The pro-apoptotic gene Bax is required for the death of ectopic primordial germ cells during their migration in the mouse embryo

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Summary

In the mouse embryo, significant numbers of primordial germ cells (PGCs) fail to migrate correctly to the genital ridges early in organogenesis. These usually die in ectopic locations. In humans, 50% of pediatric germ line tumors arise outside the gonads, and these are thought to arise from PGCs that fail to die in ectopic locations. We show that the pro-apoptotic gene Bax, previously shown to be required for germ cell death during later stages of their differentiation in the gonads, is also expressed during germ cell migration, and is required for the normal death of germ cells left in ectopic locations during and after germ cell migration. In addition, we show that Bax is downstream of the known cell survival signaling interaction mediated by the Steel factor/Kit ligand/receptor interaction. Together, these observations identify the major mechanism that removes ectopic germ cells from the embryo at early stages.

Movies available online

Key words: Mouse, Bax, Cell death, Primordial germ cells

Introduction

In the mouse embryo, primordial germ cells migrate from the posterior primitive streak during gastrulation into the definitive endoderm (Anderson et al., 2000). Although they continue to be motile, they are held somehow in the developing hindgut until E9.5 (Molyneaux et al., 2001), when they emerge into the dorsal body wall, and migrate laterally into the genital ridges. By E11.5-12.5 germ cells have colonized the genital ridges, and sexually dimorphic sex cords are appearing. Germ cell migration is a very imprecise process, and a significant proportion of germ cells get left in structures around the migratory route at all stages of this process. Germ cells are found in the allantois during migration out of the primitive streak (Anderson et al., 2000), and in the hindgut, dorsal body wall and organs surrounding the gonad at later stages of migration (Upadhyay and Zamboni, 1982). Most or all of these ectopic germ cells are thought to die. The consequences of their not doing so can be detrimental. Nearly 3% of malignant pediatric tumors are germ cell tumors. More than 50% of these, and 18% of adult germ cell tumors, arise outside the gonad, mostly in midline structures (Gobel et al., 2000). They are presumed to arise from ectopic germ cells that failed to die (Schneider et al., 2001). Whether germ cell tumors are an inevitable consequence of failure to die in ectopic locations, or whether other tumorigenic events are required in ectopic locations is unknown.

Cell death is also a feature of germ cells that do arrive successfully in the genital ridges. Gonadal male germ cells undergo a wave of apoptosis between E13.5 and E17.0 (Coucouvanis et al., 1993), followed by a second wave of cell death around the time of birth, which depletes the number of male gonadal germ cells by around 50% (Roosen-Runge and Leik, 1968). Female gonadal germ cells also die in two phases: at E13.5, and between E15.5 and birth (Bakken and McClanahan, 1978; Beaumont and Mandl, 1962; Borum, 1961; Gondos, 1978). Cell death in the mouse germ line is due to apoptosis (Coucouvanis et al., 1993; Ratts et al., 1995; Wang et al., 1998). It is not known whether the mechanism of cell death of ectopic primordial germ cells that fail to arrive in the genital ridges is the same as that occurring later in germ cell differentiation.

Several studies have implicated the Steel/Kit interaction in survival of migrating primordial germ cells. In vivo, mutations in either gene lead to decreased numbers of germ cells arriving in the gonads (Bennett, 1956; Mintz and Russell, 1957), and in culture, withdrawal of Steel factor causes decreased survival of explanted germ cells (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991), which die by apoptosis (Pesce et al., 1993). The pathway downstream of the Kit receptor in primordial germ cells is unknown. In addition, other secreted factors have been reported to increase the survival of cultured germ cells, including LIF (De Felici, 2000; Koshimizu et al., 1996) and IL4 (Cooke et al., 1996).

Bcl2 family members have been implicated in later stages of intragonadal germ cell apoptosis. Mice homozygous for mutations in the pro-apoptotic family member Bax exhibit hyperplasia in the spermatogonial layers of the seminiferous tubules, and the germ cells fail to differentiate (Knudson et al., 1995). Female $Bax^{-/-}$ mice possess three times as many primordial follicles as $Bax^{+/+}$ equivalents, and these follicles last for a greater proportion than usual of the life-cycle (Perez et al., 1999). In gonadal germ cells, the requirement for Bax for germ cell apoptosis is balanced by a requirement for the

anti-apoptotic family member Bcl2l (previously known as Bcl-x) for their survival. Mice carrying two copies of a hypomorphic allele of Bcl2l have reduced numbers of germ cells in the gonads of both sexes, and this phenotype was rescued by removing Bax, indicating that a balance of these two pro- and anti-apoptotic Bcl2 family members controls the numbers of germ cells in the developing gonads (Rucker et al., 2000). However, the numbers of germ cells that colonize the gonads are normal in embryos carrying the hypomorphic allele of Bcl2l (Rucker et al., 2000), indicating that Bcl2l is not required before this time. If this anti-apoptotic protein was required during migration, more of the migrating germ cells would die, and fewer would colonize the gonads.

