Switch from BAX-dependent to BAX-independent germ cell loss during the development of fetal mouse ovaries

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Summary
Female reproductive life is limited by the oocyte/follicle pool, which has been determined by the number of germ cells to enter meiosis and subsequent loss of oocytes. It has been suggested that apoptosis accounts for the elimination of germ cells throughout oogenesis. However, female germ cells are lost continuously while they undergo distinct cell cycles in fetal and neonatal life. No convincing evidence has yet been provided to show apoptotic death of oocytes during meiotic prophase in vivo. In this study, we examined the change in the germ cell population in mice deficient of BAX, a key proapoptotic molecule. The number of germ cells, identified by GCNA1 immunolabeling, approximately doubled in ovaries of Bax⁻/⁻ mice compared with ovaries of heterozygous Bax⁺⁻ mice and wild-type Bax⁺/+ mice by 14.5 days post coitum (d.p.c.) and remained higher up to 24.5 d.p.c. However, there was a rapid loss of germ cells in Bax⁻/⁻ ovaries, paralleling that in Bax⁺⁻ and Bax⁺/+ ovaries from 14.5-24.5 d.p.c., a period in which most germ cells entered and progressed in meiotic prophase. These results suggest that, while progressing through meiotic prophase, oocytes are eliminated by a BAX-independent mechanism.

Key words: Oocyte loss, Meiosis, Mouse ovary, Bax mutant, Apoptosis

Introduction
The primordial germ cells are first recognized in an extraembryonic site at the beginning of gastrulation and migrate into the gonad that is undergoing sexual differentiation (Ginsburg et al., 1990). During migration and for the first few days in the gonad, the germ cells proliferate actively (Tam and Snow, 1981). Subsequent sexual differentiation of germ cells is determined by the gonadal environment that harbors them (Peters, 1970; McLaren, 1995). Those in the testis are incorporated into the seminiferous tubules and arrested in the G1 phase of the mitotic cell cycle. Postnatally, they resume mitotic activity and by maintaining spermatogenenic stem cells, they continually proliferate in the entire life. The germ cells in the ovary, however, enter meiosis and become arrested at the end of prophase I at or soon after birth. They resume meiosis and undergo two consecutive meiotic divisions upon ovulation and fertilization after puberty. The germ cells in mitotic cell cycle in the ovary are known as oogonia whereas the germ cells that have entered meiosis are known as oocytes. When we cannot distinguish between these two populations, we use the general term 'germ cells'.

The oocyte pool is limited not only by the number of germ cells that enter meiosis but also by a major loss of oocytes in fetal and neonatal life (Borum, 1961; Beaumont and Mandl, 1962; Baker, 1963; McClellan et al., 2003). The cause or role of this oocyte elimination remains largely unknown. Three main hypotheses have been proposed to explain the cause of oocyte loss. According to the first hypothesis, limited amounts of trophic factors may permit the survival of only a fraction of germ cells. This hypothesis is based on the observation that various kinds of growth factors and cytokines influence the survival and proliferation of germ cells in culture (Godin and Wylie, 1991; Matsui et al., 1991; Resnick et al., 1992; Dolci et al., 1993; Cooke et al., 1993; Pesce et al., 1993, Koshimizu et al., 1995; Morita and Tilly, 1999a). Furthermore, genes such as Kit and Kit ligand are essential for the maintenance of germ cells in vivo (Mintz and Russel, 1957; Chabot et al., 1988; Huang et al., 1990; Zsebo et al., 1990; Brannan et al., 1991). However, this hypothesis is not sufficient to account for the continuous oocyte loss. For example, the Kit gene is not expressed in the oocyte at meiotic prophase (Manova and Bachvarova, 1991; Coucouvanis and Jones, 1993). According to the second hypothesis, some sort of selection mechanism may operate to eliminate oocytes with genetic or meiotic pairing errors. For example, the oocytes with telomere dysfunctions are eliminated at early stages of meiotic prophase (Liu et al., 2004). Furthermore, degeneration of oocytes is often observed at the pachytene stage, when pairing of homologous chromosomes is completed (Borum, 1961; Beaumont and Mandle, 1961; Baker, 1963; Bakken and McClanahan, 1978; Speed, 1988; Dietrich and Mulder, 1983; Burgoyne and Baker, 1985; Mittwoch and Mahadevaiah, 1992). However, it remains unknown to what extent genetic defects contribute to oocyte loss in normal development. According to the third hypothesis, the massive reduction in oocytes may serve a more altruistic purpose; oocytes may die to donate their cytoplasmic components such as mitochondria to the surviving oocytes within a cyst (Pepling and Spradling, 2001). This hypothesis is based on the observation that the major oocyte loss occurs after birth, when oocyte cysts break.
down into individual cells. Consequently, this hypothesis cannot explain the oocyte loss in fetal ovaries.

