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Absence of the proapoptotic Bax protein extends fertility and alleviates age-related health complications in female mice

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The menopausal transition in human females, which is driven by a loss of cyclic ovarian function, occurs around age 50 and is thought to underlie the emergence of an array of health problems in aging women. Although mice do not undergo a true menopause, female mice exhibit ovarian failure long before death because of chronological age and subsequently develop many of the same age-associated health complications observed in postmenopausal women. Here we show in mice that inactivation of the proapoptotic *Bax* gene, which sustains ovarian lifespan into advanced age, extends fertile potential and minimizes many age-related health problems, including bone and muscle loss, excess fat deposition, alopecia, cataracts, deafness, increased anxiety, and selective attention deficit. Further, ovariectomy studies show that the health benefits gained by aged females from Bax deficiency reflect a complex interplay between ovary-dependent and -independent pathways. Importantly, and contrary to popular belief, prolongation of ovarian function into advanced age by Bax deficiency did not lead to an increase in tumor incidence. Thus, the development of methods for postponing ovarian failure at menopause may represent an attractive option for improving the quality of life in aging females.

aging | apoptosis | menopause | ovary

Although the process of aging has been attributed in part to increased apoptosis in various tissues (1), animal models lacking cell death-regulatory genes are rarely subjected to longitudinal aging studies. In females, one of the first organ systems to fail with age is the reproductive axis, which in humans is a principal contributing factor to the onset of menopause (2). Ovarian failure, whether natural (age-related) or induced as a consequence of pathological insults, is driven by depletion of ovarian follicles, the hormonally active support structures that house oocytes, through apoptosis (3). As a consequence, dramatic changes in the endocrine activity of the female gonads ensue, which are thought to underlie the emergence of a spectrum of physiological and psychological health complications in aging females (2). Although it has been shown that aged female mice transplanted with ovaries of young donors live longer than nontransplanted control or ovariectomized females (4), it remains unclear whether postmenopausal health complications arise as a direct result of ovarian failure or simply reflect the aging process.

Despite the fact that mice do not undergo a true menopause, female mice exhaust their follicle pool long before death because of chronological age (5), similar to that seen in humans (6). Further, aging female mice exhibit many of the same health complications associated with postmenopausal life in women (see below). In a previous study, we reported that oocyte and follicle loss in female mice lacking the proapoptotic Bax protein is attenuated, leading to a dramatic prolongation of ovarian function into very advanced age (7). To better understand the consequences of Bax deficiency and sustained ovarian function on the female body with age, herein we undertook a 7-yr investigation of the aging process in mutant female mice derived from a breeding colony originally obtained from the Korsmeyer laboratory in 1996 (8) and maintained on a C57BL/6 congenic background.

Results

Outwardly, striking differences were noted in aged (≥22 mo) WT and mutant [*Bax* gene knockout (KO)] females (Fig. 1 *A* and *B*). Aged KO mice ($n = 38$) were more active and maintained a leaner body composition than WT littermates ($n = 35$; see details below). In addition, by 2 yr of age, 40% of WT females had experienced ≈30% hair loss, consistent with past observations (www.informatics.jax.org). By comparison, age-matched KO females exhibited no visible alopecia (Fig. 1 *A* and *B*). More than half (54%) of the WT females also developed cataracts by 18 mo of age (29% bilateral, 25% unilateral), whereas <3% of age-matched KO females developed this pathology. In addition, wrinkling of the skin, visible by ear assessment, was widespread in aged WT but not KO mice (Fig. 1 *A* and *B*). We also observed that approximately one-third (13 of 38) of the KO mice exhibited rectal prolapse with age, which was not seen in WT mice. In light of past findings linking *Helicobacter* to rectal prolapse (9, 10), we tested our colony and discovered that our mice were infected with this microorganism. After generation of a *Helicobacter*-free colony, rectal prolapse was no longer observed.

Reproductive performance also markedly differed among genotypes. Initially, we noted that young adult KO females frequently failed to produce litters when paired with adult WT males of proven fertility, but their breeding performance actually improved with age. During a 3-mo mating trial initiated at 2 mo of age, only 25% (5 of 20) of KO females became pregnant and delivered pups, compared with a pregnancy and delivery rate of 92% (23 of 25) over the same 3-mo period in aged-matched WT females. In contrast, during a 3-mo breeding trial initiated at 8 mo of age, >90% (22 of 24) of KO females became pregnant and delivered at least twice, with an average litter size of five pups. By comparison, and as

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Abbreviations: KO, knockout; FSH, follicle-stimulating hormone; ASR, acoustic startle response; BMD, bone mineral density; CS, conditioned stimulus; LI, latent inhibition; NPE, nonpreexposed; ovex, ovariectomy; PE, preexposed; PPI, prepulse inhibition; US, unconditioned stimulus.