In previous work, using a living marker of germ cells, we have shown that germ cells leave the hindgut between E9.0 and E9.5, and migrate laterally towards each gonad (Molyneaux et al., 2001; Molyneaux et al., 2003). By E10.5, the embryos has grown significantly in size, and a mesentery has formed that displaces the hindgut from the dorsal body wall, and increases the distance of migration. Germ cells are left at all points along the path from the hindgut to the genital ridges. Movies of slice cultures of embryos at E10.5 showed that those in the midline, or in the mesentery of the gut, at E10.5, do not migrate to the genital ridges. Instead, they disintegrate and die, while the germ cells closest to the genital ridges migrate into it and survive (Molyneaux et al., 2001). This mechanism generates a tight cluster of germ cells in and around the genital ridges by E11.5, with few ectopic germ cells.

The mechanism by which the germ cells left in the middle of the embryo are eliminated is unknown. It is likely to be different from the mechanism that maintains germ cell numbers later in the gonads, as the anti-apoptotic Bcl2l protein is not required for normal numbers of germ cells to colonize the genital ridges. To initiate germ cell death, a pro-apoptotic gene has to be activated. Bax has not previously been shown to be present during germ cell migration, but is a likely candidate for a pro-apoptotic function, as it has a role later in development in the germline. In light of the above studies, we bred mice containing a targeted allele of Bax (Knudson et al., 1995) into a line of mice carrying the Oct4ΔPE:GFP transgene (Anderson et al., 2000), which allows direct observation of germ cell behavior in living embryos or embryo slices (Molyneaux et al., 2001). We report a significantly increased number of ectopic germ cells in *Bax*$^{-/-}$ embryos. The ectopic germ cells are developmentally delayed. They retain expression of early PGC markers and retain motility. This shows that signals from the gonad regulate expression of PGC markers and inhibit their motility. *Bax*$^{-/-}$ ectopic germ cells occupy many positions in the embryo. However, they do not grow, and their numbers dwindle until by E18.5 very few can be found. As these mice have not been reported to have an increased incidence of germline tumors, the data suggest that mice have a back-up mechanism for removing embryonic migratory germ cells in ectopic locations. We also show that inactivation of Bax protects germ cells against rapid cell death in culture, and against removal of the Steel/Kit signaling interaction in culture. This shows that Bax is downstream of the Kit receptor. However, protection against cell death in culture is a short-term effect, showing that other apoptotic pathways exist in germ cells.

Materials and methods

Mice

Homozygous Oct4ΔPE:GFP^{+} mice (Anderson et al., 1999), established on an FVB background, were crossed to heterozygous Bax mutant mice on a 129SV background (Knudson et al., 1995). Littermates were crossed to create a line of mice that were Oct4ΔPE:GFP^{+} homozygous with a Bax heterozygous mutation. Bax heterozygotes were crossed to each other to give litters that contained *Bax*$^{+/+}$, *Bax*$^{+/-}$ and *Bax*$^{-/-}$ embryos. E0.5 was assumed to be noon of the morning a vaginal plug was observed. Genotyping primers for Oct4ΔPE:GFP^{+} were F-5′GGA GAG GTG AAA CCG TCC CTA GG3′ and R-5′GCA TCG CCC TCG CCC TCG C3′ producing a 250 bp fragment (Yeom et al., 1996). Bax genotyping was done using the following PCR primer set (Deckwerth et al., 1996): Bax intron 5 reverse primer (BaxIN5) 5′-GTTGACCAGAGTGGCGTAGG-3′; Neo/PGK reverse primer (NeoR) 5′-CCGCTTCCATTGCTCAGC-GG-3′; Bax exon 5 forward primer (BaxEX5) 5′-GAGCTG-ATCAGAACCATCATG-3′. Wild-type band is formed from the BaxIN5 and BaxEX5 primers giving a 304 bp fragment, and the Bax mutation yields a 507 bp band formed from the BaxIN5 and NeoR primers.