Beside the cause, understanding the mechanism of oocyte loss is equally important, particularly in attempts to increase the oocyte pool. Apoptosis, or programmed cell death, has been implicated in the death of oocytes in normal development (Morita and Tilly, 1999b; Tilly, 2001). However, the pathway, as well as the individual signaling molecules used for oocyte elimination, is largely unknown. In fact, mutation or overexpression of various molecules involved in apoptosis generated controversial results. For example, it has been reported that the number of primordial follicles in neonatal ovaries did not change when two key proapoptotic molecules, Bel2-associated X protein (BAX) or caspase 3, were absent in Bax- or caspase 3-null-mutants (Perez et al., 1999; Mattikainen et al., 2001). It has also been reported that female germ cells are less sensitive than male germ cells to the haplo-sufficiency of Bcl-x, an antiapoptotic molecule (Kasai et al., 2003). However, a role of BAX in the death of primordial germ cells is less clear (D’Elia et al., 1999; Stallock et al., 2003). Furthermore, oocytes from old females are capable of surviving in the ovary at all stages examined – with one exception: at 18 days postcoitum (d.p.c.), MVH labeling was considerably weaker in fetal ovaries.

Results

Immunolabeling of GCNA1 and MVH in Bax+/– ovaries

Immunolabeling of GCNA1 and MVH was compared in the histological sections of Bax+/– ovaries at four representative developmental stages (Fig. 1A). Intense MVH labeling was always seen in the cytoplasm of germ cells distributed over the ovary at all stages examined – with one exception: at 18 days postcoitum (d.p.c.), MVH labeling was considerably weaker in fetal ovaries. For comparison, the Bax–null mutation leads to a larger number of primordial follicles in adult ovary (Perez et al., 1999; Takai et al., 2003). Furthermore, oocytes from old females are capable of dying due to a BAX-dependent pathway (Perez et al., 2005).

A Bax–mutant mouse has been generated by replacing exons 2–5 of the Bax gene with a PGK–Neo gene, deleting several functional domains (Knudson et al., 1995). The homozygous mutant mouse is viable but displays aberrations in cell death. In the present study, we examined the change in the germ cell population in fetal and neonatal ovaries of Bax–mutant mice. We first examined the distribution of germ cells in ovarian sections by immunolabeling of the germ-cell-specific nuclear antigen 1 (GCNA1) and mouse vasa homolog (MVH; also known as DEAD box protein 4 or DDX4). It has been reported that GCNA1 is expressed exclusively in the nuclei of germ cells in fetal and neonatal gonads, whereas MVH is expressed exclusively in the cytoplasm of germ cells until later postnatal life (Enders and May, 1994; Toyooka et al., 2000). We found that GCNA1 labeling was not always nuclear, but its nuclear labeling was associated with the progression of oocytes in meiotic prophase. We then counted the total number of GCNA1-positive cells in the dissociated cells from individual ovaries. The conventional area-estimation method using histological sections may be better for fetal ovaries, in which germ cells of homogeneous size are distributed over the entire ovary. However, counting oocytes in histological sections of neonatal ovaries is less reliable because oocytes are not distributed evenly and the diameter of oocytes varies considerably (Burgoyne and Baker, 1985). Dissociation of ovarian cells can remove this obstacle. Although it is inevitable to lose a considerable number of cells during processing, this method is suitable for comparing the germ cell populations at different stages and of different genotypes. In addition, dissociated cells give us the advantage to be able to identify precisely meiotic sub-stages by immunolabeling of synaptonemal complex (SC) components (Amleh et al., 2000; McClellan et al., 2003). The results suggest a switch from BAX-dependent to BAX-independent mechanism involved in germ cell elimination during ovarian development.