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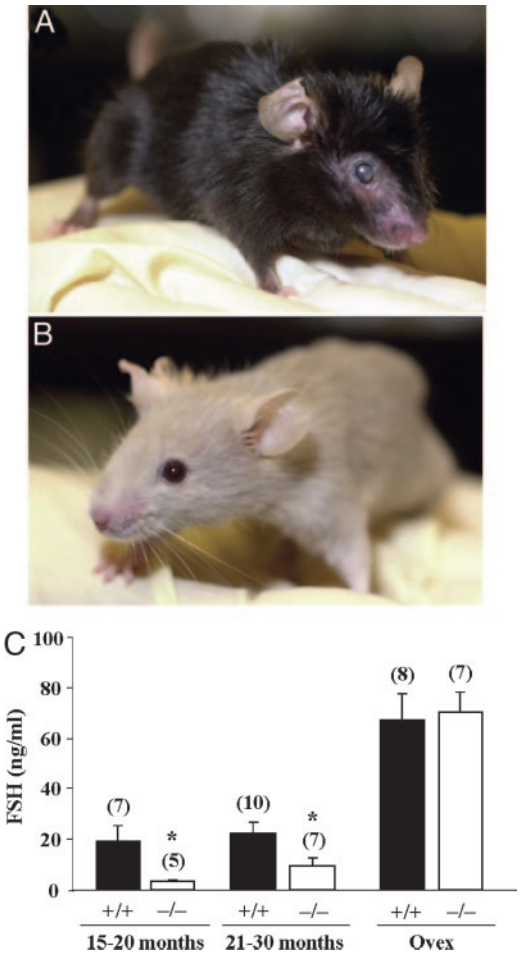

Fig. 1. Initial comparisons of aged WT and Bax-deficient female mice. Representative outward appearance of WT (*A*) and *Bax*-null (*B*) female mice at 24 mo of age. (*C*) Serum FSH levels in aging (15–20 mo) or aged (21–30 mo) WT (+/+) and KO (−/−) females or in 7-mo-old WT and KO females 4 mo after ovex. The number of mice analyzed per group is provided in parentheses above each bar (*, $P < 0.05$ vs. respective WT group).

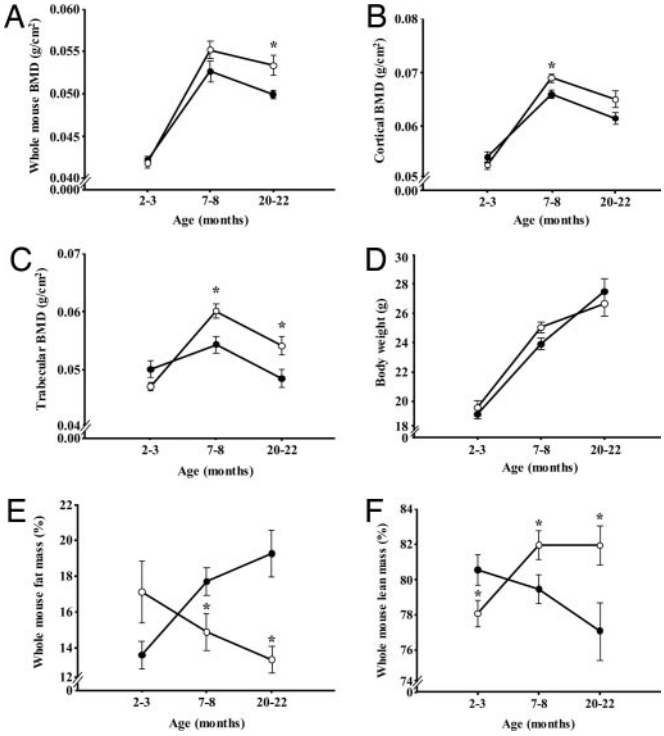

Fig. 2. Changes in body composition with age. (*A*) KO females (open circles) exhibit higher whole-animal BMD than WT mice (filled circles) at 20–22 mo of age. (*B* and *C*) Separate analysis of cortical (femoral; *B*) and trabecular (vertebral; *C*) bone shows that aged KO females have increased BMD in both types of bone. (*D–F*) Although total body weight is unaffected by genotype (*D*), KO females exhibit a significant decline in fat mass with age (*E*) while maintaining a significantly higher total lean mass (*F*). Data are the mean ± SEM with 9–14 mice per group (*, $P < 0.05$ vs. corresponding WT value).

