* (1999)

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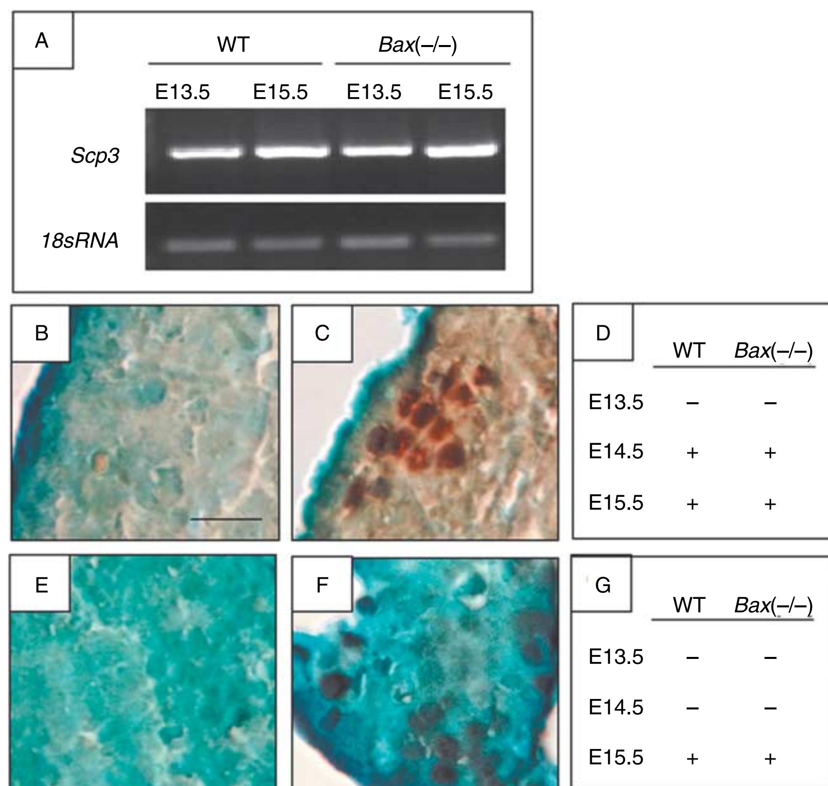

Figure 6 Effect of *Bax* deletion on the onset of meiosis as assessed by the timing of expression of meiotic markers in embryonic WT and *Bax*−/− ovaries. (A) PCR analysis of *Scp3* gene expression in ovaries at E13.5 and E15.5. (B–D) IHC analysis of SCP3 protein expression in embryonic ovaries. (B) Control IHC. (C) Representative image of SCP3-positive oocytes. (D) Table depicting the onset of SCP3 expression in embryonic ovaries. (E–G) IHC analysis of DMC1 expression in embryonic ovaries. (E) Control IHC. (F) Representative image of DMC1-positive oocytes. (G) Table depicting the onset of DMC1 expression in embryonic ovaries. Magnification, 63×. Scale bar, 30 μm.

could potentially be due then to differential classification of these follicles.

Though we saw increased primordial follicle numbers in *Bax*−/− ovaries, we also saw many more naked oocytes remaining within GCNs. GCN breakdown is thought to be due to apoptosis of oocytes within the nest (Pepling & Spradling 2001). Since we observed significantly more naked oocytes remaining within GCNs in *Bax*−/− when compared with WT ovaries, and BAX is a proapoptotic protein, we hypothesized that *Bax* deletion decreased oocyte death, and delayed the onset or slowed the process of GCN breakdown. In contrast to our hypothesis, we actually observed more oocytes dying in *Bax*−/− ovaries than in WT ovaries. There was no delay in the onset of GCN breakdown in *Bax*−/− ovaries as evidenced by the fact that more oocytes were dying at PN1 when compared with WTs. While it is possible that since only four sections per ovary were subjected to TUNEL analysis that the difference in numbers of dying oocytes could be due to regional differences in oocyte death within the ovary, we tried to use sections collected at similar depths into the ovary, and oocyte death within each ovary appeared to be fairly uniform. Therefore, the difference likely represents a real difference between the genotypes rather than differences based on region within the ovary. The fact that more oocytes were dying in the mutant ovaries is difficult to reconcile with the fact that there was a significantly smaller percentage of single oocytes present in them when compared with WTs (Fig. 2C). It is possible that since there were more oocytes in the *Bax*−/− ovaries, the increased number of oocytes that were dying represents a similar proportion of apoptotic oocytes when compared with WT. A rough calculation, dividing the mean number of TUNEL-positive oocytes per section at PN4 by the mean number of naked oocytes at PN4, shows that this is the case, with 4.9% dying in *Bax*−/− and 3.9% dying in WTs. The smaller percentage of single oocytes seen in *Bax*−/− ovaries could be due to the fact that the GCNs in these ovaries