Fig. 1. (A,B) GCNA1 and MVH immunolabeling of germ cells in (A) Bax+/– and (B) Bax–/– ovaries. The same section was labeled with fluorophore-conjugated antibodies against GCNA1 (green) and MVH (red), and the merged image is shown in the middle. Bars, 200 μm. The adjacent sections were immunostained with DAB for GCNA1 (left) and MVH (right), and shown at a lower magnification. (A) At 12.5 d.p.c., MVH-positive cells are distributed over the ovary. Fewer GCNA1-positive cells are seen in the same area. All germ cells are positive for MVH in their cytoplasm but only some cells are also positive for GCNA1 in their cytoplasm. A small number of cells are positive for GCNA1 in the nucleus (arrowheads). At 14.5 d.p.c., similar numbers of MVH- and GCNA1-positive cells are distributed over the ovary. The majority of cells are positive for both MVH and GCNA1 in the cytoplasm and nucleus, respectively. However, some cells are positive only for MVH (arrowheads). At 18.5 d.p.c., less MVH labeling is seen in many cells over the ovary compared with the labeling at previous developmental stages. Intense labeling is also seen in fewer cells in clusters. GCNA1-positive cells are distributed in similar areas as the cells with intense MVH labeling. Most cells are positive for both MVH and GCNA1. However, GCNA1 labeling often extends into the cytoplasm and overlaps with MVH labeling. Some GCNA1-positive cells without MVH labeling (arrowheads) are seen in the surface epithelium. At 24.5 d.p.c., large MVH-positive cells are prominent in the central area and smaller cells are concentrated in the peripheral area. GCNA1 labeling is weaker and mainly seen in the peripheral area. Most large MVH-positive cells in the central area show either no or only faint GCNA1 labeling (arrowheads). Faint GCNA1 labeling is also seen in the cytoplasm of smaller cells (arrow). (B) At 12.5 d.p.c., MVH-positive cells are seen clustered in the gonad. A few GCNA1-positive cells are seen in the same area. Both MVH- and GCNA1-positive cells appear to be fewer compared with the Bax+/– ovary. Double immunolabeling shows three types of germ cells: most cells are positive only for MVH in the cytoplasm whereas fewer cells are also positive for GCNA1 in the cytoplasm or the nucleus (arrowheads). At 14.5 d.p.c., a considerably higher density of both MVH- and GCNA1-positive cells is seen in the Bax+/– ovary compared with the Bax+/– ovary. Most cells showed both MVH labeling in the cytoplasm and GCNA1 labeling in the nucleus as well as the cytoplasm. A few germ cells showed only MVH labeling (arrowheads). At 18.5 d.p.c., intense MVH labeling is seen in many cells of the Bax+/– ovary. GCNA1-positive cells are also more evenly distributed in the Bax+/– ovary compared with the Bax+/– ovary. MVH labeling is only seen in the cytoplasm whereas GCNA1 labeling is seen in both the nucleus and cytoplasm. Some cells are positive only for MVH (arrowheads). At 24.5 d.p.c., distribution of MVH- and GCNA1-positive cells was similar to that observed in the Bax+/– ovary, except that smaller MVH-positive cells were crowded near the peripheral area. GCNA1 labeling diminished in the large MVH-positive cells in the central area (arrowheads). In the Bax–/– ovary a larger number of germ cells with intense GCNA1 labeling in the nucleus was seen in the surface epithelium compared with the Bax+/– ovary.
many cells, whereas intense labeling was also seen in fewer clustered cells. This result was consistent using either fluorescence or histochemical detection methods. For comparison, GCNA1 labeling varied and was limited to a subpopulation of MVH-positive cells. At 12.5 d.p.c., three types of double immunolabeling were seen. The first type of cells was positive for MVH but negative for GCNA1. The second type of cells was positive for both MVH and GCNA in the cytoplasm. The third type of cells was positive for GCNA1 in the nucleus. At 14.5 d.p.c., the proportion of cells with both

| A | Wild-type |   |   |   |   |   |   |   |
|---|-----------|---|---|---|---|---|---|---|
|   | GCNA1     |   |   |   |   |   |   |   |
|   | DAB       | FITC | Merge | Rhodamine | DAB |
|   |           |      |        |            |     |
| (dpc) |         |      |        |            |     |
| 12.5 | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) | ![Image](image5) |
| 14.5 | ![Image](image6) | ![Image](image7) | ![Image](image8) | ![Image](image9) | ![Image](image10) |
| 18.5 | ![Image](image11) | ![Image](image12) | ![Image](image13) | ![Image](image14) | ![Image](image15) |
| 24.5 | ![Image](image16) | ![Image](image17) | ![Image](image18) | ![Image](image19) | ![Image](image20) |

| B | Bax null-mutant |   |   |   |   |   |   |   |
|---|-----------------|---|---|---|---|---|---|---|
|   | GCNA1           |   |   |   |   |   |   |   |
|   | DAB             | FITC | Merge | Rhodamine | DAB |
|   |                |      |        |            |     |
| (dpc) |            |      |        |            |     |
| 12.5 | ![Image](image21) | ![Image](image22) | ![Image](image23) | ![Image](image24) | ![Image](image25) |
| 14.5 | ![Image](image26) | ![Image](image27) | ![Image](image28) | ![Image](image29) | ![Image](image30) |
| 18.5 | ![Image](image31) | ![Image](image32) | ![Image](image33) | ![Image](image34) | ![Image](image35) |
| 24.5 | ![Image](image36) | ![Image](image37) | ![Image](image38) | ![Image](image39) | ![Image](image40) |