expected, fertility decreased markedly in age-matched WT females, such that only 40% (8 of 20) became pregnant, and none of them delivered more than once (average litter size of five pups) during the 3-mo mating trial. Female fertility is known to decline with advancing age in many species, including mice and humans (11), and this has been partly attributed to increased aneuploidy in oocytes (12). Preliminary studies showed that the incidence of aneuploidy in oocytes of WT females increased from 1.4 to 4.5% between 1 ($n = 72$ oocytes) and 8 ($n = 67$ oocytes) mo of age, although this difference was not statistically different ($P > 0.05$) by χ^2 analysis. Further, the incidence of aneuploidy in oocytes of KO females remained relatively constant between 1 (3.7%, $n = 54$ oocytes) and 8 mo (3.1%, $n = 32$ oocytes) of age, and thus the observed genotype-dependent differences in reproductive capacity could not be attributed to differences in aneuploidy.

Nevertheless, KO females maintain ovarian function into very advanced age and thus do not undergo the murine equivalent of menopause (7). Even though aged KO females fail to become pregnant when housed with adult WT males of proven fertility, aged KO females ovulate mature (metaphase-II) oocytes in response to exogenous gonadotropin stimulation, which retain the capacity for fertilization and embryonic development *in vitro* (7). In evaluating the endocrine status of these animals, we observed that serum levels of follicle-stimulating hormone (FSH) were significantly higher in aging (15–20 mo) or aged (21–30 mo) WT vs. KO females (Fig. 1*C*), consistent with a maintenance of ovarian function and hence a negative feedback suppression of FSH release, in KO animals with age. In contrast, serum estradiol levels did not differ in aging or aged WT vs. KO mice [supporting information (SI) Fig. 5], probably because of marked genotype-dependent differences in the deposition of adipose tissue (discussed below), which becomes a principal site of estrogen biosynthesis in aging females (13). To then directly test whether oocytes of aged KO females can produce viable offspring, we grafted ovarian tissue from aged (24–32 mo) KO females into hemiovariectomized young adult WT females and housed these animals with heterozygous adult males. With this type of mating scheme, birth of homozygous-null offspring would occur only if the oocytes originated from the grafted KO ovarian tissue (see *SI Text* for additional details). Three WT surrogate females produced viable KO offspring (four females and one male, one of which was derived from an oocyte in grafted ovarian tissue of a 32-mo-old donor; SI Fig. 6), and all of these animals developed normally without overt evidence of abnormalities or health complications through to the time of euthanasia at 12 mo of age (data not shown).

Along with the loss of fertility, one of the most widely accepted consequences of ovarian failure at menopause is osteoporosis (14), a multifactorial age-related disease characterized by decreased bone mineral density (BMD) and deterioration of the microarchitecture of trabecular bone (15). Evaluation of bone quality in WT and KO female mice in young, mid-, and late-adult life (2–3, 7–8, and 20–22 mo of age, respectively) revealed no genotype-dependent differences in young adult animals. However, whole-animal BMD, bone mineral content, and bone area were signifi-

Table 1. Comparison of mean values (± SEM) for mechanical and structural parameters of bones between WT (+/+, $n = 12$) and KO (−/−, $n = 9$) female mice at 20–22 mo of age

Analysis	Parameter	Genotype +/+	Genotype −/−
Three-point bending (cortical bone)	Failure strength, MPa	124 ± 11	176 ± 20*
	Failure strain, %	3.8 ± 0.6	5.9 ± 0.9
	Toughness, mJ/mm^3	2.9 ± 0.7	7.1 ± 1.5*
Vertebral compression (trabecular bone)	Failure strength, MPa	5.7 ± 0.6	6.9 ± 1.0
	Failure strain, %	9.7 ± 1.3	16.4 ± 2.5*
	Toughness, mJ/mm^3	0.33 ± 0.06	0.72 ± 0.15*
Microcomputed tomography (trabecular bone)	Volume, %	12.2 ± 0.6	19.5 ± 1.7*
	Thickness, μm	24.5 ± 1.2	27.4 ± 2.1
	Trabecular number, per mm	5.1 ± 0.3	7.2 ± 0.4*
	Trabecular separation, μm	179 ± 14	116 ± 8*

*, $P < 0.05$ vs. corresponding WT value.

cantly elevated in KO females compared with WT controls in late adult life (Fig. 2*A*; SI Fig. 7). Further studies revealed that, compared with WT females, KO females exhibited increased BMD in both cortical (femoral) and trabecular (vertebral) bones (Fig. 2 *B* and *C*). Analysis of cortical bone using the three-point bending test demonstrated that aged KO females exhibited a 42% increase in failure strength, a 56% increase in failure strain, and a 148% increase in modulus of toughness vs. age-matched WT females (Table 1). Trabecular bone of aged KO mice was also more ductile than that of WT females, exhibiting a 69% increase in failure strain and a 117% increase in modulus of toughness (Table 1). In addition, microcomputed tomography revealed a 59% increase in trabecular bone volume and 41% more bone trabeculae in aged KO mice, resulting in 35% less trabecular separation in aged KO females (Table 1).