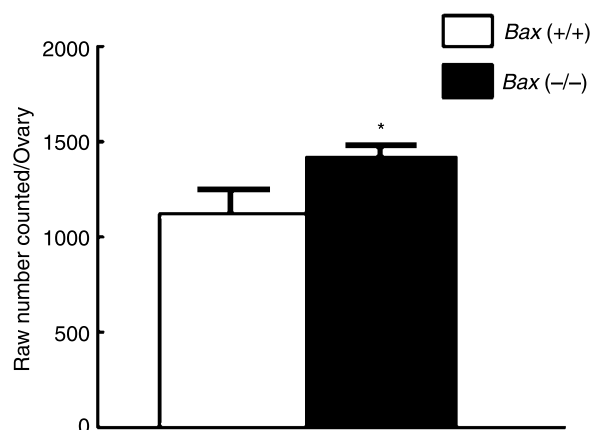

Figure 7 Effect of *Bax* deletion on germ cell numbers at E13.5 ($n=4$ WT ovaries, $n=8$ *Bax*−/− ovaries; $^*P<0.05$). Bars represent mean ± s.e.m. of the raw number of germ cells counted per ovary.

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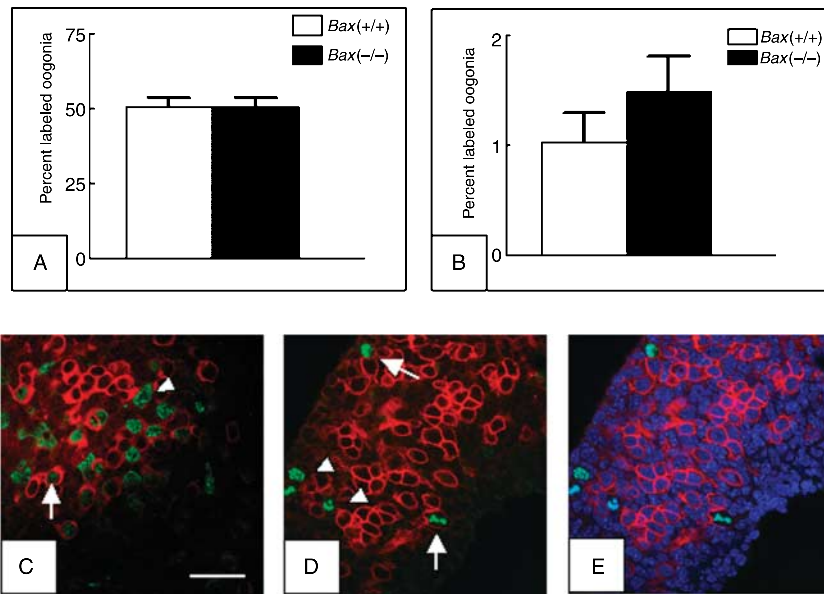

Figure 8 Effect of *Bax* deletion on cellular proliferation as assessed by wmIHC analysis of BrdU and phosphohistone H3 labeling at E12.5. (A) The BrdU labeling index in WT and *Bax*−/− ovaries (n=11 WT ovaries, n=6 *Bax*−/− ovaries; P=0.96). (B) The phosphohistone H3-labeling index in WT and *Bax*−/− ovaries (n=10 WT ovaries, n=12 Bax−/− ovaries; P=0.28). Bars represent mean±S.E.M. (C) Representative confocal image a WT ovary labeled with anti-BrdU (green) and anti-VASA (red). (D) Representative confocal image of a WT ovary labeled with anti-phosphohistone H3 (green) and anti-PECAM (red). (E) The same image as in D but with the nuclear marker TOTO-3 (blue). Arrows point to labeled germ cells, and arrowheads point to labeled somatic cells. Magnification, 63×. Scale bar, 30 μm.

appeared to be much larger than those in their WT counterparts (Fig. 2A and B). Thus, though there are more oocytes undergoing apoptosis within GCNs in *Bax*−/− ovaries, due to their larger size they may take longer time to fully undergo breakdown.