Fig. 1. See previous page for legend.
GCNA1 and MVH labeling increased, although a minority of cells still only showed MVH labeling. At 18.5 d.p.c., GCNA1 labeling was seen in the nuclei of the cells with intense MVH labeling but not in the cells with weak MVH labeling. Many GCNA1-positive cells without prominent MVH labeling were seen in or were occasionally protruding from the surface epithelium. At 24.5 d.p.c., GCNA1 labeling diminished in the nucleus of large MVH-positive cells, particularly in the central area. Weak but distinct GCNA1 labeling was seen in the cytoplasm of many other cells. The cells with intense GCNA1 labeling in the nucleus were concentrated in the peripheral area of the ovary. Much fewer GCNA1-positive cells were seen in the surface epithelium at 18.5 d.p.c. compared with 18.5 d.p.c. No distinct difference was found between Bax+/– and Bax+/+ ovaries (not shown).

The presence of germ cells with various locations of GCNA1 was confirmed in the dissociated cells from ovaries (Fig. 2). All patterns were found in ovaries at all developmental stages examined. Germ cells showing GCNA1 labeling only in the nucleus were more commonly seen in the ovary at 14.5 d.p.c., whereas germ cells showing GCNA1 labeling only in the cytoplasm predominated in the ovary at 24.5 d.p.c. Germ cells with GCNA1 labeling in both nucleus and cytoplasm were seen in ovaries at all stages.

Distribution of germ cells in the Bax+/− ovary
Distribution of germ cells, identified by immunolabeling of GCNA1 and MVH, in the Bax+/− ovary (Fig. 1B) was compared with that in the Bax+/+ ovary (Fig. 1A). At 12.5 d.p.c., considerably fewer MVH-positive cells were seen in Bax+/− ovaries. Cells positive for both GCNA1 and MVH were also fewer. At 14.5 d.p.c., by contrast, a higher density of cells labeled for both GCNA1 and MVH were seen in Bax+/− ovaries. A considerable number of germ cells also showed GCNA1 labeling in their cytoplasm. At 18.5 d.p.c., intense MVH labeling was seen in many cells distributed over the ovary. GCNA1-positive cells also appeared more than in the Bax+/+ ovary. Some cells were seen in the surface epithelium like in the Bax+/+ ovary. By double immunolabeling, a larger population of cells were positive for MVH and negative for GCNA1 in the Bax+/− ovary. At 24.5 d.p.c., large cells in the central area of the ovary showed only MVH labeling, as in the Bax+/+ ovary. However, smaller MVH-positive cells were crowded near the peripheral area, which was not seen in the Bax+/+ ovary. More cells with GCNA1 labeling in the nucleus were seen in the surface epithelium compared with the Bax+/+ ovary at this stage.

Expression of BAX proteins in the Bax+/+ ovary
Expression and localization of BAX proteins was examined by immunofluorescence labeling in the histological sections of Bax+/+ ovaries at four representative developmental stages (Fig. 3). At 12.5 d.p.c., faint BAX labeling was seen in the cytoplasm of all ovarian cells, including both somatic and germ cells. GCNA1 labeling was not apparent in the presumptive germ cells, which had large and round nuclei. At 14.5 d.p.c., more intense BAX labeling was seen in both somatic and germ cells. The latter cell type was clearly identified by GCNA1 labeling in the nucleus. Similar BAX labeling was seen in the ovary at 18.5 d.p.c. At 21.5 d.p.c., BAX labeling was particularly intense in the cytoplasm of large oocytes in the central region, whereas the labeling in somatic cells had diminished.