To determine whether these effects were due to the presence of functional ovaries or to an ovary-independent action of Bax deficiency on bone, we examined 7-mo-old female mice that underwent bilateral ovariectomy (ovex) after reaching their growth plateau at 3 mo of age. Ovex decreased whole-body BMD in both WT and KO females to a similar degree (Fig. 3*A*). Both groups of mice also showed similar decreases in cortical and trabecular BMD vs. the respective ovary-intact controls (Fig. 3 *B* and *C*). However, vertebral compression studies indicated that only KO females exhibited a significant decrease in failure strength after ovex (Fig. 3*D*), eliminating the differences in failure strength because of genotype observed in ovary-intact mice at 7 mo of age. Nonetheless, both WT and KO females showed striking alterations in the structural properties of trabecular bone after ovex (Fig. 3*E*). These findings collectively indicate that the beneficial effects of Bax deficiency on bone quality arise from a complex interplay between an ovary-independent effect of the loss of *Bax* gene function, most likely in bone, and a prominent indirect effect involving sustained ovarian function in the mutant animals. Further, although body weight did not differ among the genotypes (Fig. 2*D*), other aspects of body composition were affected by Bax deficiency with age, with the most notable being a dramatic decrease in fat deposition (Fig. 2*E*) along with an increase and maintenance of lean body mass (Fig. 2*F*). In addition, ovex-induced increases in fat mass with age were observed only in WT females (SI Fig. 8), suggesting that Bax deficiency has a direct (ovary-independent) effect on adipose deposition in females.

In a final series of experiments, we explored various aspects of behavior, learning, and memory in WT and KO females, because cognitive decline is also frequently associated with aging (2). First, mice were monitored in the open field test, which is a classical behavioral task for assessing anxiety and exploratory activity. Consistent with past studies (16), WT female mice displayed a pronounced age-related increase in anxious behavior, reflected by more frequent risk assessment and increased time spent self-grooming. These behavioral changes were not observed in KO females (Table 2). Moreover, at 15–17 mo of age, KO mice showed more active behavior than WT females, spending more time in the brightly lit central area of the open field and less time in one place, and engaging in more rearing activity (Table 2).

To next assess learning and memory capacity, WT and KO females were trained in a fear-conditioning test comprising two components, cued and contextual. Both components depend on the amygdala; however, contextual fear conditioning also involves the hippocampus (17). Animals were trained to associate a foot-shock with either a training context (contextual fear conditioning) or a tone (cued fear conditioning), which then triggers a conditional fear response resulting in increased freezing. In the contextual fear memory test, aged (15–17 mo) KO

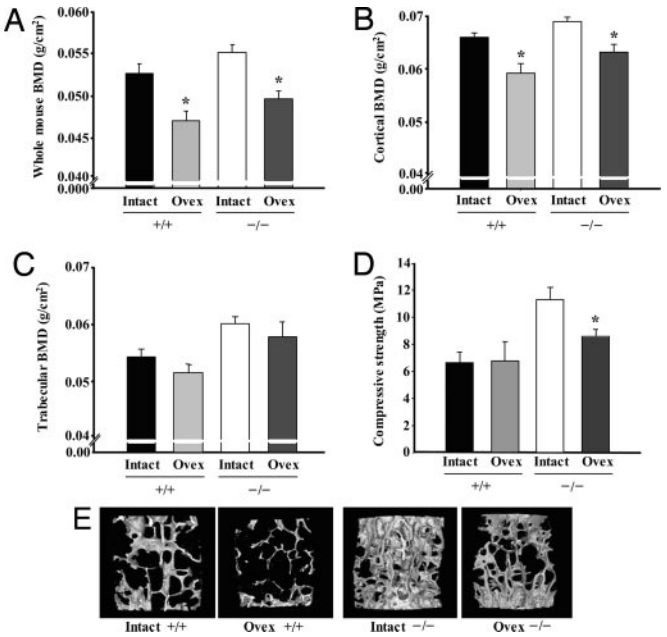

Fig. 3. Impact of the loss of ovarian function on body composition. (*A–C*) Ovex reduces whole mouse (*A*) and cortical (*B*), but not trabecular (*C*), BMD compared with age-matched ovary-intact controls irrespective of genotype (WT, +/+; KO, −/−). (*D* and *E*) Although ovex reduces compressive strength of trabecular bone only in KO females (*D*), trabecular bone structure is negatively affected by ovex in both genotypes (*E*). Data in *A–D* are the mean ± SEM with 7–10 mice per group (*, $P < 0.05$ vs. corresponding ovary-intact value), whereas *E* depicts representative tomographic scans.

