

Prolongation of ovarian lifespan into advanced chronological age by *Bax*-deficiency

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Female mammals are endowed with a finite number of oocytes at birth, each enclosed by a single layer of somatic (granulosa) cells in a primordial follicle^{1,2}. The fate of most follicles is atretic degeneration^{1,3}, a process that culminates in near exhaustion of the oocyte reserve at approximately the fifth decade of life in women, leading to menopause^{4,5}. Apoptosis has a fundamental role in follicular atresia^{6,7}, and recent studies have shown that *Bax*, which is expressed in both granulosa cells^{8,9} and oocytes¹⁰, may be central to ovarian cell death^{6–12}. Here we show that young adult female *Bax*^{-/-} mice possess threefold more primordial follicles in their ovarian reserve than their wild-type sisters, and this surfeit of follicles is maintained in advanced chronological age, such that 20–22-month-old female *Bax*^{-/-} mice possess hundreds of follicles at all developmental stages and exhibit ovarian steroid-driven uterine hypertrophy. These observations contrast with the ovarian and uterine atrophy seen in aged wild-type female mice. Aged female *Bax*^{-/-} mice fail to become pregnant when housed with young adult males; however, metaphase II oocytes can be retrieved from, and corpora lutea form in, ovaries of aged *Bax*^{-/-} females following superovulation with exogenous gonadotropins, and some oocytes are competent for *in vitro* fertilization and early embryogenesis. Therefore, ovarian lifespan can be extended by selectively disrupting *Bax* function, but other aspects of normal reproductive performance remain defective in aged *Bax*^{-/-} female mice.

The first clue that *Bax*-deficiency led to sustained ovarian function in aged mice was provided by the findings of gross uterine hypertrophy in 20–22-month-old (days 581–640 postpartum) *Bax*^{-/-} female mice (Fig. 1*b,d,f*), compared with wild-type age-matched females (Fig. 1*a,c,e*). Subsequent morphological analyses of the ovaries of aged female *Bax*^{-/-} mice confirmed the presence of follicles at all stages of development, including numerous large estrogenic antral follicles housing intact oocytes, resembling an ovarian architecture seen in young adult female mice (Fig. 2*b–i*). In comparison, and as anticipated¹³, ovaries of aged wild-type female mice primarily possessed stromal tissue devoid of follicles (Fig. 2*a*). Corpora lutea were absent from ovaries of aged *Bax*-deficient mice (Fig. 2*b–i*). Furthermore, long-term housing of aged female *Bax*^{-/-} mice with young adult wild-type males did not yield pregnancies (data not shown), probably due to the fact that these aged mice fail to ovulate normally and do not produce corpora lutea needed for pregnancy. As young adult female *Bax*^{-/-} mice exhibit relatively normal ovarian development¹¹ and become pregnant after housing with adult wild-type males (C.M.K. and S.J.K., unpublished data), the

inability of aged female *Bax*^{-/-} mice to become pregnant by natural means is probably due to the normal age-related decline in hypothalamic-hypophyseal function known to be important in female rodent reproductive senescence⁴.

This conclusion is in agreement with the fact that aged female *Bax*^{-/-} mice could be superovulated with exogenous gonad-