Loss of germ cells in Bax-mutant ovaries
The total number of MVH-positive cells was counted in the dissociated cells from ovaries of Bax−/−, Bax+/− and Bax+/+ females at 12.5 d.p.c. because GCNA1 labeling did not represent the total germ cell population at this stage; the mean values of total MVH-positive cells were 1545±291 (n=6), 976±104 (n=9), and 1863±657 (n=3), respectively. No statistical difference was found among these values. From 14.5 to 24.5 d.p.c., the total numbers of GCNA1-positive cells in chromosome-spreading preparations were compared between the ovaries of the three genotypes (Fig. 4). Significantly larger numbers of GCNA1-positive cells were seen in Bax−/− ovaries compared with Bax+/+ or Bax+/− ovaries at 14.5 and 18.5 d.p.c. (P<0.05). However, there was a rapid decline in the number of GCNA1-positive cells in the ovaries of the all three genotypes from 14.5 to 24.5 d.p.c. At 24.5 d.p.c., the number of MVH-positive cells in dissociated ovarian cells was also counted. The mean number in Bax−/− ovaries (2223±272, n=5) was significantly larger (P<0.01) than that in Bax+/− ovaries (1195±147, n=8), but not significantly different from the number in Bax+/+ ovaries (1096±534, n=4).

Meiotic progression in Bax-mutant ovaries
Meiotic progression in germ cells was examined in chromosome spreading preparations with immunolabeling of SC components. It was anticipated that SC-component labeling would be negative in the oogonium and would show characteristic patterns of meiotic prophase sub-stages in the oocyte. Typically, SC-component labeling appeared along the axial element of sister chromosomes at the leptotene stage, became thicker along the pairing cores of homologous chromosomes at the zygotene stage, completed thickening along the pairing cores at the pachytene stage and finally disappeared at the diplotene stage (McClellan et al., 2003). Similar proportions of GCNA1-positive cells at various cell cycle stages were observed in the ovaries of the three genotypes.
Oocyte loss in Bax-mutant ovaries at 14.5 d.p.c. (Fig. 5). The majority of GCNA1-positive cells were SC-component-negative whereas approximately 15% had reached the leptotene stage. At 18.5 d.p.c., over 90% of GCNA1-positive cells had entered meiotic prophase and its majority had reached the pachytene stage in the ovaries of the all three genotypes.

Discussion

GCNA1 is expressed in a subpopulation of germ cells

Both GCNA1 and MVH are known to be the markers for germ cells throughout fetal gonadal development (Enders and May, 1994; Toyooka et al., 2000). However, their expression patterns had not been systematically compared. We found that the expression of GCNA1 was delayed compared with that of MVH in fetal mouse ovaries of the B6 genetic background. Therefore, many germ cells were labeled positive for MVH and negative for GCNA1 in ovaries at 12.5 d.p.c. Furthermore, GCNA1 labeling was often seen in the cytoplasm rather than the nucleus at early developmental stages, suggesting that GCNA1 had not yet been transported to the nucleus. By 14.5 d.p.c., most germ cells were labeled positive for both markers, showing MVH labeling in the cytoplasm and GCNA1 in the nucleus. At 18.5 d.p.c., three distinct labeling patterns were seen. Almost all GCNA1-positive cells were also positive for MVH. A large number of these cells showed GCNA1 labeling in the cytoplasm in addition to the nucleus. However, many cells showed considerably weaker labeling for MVH and none for GCNA1. SC components, reliable indicators of meiotic prophase, were seen only in the cells with GCNA1 labeling in the nucleus (McClellan et al., 2003) (our unpublished observations). Therefore, GCNA1 expression in the nuclei of germ cells appears to coincide with their progression in meiotic prophase. Interestingly, many GCNA1-positive cells were seen in and, occasionally, protruding from the surface epithelium in ovaries at 18.5 d.p.c. These cells were often negative for MVH. It has been suggested that the extrusion from the surface epithelium is a mechanism of oocyte elimination in developing ovaries in mouse and human (reviewed by Makabe et al., 1991).

By 24.5 d.p.c., GCNA1 labeling diminished in the nuclei of large oocytes, which were most probably at the diplotene or dictyate stage, in the central area of the ovary. SC-component labeling was not seen beyond the early diplotene stage and, therefore, not very useful for identifying meiotic stages. In addition, we found many cells with GCNA1 labeling only in the cytoplasm, overlapping with MVH labeling. It is conceivable that GCNA1 was expressed but was not transported to the nucleus in the oocyte at late meiotic prophase. The cells with GCNA1 labeling in the nucleus were mainly seen in the peripheral area of the ovary, where germ...
cells usually enter and progress in meiotic prophase later in development. These observations suggest that the germ cell populations in fetal and neonatal mouse ovaries are more heterogeneous than previously thought. GCNA1 labeling in the nucleus identifies only a subpopulation of germ cells, but most probably represents cells that progress through meiotic prophase.