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Table 2. Comparison of mean values (± SEM, n = 9–17 mice per group) for behavioral observations in the open field test between WT (+/+) and KO (−/−) female mice

	Two to three mo of age		Fifteen to seventeen mo of age	
Parameter	+/+	−/−	+/+	−/−
Time spent on the periphery, s	132.1 ± 23.2	146.3 ± 12.8	126.0 ± 15.3	133.9 ± 11.7
Time spent in the center, s	22.5 ± 5.7	36.6 ± 10.2	20.4 ± 4.7	52.7 ± 6.8*†
Risk assessment	2.0 ± 0.4	3.0 ± 1.2	5.1 ± 0.8†	2.9 ± 0.9*
Time spent self-grooming, s	3.9 ± 0.07	4.6 ± 0.7	13.5 ± 4.9†	4.8 ± 0.7
Time spent freezing, s	3.4 ± 0.9	3.1 ± 0.7	3.2 ± 0.6	1.2 ± 0.6†‡
Horizontal activity	592.5 ± 103.0	750.8 ± 82.0	612.7 ± 50.6	697.5 ± 55.3
Vertical activity	49.4 ± 13.0	36.6 ± 10.2	38.7 ± 6.4	63.5 ± 9.8‡

*, $P < 0.01$ vs. corresponding age-matched WT value.
†, $P < 0.05$ within each genotype comparing young adult (2–3 mo) and aged (15–17 mo) female mice.
‡, $P < 0.05$ vs. corresponding age-matched WT value.

females displayed significantly less freezing when compared with age-matched WT animals (Fig. 4*A*), whereas no differences were observed in the cued memory test (Fig. 4*B*). Because pain sensitivity and motor ability were similar in WT and KO females (SI Fig. 9), the contextual memory deficit detected in aged KO females most likely reflects hippocampal disorganization and dysfunction because of excess neurons that fail to be eliminated by apoptosis in Bax-deficient mice (18).

To further investigate cognitive function, latent inhibition (LI) and prepulse inhibition (PPI) tests were used. LI is the degree to which preexposure to a conditioned stimulus (CS) decreases the meaning of that stimulus when it is later paired with an unconditioned stimulus (US) and thus reflects the animal's ability to ignore irrelevant stimuli. Suppression ratios in aged animals not preexposed (NPE) to a CS were similar; however, only aged KO females developed LI after CS preexposure (Fig. 4*C*), indicating an improved selective attention over their age-matched WT female siblings. The PPI test involves attenuation of the acoustic startle response (ASR) by a weak stimulus (prepulse) executed a short time before the startle stimulus. Although no significant difference in ASR to a 120-dB startle stimulus was observed between groups in young adult life (2–3 mo), the age-related decline in ASR observed in WT mice was significantly attenuated in KO females (Fig. 4*D*). Further, when examining the percent PPI as a function of prepulse intensity, KO mice maintained a stable level of PPI with age compared with the significant age-related decline in PPI noted in WT females (Fig. 4*E*). Because PPI depends on hearing (19), and aging C57BL/6 mice are prone to high-frequency hearing loss (www.informatics.jax.org), we next applied a wider range of startle intensities to aged WT and KO females. With the exception of the two lowest intensities, the ASR was significantly higher in KO vs. WT females at all other intensities (Fig. 4*F*). Thus, the maintenance of PPI in KO females with age likely reflects a reduction in age-related hearing loss. This conclusion agrees with the known effect of Bax deficiency on eliminating death of trigeminal sensory ganglion neurons (18), which project to the cochlea and are associated with hearing function (20).