The fact that we saw a similar proportion of oocytes dying following *Bax* deletion when compared with WT ovaries suggests that BAX is dispensable for oocyte death in the neonate during GCN breakdown and follicle formation. It is likely that other pro-apoptotic Bcl-2 family members compensate for the absence of BAX to promote oocyte death. Specifically, BAK has been shown in a variety of cell types to have a redundant function with BAX, and in some cell types the deletion of both factors is required to affect the cell's ability to undergo 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 oocyte death in the neonate and facilitate GCN breakdown and follicle formation.

Since a reduction in the amount of oocyte death occurring in neonatal *Bax*−/− ovaries could not explain the observed increased follicular endowment, we investigated whether a reduction in oocyte death during germ cell attrition in the embryonic ovary could explain it. This notion is supported by several studies that demonstrate an involvement of BAX in oocyte death at this time. Specifically, De Felici *et al.* (1999) showed that BAX is upregulated during germ cell attrition and in cultured fetal oocytes undergoing apoptosis. Further, BAX is involved, though not required, for the death of ectopic PGCs that fail to correctly migrate to the ovaries (Stallock *et al.* 2003). Deletion of *Bax* also rescues loss of BCL-XL function (Rucker *et al.* 2000). BCL-XL is an anti-apoptotic BCL-2 family member, and loss of function was shown to decrease oocyte survival beginning at E13.5. Co-deletion of *Bax* rescued this phenotype (Rucker *et al.* 2000). Despite these data supporting a role for BAX in promoting oocyte death during attrition, our data do not support the hypothesis that *Bax* deletion enhances oocyte survival during germ cell attrition. Similar to what we observed in neonatal ovaries, there was actually more death occurring in *Bax*−/− ovaries when compared with WT ovaries at E15.5. TUNEL analysis demonstrated that increased apoptosis at E15.5, evidenced by increased 3′-end labeling of isolated DNA, was due to oocyte death, rather than somatic cell death, which is consistent with other reports (De Pol *et al.* 1997, Pepling & Spradling 2001). It is notable that 3′-end labeling experiments did not yield a typical laddering pattern in the gels characteristic of apoptosis. This is a consistent phenomenon that we observe using embryonic ovaries and similar to what has been observed in other studies (Ratts *et al.* 1995). One possible explanation for this phenomenon could be that the endonuclease system is not fully matured or functional in the embryonic ovary leading to an absence of clear DNA laddering. This demonstrates that as in oocytes in the neonate, BAX is dispensable for oocyte death in the embryo. For the same reason discussed earlier, it is likely that a redundant factor, for example BAK, acts to promote oocyte death in its absence.

Interestingly, despite the fact that in our study, *Bax* deletion did not reduce oocyte death within the ovary, *Bax* deletion was previously observed to delay the death of ectopic germ cells (Stallock *et al.* 2003). This suggests that germ cell death during attrition is not an autonomous process, and that an atretogenic factor(s) produced within the ovary, and not in ectopic locations, is

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important for promoting oocyte death during attrition, seemingly utilizing a BAX-independent or redundant pathway. This may speak to the mechanism by which germ cell attrition occurs. *Bax*−/− oocytes are seen to be more viable than WT oocytes following growth factor withdrawal (De Felici *et al.* 1999, Stallock *et al.* 2003). If growth factor withdrawal were the mechanism by which germ cell attrition was initiated, then increased death at E15.5 would not be expected. Rather, it would likely be delayed in *Bax*−/− ovaries as observed in ectopic locations. Therefore, it is more likely that germ cell attrition is an active process.