**BAX proteins are expressed in both germ and somatic cells in fetal and neonatal ovaries**

We confirmed that BAX was expressed in female germ cells as well as ovarian somatic cells from fetal to neonatal life. The labeling intensity increased from 12.5 to 14.5 d.p.c. in both types of cells, and then diminished in somatic cells afterwards. Therefore, any change in the cell death pathway during normal ovarian development cannot be attributed to the absence of BAX expression.

**Bax null-mutation increases oogonia population**

At 12.5 d.p.c., both MVH- and GCNA1-positive cells in the histological sections of Bax+/– ovaries appeared lower in number, compared with the Bax+/+ ovaries. However, the total number of MVH-positive cells counted in the dissociate cell preparations did not differ among the ovaries of the three genotypes. By contrast, many more MVH- and GCNA1-positive cells were seen in the histological sections of Bax–/– ovaries compared with Bax+/+ ovaries at 14.5 d.p.c. The total number of GCNA1-positive cells counted in chromosomespreading preparations from the Bax–/– ovary was also significantly higher than that from the Bax+/+ or Bax+/– ovaries. The majority of germ cells were in the mitotic cell cycle until this developmental stage. Accordingly, we speculate that, when oogonia rapidly proliferate in the fetal ovary, approximately half of them die by a BAX-dependent apoptotic pathway. This conclusion is consistent with previous reports concerning the death of primordial germ cells in gonads as well as in ectopic locations (De Felici et al., 1999; Rucker et al., 2000; Stallock et al., 2003). Our results also indicated that an increase in the GCNA1-positive cell population at the onset of meiosis was maintained throughout fetal development in the Bax+/+ mouse. This finding does not agree with the hypothesis that limited amounts of trophic factors permit the survival of only a fraction of germ cells; the fetal ovary appears to be capable of maintaining an excessive number of germ cells at least until neonatal life.

The results by Perez et al. (Perez et al., 1999) did not show any difference in the number of primordial and primary follicles between Bax+/+ and Bax–/– female mice at 4 days after birth. As Perez et al. have observed that the numbers of both primordial and primary follicles in Bax+/+ ovaries exceeded those in Bax+/– ovaries at 42 days after birth, they concluded that Bax deficiency renders granulosa cells and oocytes resistant to apoptosis, thereby reducing the rate of primordial and primary follicle atresia in postnatal life. Our results indicate that the number of germ cells in Bax+/+ ovaries remained higher than in Bax+/– ovaries up to 5 days after birth. The discrepancy between our results and those described by Perez et al. may have been caused by different methods for counting germ cells. If our results are correct, it is conceivable that the larger number of oocytes in the neonatal Bax+/+ female may contribute to longer reproductive life.

**Oocytes in the progression of meiotic prophase are eliminated despite BAX deficiency**

Despite the differences in the total numbers of GCNA1-positive cells, there were similar declines in the GCNA1-positive cell populations in the ovaries of the three genotypes from 14.5 to 24.5 d.p.c. These results indicate that GCNA1-positive cells were eliminated despite BAX deficiency. It has been reported that the germ cells in ectopic locations increase in number at 12.5 to 14.5 d.p.c. but they disappear by 18.5 d.p.c. in the Bax+/– mouse (Stallock et al., 2003). We propose that not only the germ cells in ectopic locations but also those in the ovary are eliminated by a BAX-independent mechanism.

**An excessive number of germ cells enter meiosis in the Bax–/– ovary**

Almost all GCNA1-positive cells entered meiosis in the ovaries of all three genotypes between 14.5 and 18.5 d.p.c., regardless of the number of cells at the start. We did not observe any abnormality in the pairing of homologous chromosomes at the pachytene stage in the Bax–/– ovary (data not shown). The fetal ovary appeared to be capable of processing the excessive number of germ cells to enter meiosis. It must be noted that MVH-positive cells without GCNA1 labeling were more often seen in the Bax+/+ ovary than in the Bax+/– ovary at 18.5 d.p.c. However, the germ cells with GCNA1 labeling in the nucleus were more often seen in the peripheral area of the Bax–/– ovary than in the Bax+/– ovary at 24.5 d.p.c. Therefore, we cannot rule out the possibility that the fate of oocytes may have deviated from normal development in the presence of an excessive number of oocytes in the Bax–/– ovary.

**Concluding remarks**

Based on our current findings, we conclude that a large number of oogonia is eliminated by a BAX-dependent apoptotic pathway whereas oocytes during the progression of meiotic prophase are eliminated by a different mechanism. We cannot exclude the possibility that oocytes are eliminated by another pathway of apoptosis, particularly in BAX deficiency. Nonetheless, our results clearly indicate that oogonia and oocytes use different mechanisms for their elimination. Further studies are needed to fully understand oocyte loss in normal female development.