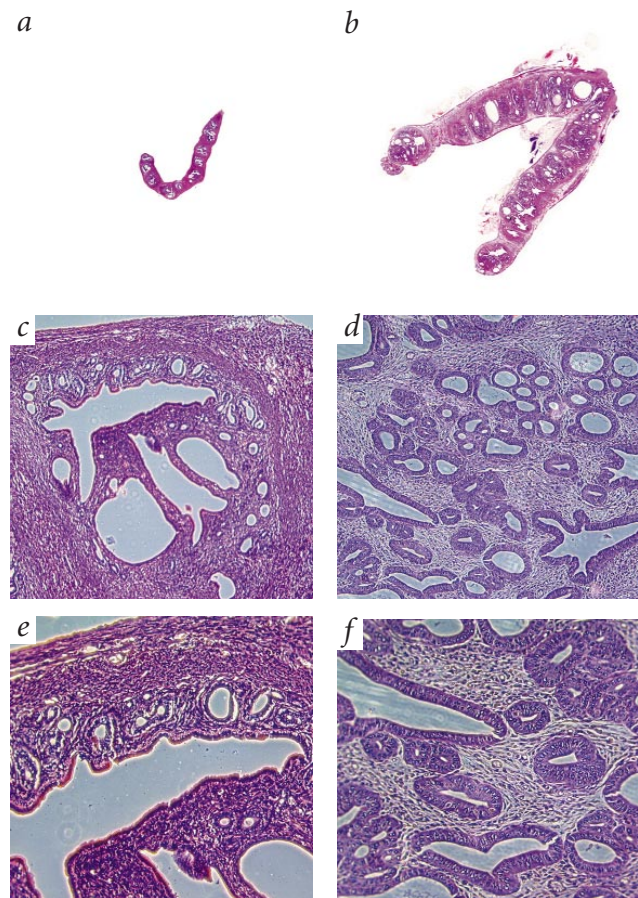
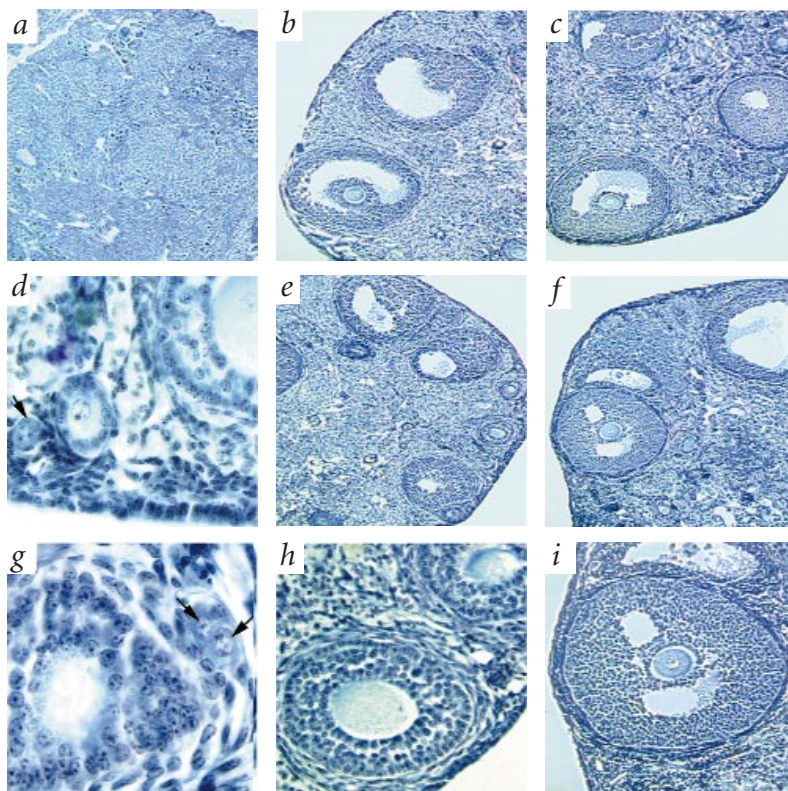


Fig. 1 Uterine histology in aged female wild-type and *Bax*^{-/-} mice. **a,c,e**, Haematoxylin and eosin staining of uterine tissues from a wild-type female mouse at 581 d, showing the expected overall atrophic nature of the tissue, decreased number of glands and shrunken epithelial cells. **b,d,f**, Comparative analysis of uterine tissues from a female *Bax*^{-/-} mouse at 640 d, showing hypertrophy of the tissue, increased numbers of glands and enlarged epithelial cells, all indicative of ovarian steroid-driven growth. Original magnifications: **a,b**, $\times 0$; **c,d**, $\times 100$; **e,f**, $\times 400$.

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Fig. 2 Ovarian histology in aged female wild-type and *Bax*^{-/-} mice. **a**, Morphology of a senescent ovary from a wild-type female mouse at 620 d, showing a gonad devoid of functional structures. **b–i**, Comparative morphology of the ovaries of *Bax*^{-/-} female mice at 620 and 640 d, revealing a maintenance of oocyte-containing follicles at all developmental stages, including mature antral (**b,c,e,f,i**), maturing preantral (**g,h**), primary and primordial (**d,g**; arrows, primordial follicles) follicles. Original magnifications: **a–c,e,f**, ×40; **i**, ×100; **h**, ×200; **d,g**, ×400.



otropin priming and were capable of forming histologically normal corpora lutea following such treatment (Fig. 3). Retrieval of ovulated oocytes from the oviducts of superovulated aged *Bax*^{-/-} females indicated that a mixture of normal (cumulus cell-enclosed) and abnormal (cumulus cell-free and/or degenerated) oocytes was present; however, the total number of oocytes retrieved was low compared with the superovulation rates obtained with young adult wild-type mice treated in parallel (Table 1). Nonetheless, *in vitro* fertilization and embryo culture of the limited number of cumulus cell-enclosed oocytes from aged *Bax*^{-/-} females revealed that some germ cells retained competency for fertilization and early embryo development (Table 1). These findings argue that at least some oocytes of aged *Bax*^{-/-} females are competent to produce embryos when assisted reproductive technologies are employed.