Our data suggest that the regulatory role of *Bax* during follicular endowment occurs prior to germ cell attrition. Therefore, we proposed several hypotheses to explain the increased follicle numbers observed following *Bax* deletion. One hypothesis that we did not test was that *Bax* deletion enhances the survival of premeiotic oogonia, because oocyte death has largely been observed to begin only after they have entered meiosis (Coucouvanis *et al.* 1993, Ratts *et al.* 1995). Enhanced survival of germ cells in a stable population could not explain the increased number of oocytes that we observed in embryonic and PN life. Thus, the first hypothesis we tested was that *Bax* deletion alters PGC allocation. We did not see an effect of *Bax* deletion on PGC allocation. Next, we looked at whether *Bax* deletion delays the onset of meiosis. The rationale behind this hypothesis was that if there were a delay in the onset of meiosis, the proliferative period would be lengthened. Further, *Bax* deletion has been shown to lead to meiotic defects in males (Knudson *et al.* 1995). While *Bax*−/− females are fertile, and oocytes are thus able to normally progress through meiosis, that does not preclude the possibility of a more subtle effect of *Bax* deletion on meiosis in oocytes, for example, a delayed onset. That said, we did not observe a delay in the onset of meiosis in *Bax*−/− oocytes when compared with WT oocytes. Based on the expression of the markers SCP3, which is involved in the formation and stability of the synaptonemal complex produced during zygotene and pachytene of prophase I (reviewed in Champion & Hawley 2002) and DMC1, which is a germ cell-specific protein involved in DNA repair during recombination (Pittman *et al.* 1998, Yoshida *et al.* 1998), *Bax*−/− germ cells entered meiosis at an appropriate time.

Finally, we tested the hypothesis that *Bax* deletion enhances oogonia proliferation. A large amount of recent data has demonstrated a role for BCL-2 family members in regulating cellular proliferation (reviewed in Bonnefoy-Berard *et al.* 2004). For example, BCL-2 has been shown to have an inhibitory effect on cell cycle progression in a variety of cell types (Linette *et al.* 1996, Mazel *et al.* 1996, O'Reilly *et al.* 1996, Vairo *et al.* 1996, Deng *et al.* 2004). Conversely, Knudson *et al.* (2001) showed that overexpression of *Bax* enhances proliferation of thymocytes. However, our data do not support the hypothesis that *Bax* deletion increases proliferation of oogonia.

The fact that we observed significantly more germ cells present within *Bax*−/− when compared with WT ovaries at E13.5, the time at which proliferation ceases and germ cells begin to enter meiosis (McLaren 1988), and a time before the start of germ cell attrition (Coucouvanis *et al.* 1993, Ratts *et al.* 1995), is highly suggestive of a role for *Bax* in the regulation of germ cell function prior to the onset of meiosis. That oogonia proliferation was unaltered by *Bax* deletion at E12.5, and oogonia death is rare prior to meiosis (Coucouvanis *et al.* 1993, Ratts *et al.* 1995), *Bax*'s regulatory role likely takes place prior to germ cell colonization of the gonad. We further showed that *Bax* deletion does not affect the size of the initial PGC allocation. Therefore, it appears that *Bax* is an important regulator of PGC function during migration. Stallock *et al.* (2003) showed that *Bax* is expressed in migratory PGCs and therefore, it could be that *Bax* regulates either the survival or proliferation of PGCs during their migration to the developing gonad.

In conclusion, we have shown that BAX is an important negative regulator of follicular endowment in mice, such that in its absence, follicular endowment is increased. Perez *et al.* (1999) demonstrated that *Bax*−/− females have prolonged reproductive longevity due to reduced atresia of immature follicles. Our study suggests that another reason for prolonged reproductive lifespan in *Bax*−/− mice is an increased follicular endowment. The mechanism by which BAX acts to regulate follicular endowment is unclear, but we have shown that it is independent of changes in germ cell viability. This study has implications in terms of the interpretation of previous results regarding the role of apoptotic regulatory genes during follicular endowment. For example, deletion of *Bcl-2* was shown to decrease the follicular endowment (Ratts *et al.* 1995), whereas overexpression of *Bcl-2* was shown to increase it (Flaws *et al.* 2001). It was logically assumed that these results were due to altered germ cell viability during development. However, in the light of this study, it is possible that BCL-2, like BAX, may regulate follicular endowment independent of regulating germ cell apoptosis. A comparison of these studies could yield potential clues as to how these factors regulate germ cell function, and how germ cell death is regulated during development.

Acknowledgements

This work was supported by NIH 38 955, T32HD07170. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 13 October 2006
First decision 21 November 2006
Accepted 26 January 2007

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