**Materials and Methods**

**Animals**

All animal experiments were conducted in accordance with the *Guide to the Care and Use of Experimental Animal* issued by the Canadian Council on Animal Care and with the approval by the Animal Research Committee of McGill University. A breeding pair of Bax mutant mice on the B6 genetic background was purchased from the Jackson Laboratory (Bar Harbor, ME), and propagated in our mouse colony. Bax–/– male and female mice were crossed to produce Bax–/–, Bax+/– and Bax+/+ offspring. The gestation age was defined as days postcoitum (d.p.c.), assuming that the copulation occurred at 1:00 AM. Although delivery usually occurred at 19.5 d.p.c., we used the same method for counting the ages of newborn mice.

**Isolation of ovaries**

Pregnant females were sacrificed at 12.5, 14.5 and 18.5 d.p.c. and their fetuses were removed. Newborn mice were removed at 24.5 d.p.c. and their mothers kept for next experiments. Ovaries, with or without the adjacent mesonephroi, were collected and kept in Eagle’s minimum essential medium containing Hank’s salts supplemented with 0.25 mM Hepes buffer (MEM-H) (GIBCO/Life Science, Long Island, NY), and a piece of liver was taken from each mouse and stored at –20°C for genotyping. PCR amplification of the endogenous Bax gene and the Neo insert was performed under the conditions and primers described in the protocol from the Jackson Laboratory. Examples of genotyping are shown in Fig. 6.
Fig. 6. Identification of Bax genotypes by PCR. Samples 1 and 2 are from Bax<sup>−/−</sup> mice, resulting in a 200 bp single band, amplified from the Neo cassette inserted to mutate the Bax gene. Samples 3 and 4 are from Bax<sup>+/−</sup> mice, resulting in a 304 bp single band amplified from the endogenous Bax gene. Sample 5 is from a Bax<sup>+++</sup> mouse, resulting in two bands (200 and 304 bp). Sample 6 is a control from a Bax<sup>−/−</sup> mouse. S, standard 100 bp DNA marker.

Preparation of histological sections
Immediately after isolation, samples of ovaries with attached mesonephroi taken between 12.5 and 14.5 d.p.c. or samples of ovaries without mesonephroi from later developmental stages were fixed in 2% formaldehyde in microtube-stabilizing buffer (Messinger and Albertini, 1991) for 1 hour at room temperature, rinsed in phosphate buffered saline (PBS) and stored in 70% ethanol at 4°C. The fixed ovaries were embedded in paraffin and sectioned at 5-μm using a standard protocol. Serial sections from each ovary were placed on histology slides. The slides were kept in glass jars with silica gel at 4°C and used within a month. At least two ovaries from two litters of each genotype were examined at every developmental stage.

Double immunofluorescence of GCNA1 and MVH
Slides of single sections from the middle part of each ovary were deparaffinized and treated for antigen retrieval as described previously (Taketo et al., 2005). After three washes in holding buffer (HB) (Dobson et al., 1994), the slides were incubated with rat monoclonal anti-GCNA1 antibody at 1:50 at room temperature for 1 hour. After three washes in HB, the slides were incubated with goat anti-rat IgM antibody conjugated to biotin and goat anti-rabbit antibody conjugated to Rhodamine (Pierce), both at 1:1000 at room temperature for 1 hour. After three washes in HB, the slides were incubated with rabbit polyclonal antibody against MVH at 1:1000 for 1 hour. After three washes in HB, the slides were incubated with avidin conjugated to FITC and goat anti-rabbit antibody conjugated to Rhodamine (Pierce), both at 1:1000, for 30 minutes. After three washes in HB, the slides were incubated with goat anti-rabbit antibody conjugated to Rhodamine and goat anti-rat antibody conjugated to FITC (Pierce), both at 1:1000 for 30 minutes. The slides were then washed and mounted as described above.

Double immunofluorescence of BAX and GCNA1
Four sections from the middle part of each Bax<sup>−/−</sup> ovary were deparaffinized and treated as described above. After three washes in HB, the slides were incubated with rabbit anti-BAX antibody (sc-526, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 and rat monoclonal anti-GCNA1 antibody at 1:50 at room temperature for 1 hour. After three washes in HB, the slides were incubated with goat anti-rabbit antibody conjugated to Rhodamine and goat anti-rat antibody conjugated to FITC (Pierce), both at 1:1000 for 30 minutes. The slides were then washed and mounted as described above.