Discussion

This work has uncovered several aspects of the aging process in female mammals not previously known or fully appreciated. For example, although much has been made of the negative correlation between maternal age and oocyte quality, oocytes within ovaries whose function is sustained into very old age by Bax deficiency remain fully competent to be fertilized and to produce normal viable offspring if a suitable, i.e., young, endocrine environment is provided. Such findings agree well with very recent data from studies of the male germ line, which indicate that dysfunction of the gonadal microenvironment rather than the germ cells per se is a key aspect of age-related spermatogenic failure (21). The data presented also provide insight into the importance of both chronological age and loss of ovarian function to the deterioration of cortical

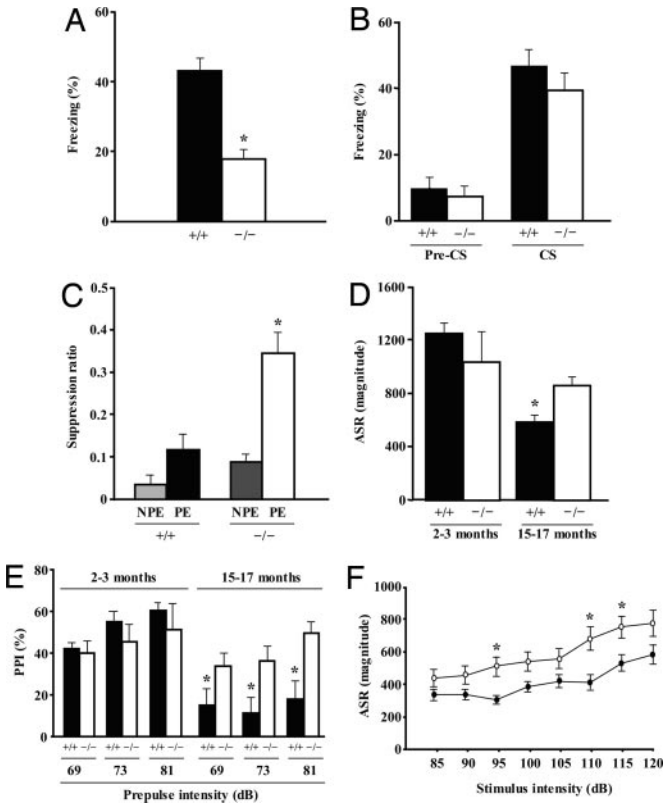

Fig. 4. Bax deficiency affects age-related changes in behavior, cognitive function, and sensory perception. (*A* and *B*) Aged (15–17 mo) KO (−/−) females exhibit a deficit (lower freezing values) in contextual (*A*) but not cued (*B*) fear memory when compared with age-matched WT (+/+) animals (*, $P < 0.001$ vs. corresponding WT value). (*C*) Suppression ratios are comparable among genotypes in aged animals not preexposed to a CS; however, after preexposure to a CS, LI was present in aged KO but not WT females (*, $P < 0.01$ vs. respective NPE value). (*D*) In response to 120 dB, aged WT females exhibit a markedly decreased ASR compared with young adult (2–3 mo) WT mice (*, $P < 0.001$), whereas no significant change in ASR is detected in KO females with age. (*E*) In young adult WT and KO females, the percent PPI as a function of prepulse intensity is comparable at all three prepulses; however, aged KO mice exhibit a maintenance of PPI compared with their aged WT littermates (*, $P < 0.05$ vs. corresponding WT response). (*F*) The ASR to a wide range of startle intensities in aged WT (filled circles) and KO (open circles) female mice (*, $P < 0.05$ vs. corresponding WT value). Data are the mean ± SEM with 9–17 mice per group.

and trabecular bone in aging females. Similarly, although increased adipose deposition results from the absence of functional ovaries, ovary-independent pathways influenced directly by Bax also contribute to fat accumulation with age. In addition, striking improvements in several behavioral domains that relate to cognition, anxiety, and sensory perception were observed in aged Bax-deficient females.

Although postmenopausal health problems have been most often attributed to a loss of estrogen production, the reproductive hormone assessments indicate this may not be the case, at least in aging female mice. Along these lines, a very recent study has reported that age-related bone loss in females is actually because of the elevated secretion of FSH, and a direct catabolic effect of FSH on bone, rather than a decline in ovarian estrogen production (22). Further, the actions of FSH appear to require TNF-α as a mediator, possibly by its ability to expand the number of osteoclast precursors in bone marrow (23). Our data fully agree with this in that the genotype-dependent differences in BMD with age were correlated with differences in the circulating levels of FSH and not estradiol. The estradiol levels detected in the blood of aged WT females likely reflect elevated peripheral production of this steroid from the increased adipose deposition noted in WT, but not KO, females. Indeed, a shift from ovarian to peripheral estrogen production has been documented in postmenopausal women, accompanied by increased expression and activity of aromatase in fat tissue (13, 24, 25). Thus, our data add support to the emerging concept that postmenopausal increases in circulating FSH, independent of changes in the levels or site of estrogen production, are responsible for age-related bone loss in females (26). Finally, it should be noted that the low level of FSH secretion detected in aged KO animals was not because of a defect at the level of the pituitary caused by Bax deficiency, since a comparable hypersecretion of FSH was detected in WT and KO females following ovex (Fig. 1*C*).