Organ culture

Embryos were obtained by euthanizing pregnant females with $C0_2$, followed by cervical dislocation, and immediate extraction of the uterus from the female. Embryos were removed from the uterus and extra-embryonic tissue in 1×PBS + 2% FCS, and then placed in Hepes-buffered DMEM/F-12 medium plus 100 U/ml penicillin/streptomycin (Gibco BRL), which we designate as DF-12 media. Slices were cut from the trunk of embryos using a scalpel. Slices in ~50 μl of DF-12 were placed into millicell CM organ culture chambers (Millipore) pre-coated with collagen IV (Becton Dickinson). The millicell organ chambers were placed into 50 mm glass-bottomed culture dishes (Willco Wells, the Netherlands), and the dishes were filled with ~2-3 ml DF-12 medium. Filming was carried out with a Zeiss LSM510 confocal system attached to a Zeiss axiovert inverted scope. Images were captured during filming every 7 minutes for 700 minutes. Organ cultures were maintained at 37°C during filming by placing the culture dish in a heating stage (Zeiss), and creating a humidity chamber with wet paper towels placed in a 100 mm culture dish fastened over the organ culture dish.

Wholemounts, cryosections and antibody staining

Gonads were extracted from pregnant females as stated above. Whole embryos were fixed in 4% PFA (Sigma) overnight at 4°C, then washed twice with PBS. For cryosections, embryos were submerged in 20% sucrose and embedded in OCT medium. For wholemounts, gonads or partial embryos were placed in PBS + 0.1% TritonX100 (Fisher) overnight at 4°C. SSEA1 antibody (The Developmental Studies Hybridoma Bank, University of Iowa), an IgM mouse monoclonal antibody, was used at a 1:100 dilution (4°C overnight). Samples were washed five times for 1 hour in PBS+0.1% Tx-100 and exposed to the secondary antibody, an anti-mouse IgM Cy5 conjugate (Jackson ImmunoResearch) at a 1:100 dilution (4°C overnight). Samples were washed as described above and mounted in 80% glycerol. Alkaline phosphatase staining followed the protocol outlined by Hogan et al. (Hogan et al., 1994). Whole gonads were exposed to AP staining solution for 12 minutes prior to being washed in PBS and cleared overnight in 80% glycerol. AP-stained gonads were viewed with the Cy3 channel on the LSM510 confocal microscope.

Cell culture

Feeder layers consisted of either irradiated MEF cells or irradiated STO cells plated in a 12-well organ culture plate (Becton Dickinson) at ~80% confluency in 1.25 ml of DMEM (Gibco BRL) + 10% FCS (Gibco BRL) + 2 mM reconstituted glutamine (Gibco BRL) + 1×

antimycotic/antibiotic (Gibco BRL), which we designated MEFs Medium. For the E10.5 PGC culture experiments, the area between forelimb and hindlimb buds was isolated using a scalpel, and the neural tube and notochord were excised and discarded. In the E12.5 experiments, whole gonads were isolated along with the dorsal aorta and midline mesenchymal tissue. Hindlimb and tail tissue was frozen for genotyping. PGC-containing tissue was trypsinized (Gibco BRL) for a 12 minute interval, triturated and added to medium-laden feeder cell plates (one embryo per well). Initial PGC counts were calculated using a hemacytometer (Fisher). All counts were done on a Zeiss Axiovert 100M inverted fluorescence microscope. Genotyping of embryos was carried out after the experiment and all counts had been concluded. ACK-2 (22.5 mg/ml stock) and J2.1 (20.2 mg/ml stock) were a kind gift from Dr Fred Finkelman (Veterans Hospital, Cincinnati, OH). ACK-2 and J1.2 were diluted to the respective amounts in MEFs medium and added directly to the feeder cells prior to addition of trypsinized gonads. AP staining of cultured PGCs was done with incubation of cell culture wells in AP staining solution (described above) for 4 minutes, and images were taken with the LSM510 confocal microscope. AP staining was visualized with the Cy3 channel on the LSM 510 confocal microscope.