Double immunofluorescence of GCNA1 and SC components
Slides with chromosome-spread preparations were washed in HB and incubated with both the anti-GCNA1 antibody at 1:50 and rabbit polyclonal antibody against SC components (Dobson et al., 1994) at 1:1000 at room temperature overnight. The next day, the slides were washed and incubated with goat anti-rat antibody conjugated to FITC and goat anti-rabbit antibody conjugated to biotin, both at 1:1000, for 30 minutes. After three washes in HB, the slides were incubated with an avidin conjugated to Cy3 (Pierce). Finally, slides were washed in PBS, ddH<sub>2</sub>O and mounted in Prolong Antifade mounting solution (Molecular Probes, Eugene, OR) containing 0.4 g/ml 4',6-diamidino-2-phenyldino dehydrochloride (DAPI) (Boehringer Mannheim, Germany). The labeling was examined under an epifluorescence microscope (Zeiss Axiopt, Germany). All images were captured with a digital camera (Retiga 1300, QImaging, Burnaby, BC, Canada) and processed with Northern Eclipse digital imaging software, version 6.0 (Empix Imaging, Mississauga, ON, Canada).

Immunohistochemistry
After antigen retrieval as described above, the adjacent sections of the doubly GCNA1- and MVH-immunolabeled sections were incubated in 3% H<sub>2</sub>O<sub>2</sub> for 5 minutes, washed and incubated with either the anti-GCNA1 antibody at 1:50 or anti-MVH antibody at 1:1000 at room temperature for 1 hour. After three washes in HB, the slides were incubated with either goat anti-rat or goat anti-rabbit IgG antibody, both conjugated to biotin (Pierce), at 1:1000 for 30 minutes. After three washes in HB, the slides were incubated with the avidin-biotin horseradish-peroxidase complex from ABC Vectastain Kit according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). The slides were washed with PBS and incubated with 0.2 mg/ml 3,3-diamobenzidine (DAB) and 0.005% H<sub>2</sub>O<sub>2</sub> (both provided in a kit from Sigma-Aldrich Canada) for 7 minutes. The slides were rinsed with ddH<sub>2</sub>O and dehydrated for mounting in Histoclay (Clay Adams, Franklin Lakes, NJ). The labeling was examined in a transmission microscope (Zeiss Axiopt, Germany). All images were captured and processed as described above.

Dissociated cells/chromosome spreading preparations
Immediately after isolation, ovaries without mesonephroi were subjected to chromosome-spread preparations as previously described (McClellan et al., 2003). In brief, ovaries were placed individually in microfuge tubes and treated with 0.05% collagenase in MEM-H followed by 0.25% trypsin in Rinaldini solution (Rinaldini, 1959). After trypsin inactivation and two washes in MEM-H and one in PBS, each ovary was dissociated by repeated pipetting in 100 μl PBS and centrifuged for 3 minutes at 300 g. For chromosome-spread preparations, the pellet was resuspended in 20-80 μl (depending on the developmental stage) of MEM-H and kept at room temperature. A 20 μl aliquot of cell suspension was applied to each well of cytocentrifuge chamber (Thermo IEC, Needham Heights, MA) containing 400 μl of 0.5% NaCl solution (pH 8.0), placed on a histology slide and incubated for 5 minutes. The cells were spun down onto the slide, followed by fixation and three washes in 0.2% Photofto (Kodak, Eastman, NY) in H<sub>2</sub>O. Slides were then vacuum-dried and stored in boxes with silica gel at −20°C, and boxes were sealed. To preserve cytoplasmic structures, dissociated cells were centrifuged as described above and 100 μl of 2% formaldehyde fixative (as described for histological sections) was added to the pellet. After the tube was left for 5 minutes and centrifuged for 5 minutes at 600 g, fresh fixative was added to the pellet and centrifugation was repeated. After washing with 100 μl PBS, the pellet was resuspended in 50 μl PBS and applied to 400 μl PBS in each well of cytocentrifuge chamber. The cells were spun down, washed three times in 0.2% Photofto, and stored as described above. These slides were processed for double immunofluorescence labeling of GCNA1 and MVH, or MVH alone, as described above without antigen retrieval.

Statistical analyses
For estimating the total number of germ cells, at least three litters were examined and the mean and s.e.m. were calculated in each group. The percentages of meiotic substages were estimated by the same method. Student’s t-test was used to compare the results among the three genotypes of Bax mutants.

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