Somewhat surprisingly, despite the apparently increased quality of life of aging females lacking Bax, no significant differences in overall lifespan were found between KO (22.3 $\pm$ 5.5 mo, $n = 30$) and WT (26.7 $\pm$ 4.8 mo, $n = 32$) animals ($P > 0.05$). In fact, the absence of lifespan extension in KO females, which remain extremely lean with age, contrasts a past study of mice with a fat-specific insulin receptor KO that concluded, "leanness, not food restriction, is a key contributor to extended longevity" in caloric-restricted mice (27). Of final note, and in full agreement with past work from others (28), we did not observe an increase in tumor incidence in aged KO females (data not shown; see *SI Text*). Although these findings contrast inferences from clinical studies that efforts to sustain ovarian function in aging females might predispose them to cancer, particularly in steroid-sensitive tissues such as the breast (29), these data actually align well with very recent observations from the Women's Health Initiative indicating that estrogen supplementation in women after menopause does not increase, but may actually decrease, breast cancer risk (30). Therefore, development of methods for postponing ovarian failure at menopause may represent an attractive option for improving the quality of life in aging females.

Materials and Methods

Animals. WT and KO female mice were generated by mating of *Bax*-heterozygous animals, as detailed (8). All animal procedures were approved by the respective institutional animal care and use committees of Massachusetts General Hospital, Michigan State University, and Mount Sinai Hospital.

Aneuploidy Analysis. Chromosome preparations from single oocytes were made by using the air-dried technique of Tarkowski (31) and then analyzed as detailed (ref. 32; see also *SI Text* for additional details).

Ovarian Transplantation and Mating Trials. As described (33), ovaries were aseptically collected from aged (24–32 mo) KO females, placed in Petri dishes containing 2 ml of modified human tubal fluid (Irvine Scientific, Santa Ana, CA), and dissected free from the fat pad and bursa. WT female mice (from the same colony) at 21–23 d of age were anesthetized, and the surgical field was prepared for aseptic surgery. One ovary was removed through a single dorsal skin incision across the lumbar area, and a transplant consisting of one-half of an ovary from an aged KO donor was then placed inside the empty ovarian bursa. The incision was closed with surgical clips, and 2 wk after surgery, all recipients were placed in mating trials with adult *Bax*-heterozygous males of proven fertility.

Body Composition. WT and KO female mice were randomly assigned to three age groups for analysis: 2–3, 7–8, or 20–22 mo of age. Additional WT and KO mice were subjected to ovex at 3 mo of age and euthanized with age-matched ovary-intact females at 7 mo of age for comparative studies. Dual-energy x-ray absorptiometry (DEXA), using a PIXImus mouse densitometer (GE Medical Systems Lunar, Madison, WI), was performed to determine whole-mouse BMD, bone mineral content, total bone area, total fat mass, and total lean mass (34). Additional DEXA scans were performed on the excised lumbar vertebrae and femora to determine BMD of specific bone regions. Evaluation of the mechanical properties of cortical bone was based on subjecting excised right femora to failure tests using the three-point bending procedure (35). Fourth or fifth lumbar vertebrae were subjected to failure tests or analyzed by using microcomputed tomography (eXplore Locus SP Specimen Scanner and MicroView CT visualization software; GE Medical Systems) to evaluate trabecular bone properties (architecture, volume, thickness, density, and separation).

Hormone Assessments. Serum samples collected from WT and KO female mice between 15–20 and 21–30 mo of age were analyzed by highly specific radioimmunoassays for FSH and estradiol through the facilities of National Hormone and Pituitary Program directed by A. F. Parlow (Harbor–University of California Los Angeles Medical Center, Torrance, CA).

Open-Field Observations (Behavioral Studies). Each mouse was individually placed in the center of a brightly lit open field equipped with infrared sensors to automatically record horizontal and vertical movements (Ugo Basile, Malvern, PA). The following parameters were recorded over a 5-min interval: number of interruptions of the horizontal and vertical sensors, duration of passive behavioral episodes (sitting in one place without any visible movements or "freezing"), duration of self-grooming, number of risk assessments (behavior involving the mouse stretching its body from corners/wall toward the center), duration of exploratory activity, and duration of active walking in the center area (5 cm from the walls) vs. activity close to the walls (within 5 cm of the walls).