RT-PCR

PGC isolation and reverse transcription was performed as previously described (Molyneaux et al., 2003). PGC and somatic cDNAs were diluted 1:10 and 1 μl used for PCR (25 μl). RedMix Plus (PGC Scientific) was used as the source of Taq, buffer and dNTPs. Final primer concentrations were 0.4 μM. PCR consisted of an initial denaturing step of 5 minutes at 95°C; followed by 5 cycles of 30 seconds at 95°C, 1 minute at 65°C, 30 seconds at 72°C; followed by 35 cycles of 30 seconds at 95°C, 1 minute at 60°C, 30 seconds at 72°C; followed by a 10 minute extension step at 72°C. Bax primers (Rucker at al., 2000) generate a 162 bp product: BaxF, 5′-ATG-CGTCCACCAAGAAGCTGAG-3′; BaxR, 5′-CCCCAGTTGAAG-TTGCCATCAG-3′.

Results

Bax is expressed in germ cells during migration

In previous studies, Bax has been shown to be involved in maintenance of germ cell numbers in the gonad. However, Bax expression has not previously been demonstrated in germ cells during migration (Felici et al., 1999). Fig. 1 shows by RT-PCR that Bax mRNA is present in E10.5 germ cells. PGCs were prepared by flow cytometry from the Oct4ΔPE:GFP strain of mice (Anderson et al., 1999) and the purity of the sort was confirmed by RT-PCR for germ cell markers (Kit, Stag3) and somatic markers (Steel, cystatin 3 and Sparc) (data not shown).

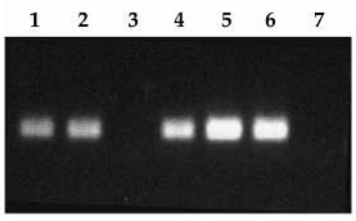

Fig. 1. Bax is expressed in migratory and post-migratory PGCs. Lane 1, E10.5 PGCs (gfp+); lane 2, E12.5 PGCs (gfp+); lane 3, E12.5 PGC cDNA RT-; lane 4, E12.5 whole embryo cDNA (+ control); lane 5, E11.5 PGCs (gfp+); lane 6, E11.5 somatic tissue from the genital ridge (gfp–); lane 7, E11.5 somatic tissue RT-. PGCs were prepared by flow cytometry and the purity of the sort was confirmed by RT-PCR for germ cell markers (Kit, Stag3) and somatic markers (Steel, cystatin 3 and Sparc) (data not shown).

Ectopic germ cells lacking Bax do not die in slice cultures of migrating primordial germ cells

Bax mutant mice (Knudsen et al., 1995) were bred into the Oct4ΔPE:GFP strain of mice (Anderson et al., 1999) to create a double transgenic line of mice that enabled direct observation of wild-type and mutant germ cells in living embryo cultures.

Slice cultures taken from Oct4ΔPE:GFP embryos have been useful in studying the behavior of living germ cells during migration (Molyneaux et al., 2001). Using this method, we found previously that during the E10.5 to E11 period, germ cells have already emerged from the hindgut, and migrate laterally both singly and in aggregating clusters, to the gonadal ridges; while germ cells remaining distant from the genital ridges, in the midline or in the mesentery, fragment and die (Molyneaux et al., 2001). To identify the role of Bax in this process, we filmed slices dissected from $Bax^{-/-}$, $Bax^{+/-}$ and $Bax^{+/+}$ littermates. We crossed $Bax^{+/-}$ Oct4DPE:$GFP^{+/+}$ animals, harvested embryos at E10.5 and cut two transverse