Morphometric analysis of the number of non-atretic follicles in serially sectioned ovaries indicated that wild-type females between 581 and 640 days of age lacked germ cells or possessed very few immature follicles per ovary (Fig. 4a). These data were

in contrast with the large numbers of primordial and growing follicles maintained in aged female *Bax*^{-/-} mice (Fig. 4a). To elucidate the basis of the sustained follicle endowment and ovarian function, we next determined if the ovarian follicle reserve in female *Bax*^{-/-} mice was larger than that observed in age-matched wild-type females at earlier ages. Morphometric analysis of non-atretic follicle numbers in wild-type and *Bax*^{-/-} mice shortly after puberty (day 42 postpartum) revealed approximately threefold greater numbers of non-atretic primordial follicles in female *Bax*^{-/-} mice compared with that of their wild-type sisters (Fig. 4b), indicating that the surfeit of follicles present in aged female *Bax*^{-/-} mice has been maintained since early adult life.

As perinatal germ cell attrition ceases at day 4 postpartum in female mice¹⁴, and primordial follicle numbers are at peak levels in postnatal life, we next examined follicle endowment in wild-type and *Bax*^{-/-} female mice shortly after birth to determine if mutants are indeed provided with a larger starting reserve of germ cells. Neonatal wild-type and female *Bax*^{-/-} mice possessed the same numbers of non-atretic primordial follicles as well as equivalent numbers of non-atretic primary follicles (Fig. 4c). Therefore, the follicle surplus noted at later ages and the extended ovarian lifespan in female *Bax*^{-/-} mice could not be attributed to a greater initial endowment of oocytes. A second possibility is that *Bax* deficiency renders granulosa cells and oocytes resistant to apoptosis during ovarian development, thereby reducing the rate of primordial and primary follicle atre-

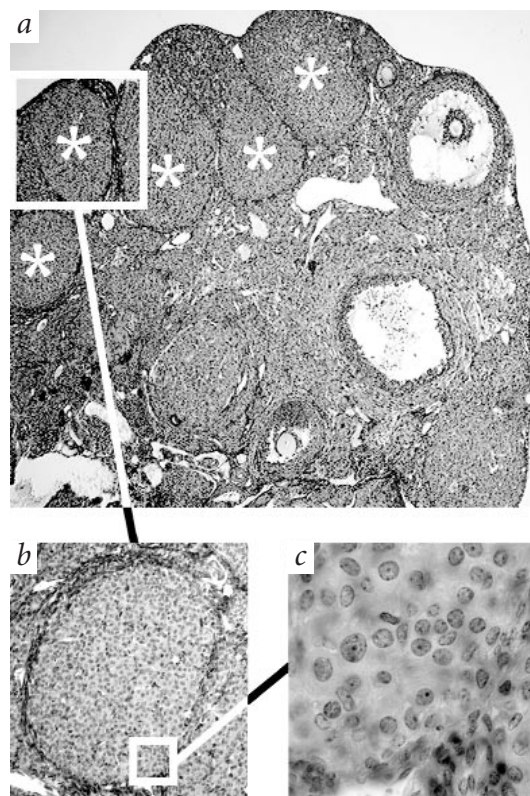


Fig. 3 Ovarian histology of aged *Bax*^{-/-} female mice following superovulation. **a**, Low-power magnification (×50) indicating the multiple corpora lutea (asterisks) present in the ovary of an aged *Bax*^{-/-} female mouse following exogenous gonadotropin priming to induce ovulation of the large antral follicles observed before hormone treatment (Fig. 2). **b,c**, Increasing magnifications of the morphology of a single corpus luteum from the ovary depicted in (a) (original magnifications: ×200 and ×1,000, respectively). These photomicrographs are representative of ovaries collected from the superovulated aged *Bax*^{-/-} female mice used to obtain oocytes for the *in vitro* fertilization and embryo development assays (Table 1).