Contextual and Cued Fear Conditioning. The fear-conditioning experiment was performed in sound-attenuated chamber (Med Associates, St. Albans, VT) equipped with a computer-controlled fear conditioning system (Actimetrics, Wilmette, IL). The conditioning procedure consisted of placing each mouse inside the chamber and allowing it 3 min of baseline exploratory activity. A 3.6-kHz pulsated tone (75 dB), which served as a CS, was then presented for 20 s, followed by a foot-shock (2 s, 0.6 mA), which served as a US. Two more CS–US pairings were presented with 2-min intertrial intervals. The mouse was removed from the chamber 30 s after the last CS–US pairing and returned to its home cage. The distance traveled in pixels between successive frames shot at three frames per second during the foot-shock interval was used as a measurement of the unconditional response of each mouse. Using known reference marks in the box, these values were converted into real

distance (centimeters) and then into velocity (centimeters per second).

To evaluate contextual fear memory, the mouse was placed back into the training context 24 h after training, and freezing behavior was measured for 5 min in the absence of tone or shock. Fear memory to the tone was evaluated 24 h after the context test. The tone test consisted of two phases. In the first phase (pre-CS), the mouse was placed in a novel context (novel odor, lighting, cage floor, and visual cues), and freezing behavior was measured for 3 min in the absence of the tone. In the second phase (CS), the tone was presented, and freezing behavior was measured for another 3 min. Freezing behavior, defined as the complete absence of any movement except for respiration and heartbeat (36), was measured during the context and cued conditioning tests at 0.25-s intervals by using FreezeFrame automated fear conditioning software (Actimetrics).

LI. Before beginning the LI experiments, mice were weighed, and water was removed from the cages for 24 h. During the first 5 d, water-deprived mice were trained to drink in the sound-attenuated chamber for 15 min/d. The mice in each age group were further separated into two groups: NPE and preexposed (PE). The PE mice received 40 white-noise tones as CS (85 dB, 10-s duration, 60-s interstimulus interval), whereas the NPE group received no stimuli during an equivalent period in the chamber. The next day, all mice received two CS/foot-shock (1 s, 0.37 mA) pairings given 5 min apart, with the shock given immediately after the CS. On the test day, the CS was presented after mice had completed 75 licks, and it lasted until lick 101. During the test, the following observations were recorded: time to first lick, time to complete licks 50–75 (before CS onset, period A), and time to complete licks 76–101 (after CS onset, period B). The degree of lick suppression was expressed as a suppression ratio, A/(A + B). A lower suppression ratio indicates a stronger suppression of drinking, and LI is defined as a significantly higher suppression ratio score in the PE compared with the NPE mice. There were eight experimental groups in a $2 \times 2 \times 2$ design with main factors of PE (0 or 40), genotype (WT or KO), and age (2–3 or 15–17 mo).

PPI. For PPI, five types of trials were performed in the sound-attenuated chamber: startle-pulse-alone trials (sp), which consisted of a single tone burst (10 kHz, 120 dB, 40 ms); three prepulse-plus-startle trials (pp-sp) that preceded the startle pulse by 100 ms and consisted of prepulses (10 kHz, 20 ms) at 69, 73, or 81 dB (pp69sp, pp73sp, and pp81sp); and no-stimulus trials (ns), which consisted of background noise only (65 dB). Sessions were structured as follows: (*i*) a 15-min acclimation to background noise levels; (*ii*) five sp trials; (*iii*) 10 blocks each containing all five trials (sp, pp69sp, pp73sp, pp81sp, and ns) in pseudorandom order; and (*iv*) five sp trials. Intertrial intervals were set between 12 and 30 s. The startle level was quantified by force intensity through a floor-plate sensor. For each mouse, the PPI at each prepulse was expressed as a percentage of the amplitude of the mean startle response alone for that mouse and the startle response to pulse alone.

ASR. Each mouse was placed in the sound-attenuated chamber and allowed to acclimate for 5 min, after which 80 startle stimuli of varying intensities were presented, with a duration of 50 ms and a variable interstimulus interval of 20–30 s. The following stimulus intensities were applied: 85, 90, 95, 100, 105, 110, 115, and 120 dB. Ten trials were presented in three blocks of eight stimuli and in pseudorandom order within a block.

Longevity Studies. WT and KO female mice were housed and maintained under identical conditions without any experimental manipulation, and lifespan was recorded. Animals that died prematurely before reaching at least one-half of normal lifespan (i.e., <12 mo of age) (WT, $n = 3$; KO, $n = 8$) were excluded from the analyses.

Data Analysis. All experiments were independently replicated with at least four mice per group (see text and legends for specific numbers in each experiment), and the data shown in text, graph, or table form represent the mean ± SEM of combined results. Depending on the experiment, data analysis was performed by one-way, two-way, or multiple ANOVA, followed by *t* tests, least-significant difference post hoc comparisons, or Tukey least significant difference post hoc tests (see *SI Text* for additional details).

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