Table 1 • *In vitro* fertilization rates and embryo development with oocytes from aged *Bax*^{-/-} mice

	n*	Total number of oocytes retrieved	Total number of COC retrieved	Number of two-cell embryos	Number of blastocysts
WT	3	90 (33, 29, 28)	80 (29, 27, 24)	58 (15, 26, 17)	31 (11, 9, 11)
<i>Bax</i> ^{-/-}	3	18 (9, 5, 4)	5 (1, 1, 3)	2 (0, 1, 1)	1 (0, 0, 1)

Data are compiled from a parallel analysis of aged (581–640 d postpartum) *Bax*^{-/-} female mice with young adult wild-type (WT) female mice as positive controls. One of the two-cell embryos obtained by *in vitro* fertilization of oocytes from aged *Bax*^{-/-} females failed to progress past this stage of development. The total number of oocytes retrieved represents both normal and abnormal oocytes, whereas the total number of COC retrieved represents normal cumulus-oocyte complexes. *The values for each endpoint per mouse are provided in parentheses.

sia in postnatal life. We confirmed this by histomorphometric analysis of the numbers of atretic follicles per ovary in age-matched (day 42 postpartum) wild-type and *Bax*^{-/-} female mice, which indicated that the incidence of atresia of primordial and primary follicles was significantly higher in wild-type compared with *Bax*^{-/-} females (Fig. 5).

Our data indicate that a single genetic manipulation, *Bax* inactivation, can produce a surplus of non-atretic primordial follicles in young adult females by maintaining survival of the oocyte-

containing follicle reserve endowed at birth. Furthermore, *Bax* inactivation extends the functional lifespan of the female gonads well into advanced chronological age, providing evidence that ovarian senescence can be delayed, if not entirely prevented. Although mice do not undergo a 'formal' menopause *per se*, female rodents do enter an extended period of reproductive acyclicity followed by complete follicle exhaustion several months before death from chronological age¹³. Moreover, elevated *Bax* levels are known to be associated with the initiation of apoptotic cell death in the human ovary⁹, in agreement with data regarding *Bax* expression and ovarian cell demise in rodent models^{8,11,12}. Consequently, *Bax*^{-/-} mice should provide a powerful genetic model to further characterize the molecular basis of female germ cell depletion associated with menopause.

Methods

Mice. Wild-type and *Bax*^{-/-} female mice (C57Bl/6) were generated by heterozygote breedings and genotyped from tail-snip genomic DNA as described¹¹. All animal work was conducted using protocols approved by Institutional Animal Care and Use Committees of the Massachusetts General Hospital and the Washington University School of Medicine.

Histology and morphometrics. Ovarian and uterine tissues were collected from wild-type and *Bax*^{-/-} female mice at the indicated ages, fixed (0.34 N glacial acetic acid, 10% formalin, 28% ethanol) and paraffin-

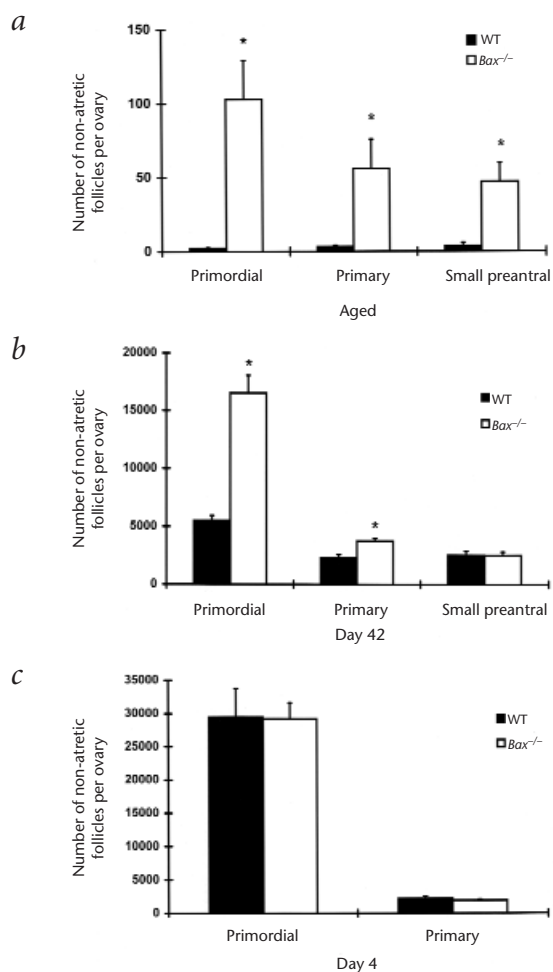


Fig. 4 Non-atretic follicle numbers in wild-type and *Bax*^{-/-} female mice throughout life. **a**, Numbers of non-atretic primordial, primary and small preantral follicles remaining in the ovaries of age-matched wild-type (WT) and *Bax*^{-/-} female mice between 581–640 d postpartum (mean±s.e.m., n=3 mice per group; **P*<0.05 versus respective wild-type value). **b**, Morphometric analysis of the numbers of non-atretic primordial, primary and small preantral follicles in wild-type and *Bax*^{-/-} female mice at 42 d postpartum (mean±s.e.m., n=3 mice per group; **P*<0.05 versus respective wild-type value). **c**, Numbers of non-atretic primordial and primary follicles in ovaries of wild-type and *Bax*^{-/-} female mice at day 4 postpartum (mean±s.e.m., n=4 mice per group).

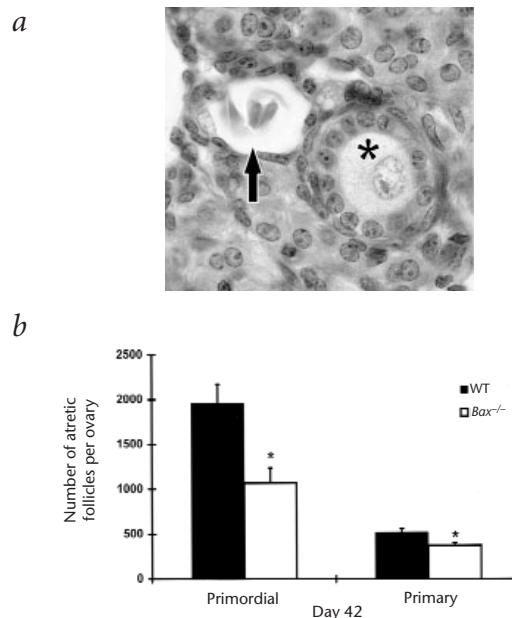


Fig. 5 Histomorphometric analysis of atretic follicles in wild-type and *Bax*^{-/-} female mice. **a**, Representative photomicrograph (original magnification, ×1,000) showing a healthy primary follicle with a normal oocyte (asterisk) lying adjacent to an atretic primordial follicle (degenerated oocyte, arrow). **b**, Numbers of atretic primordial and primary follicles present in ovaries of wild-type (WT) and *Bax*^{-/-} female mice at 42 d postpartum (mean±s.e.m., n=3 mice per group; **P*<0.05 versus respective wild-type value).

embedded. Serial ovarian sections (8 µm) were mounted in order on glass microscope slides. After staining with modified Lee's picric methyl blue, we estimated the numbers of oocyte-containing primordial, primary and small preantral follicles in a stratified randomized sampling of the entire ovary as described^{12,14,15}. On the basis of oocyte morphology^{1,2} (normal or intact, healthy follicle; degenerating or absent, atretic follicle), we determined the health status of primordial and primary follicles present in ovaries of wild-type and *Bax*^{-/-} female mice at 42 postpartum. For studies of aged female mice, we analysed every ovarian section to ensure that no follicles of any developmental stage were missed. Uterine tissues were sectioned, stained with haematoxylin and eosin and analysed by conventional light microscopy.

In vitro fertilization and embryo cultures. Young adult wild-type (positive controls) or aged *Bax*^{-/-} female mice were superovulated with a single subcutaneous injection of equine chorionic gonadotropin (10 IU; Professional Compounding Centers of America) followed by a single subcutaneous injection of human chorionic gonadotropin (Serono Laboratories) after 48 h. Ovulated oocytes were collected from the ampullae of the oviducts 16 h after hCG injection and graded as normal (cumulus cell-enclosed, metaphase II) or abnormal (cumulus cell-free or degenerated). Normal oocytes were then transferred to 50-µl drops of human tubal fluid medium (HTF; Irvine Scientific) supplemented with 0.5% (w:v) bovine

serum albumin (Fraction V, fatty acid-free; Sigma), suspended in pre-warmed mineral oil (microdrop cultures) and combined with sperm (10 µl; from a stock of 5×10⁴ capacitated epididymal sperm/ml) collected from young adult wild-type male mice. *In vitro* fertilizations were carried out for 4 h at 37 °C in a humidified incubator gassed with a mixture of 95% air and 5% CO₂, after which the oocytes were carefully removed and washed three times with fresh HTF. Embryos were then maintained *in vitro* in microdrops (25 µl) of HTF culture medium suspended in mineral oil at 37 °C for up to 96 h. Every 24 h, dishes were removed, quickly examined for progression of embryonic development by inverted light microscopy and returned to the incubator¹².

Acknowledgements

We thank I. Schiff for critical reading of the manuscript before its submission. C.M.K. was supported as a Pfizer Postdoctoral Fellow and G.I.P. was supported in part by the Massachusetts General Hospital Fund for Medical Discovery and the Harvard Medical School Janet M. McArthur Fellowship. This study was supported by research grants from the National Institutes of Health to J.L.T. (R01-AG12279, R01-HD34226) and S.J.K. (R01-CA49712), and by Vincent Memorial Research Funds.

Received 2 September; accepted 15 December 1998.

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