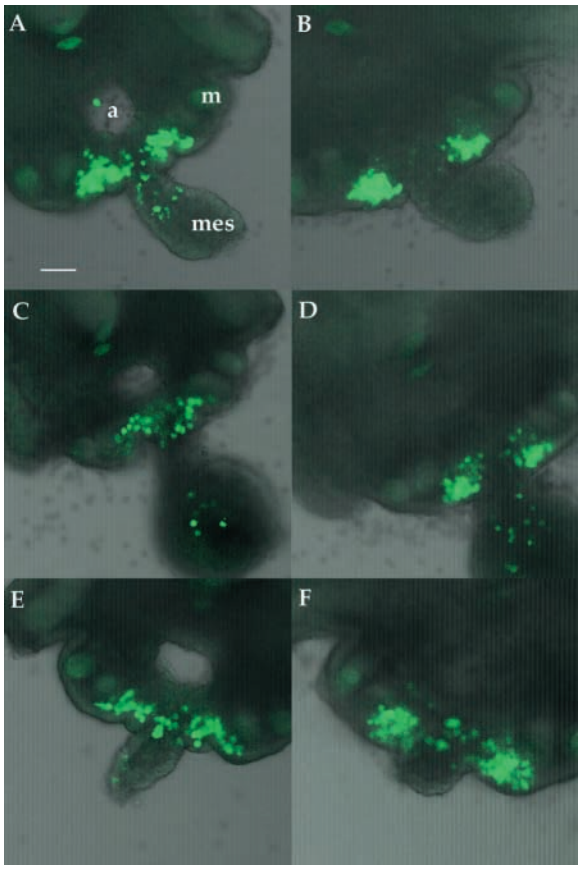

Fig. 2. The survival of PGCs in the midaxial region of the embryo in $Bax^{-/-}$ embryos at E10.5. (A,B) PGCs at 0 (A) and 700 (B) minutes in a slice from an E10.5 $Bax^{+/+}$ embryo. a, aorta; m, mesonephric duct; mes, mesentery of the gut. PGCs in the mesentery and midaxial region near the aorta fragment and die during the culture period. (C-F) Slices from $Bax^{-/-}$ embryos, showing that after 700 minutes of culture, germ cells distant from the genital ridges do not fragment and die. C and E show slices at 0 minutes; D and F show the same slices at 700 minutes. Scale bar: 50 μm.

200 μm slices through each embryo. Slices from an entire litter were filmed simultaneously for a 12 hour period. The cranial end of each embryo was genotyped by PCR. The genetic constitution of one such litter was: 2×*Bax*$^{+/+}$, 3×*Bax*$^{+/-}$ and 4×homozygote *Bax*$^{-/-}$. Fig. 2 shows images from different time-points of movies from the wild-type (Fig. 2A,B) and *Bax*$^{-/-}$ (Fig. 2C-F) slices. In the wild type, germ cells that failed to migrate with the main group of germ cells, and instead remained in the mesentery and midline area, fragmented and disappeared during the 700 minute time period (4/4 slices), leaving the central area of the embryo clear of germ cells. Those in the *Bax*$^{-/-}$ slices survived (7/8 slices). Movies are available online at http://dev.biologists.org/supplemental/.

Bax$^{-/-}$ embryos have many surviving ectopic germ cells after the migratory period, which later disappear

The distribution of germ cells was examined in cryostat sections from *Bax*$^{+/+}$, *Bax*$^{+/-}$ and Bax$^{-/-}$ embryos at E13.5 and E14.5. At E13.5, a variable number (up to 100) ectopic germ cells could be found in both *Bax*$^{+/+}$ and *Bax*$^{-/-}$ embryos. These were located immediately surrounding the forming gonad and in the midline (Fig. 3). In *Bax*$^{-/-}$ embryos, additional ectopic groups of germ cells were found in several locations, including midline structures, the spinal cord and in the mesenteries of the gut. In each case, ectopic GFP+ cells were positive for the germ cell markers SSEA1 and alkaline phosphatase. The data shown in Fig. 3 are from a single litter. Similar data were found for two other litters (not shown). By E14.5, there were very clear differences in germ cell distribution between *Bax*$^{+/+}$ and *Bax*$^{-/-}$ embryos. Only two out of 21 Bax$^{+/+}$ embryos had any identifiable ectopic germ cells. These were found in the capsule of the gonad. By contrast, 12 out of 12 *Bax*$^{-/-}$ embryos had large numbers of ectopic germ cells (100-1000+), within the capsule of the gonad and surrounding midline structures (Fig. 4). *Bax*$^{+/-}$ embryos (21 out of 21) also had ectopic germ cells. Although quantitation was not attempted, there were many fewer ectopic germ cells seen in *Bax*$^{+/-}$ embryos, suggesting a gene dose requirement for Bax at this stage. Ectopic germ cells at E13.5 and E14.5 retained markers that are downregulated in the gonads. Fig. 4C,D show whole dissected E14.5 gonads, together with their mesenteries, that were stained for alkaline phosphatase. The gonadal germ cells, identified by GFP, had downregulated alkaline phosphatase, while the ectopic germ cells in the mesentery of the gonad still retained it. Fig. 4E,F show that the ectopic germ cells still retained the carbohydrate germ cell marker SSEA1. By E15.5, there were no ectopic germ cells found in *Bax*$^{+/+}$ embryos (eight out of eight embryos from the three litters examined), and the numbers seen in *Bax*$^{-/-}$ embryos were less than at E14.5 (10 out of 10 embryos). Quantitation was not possible in

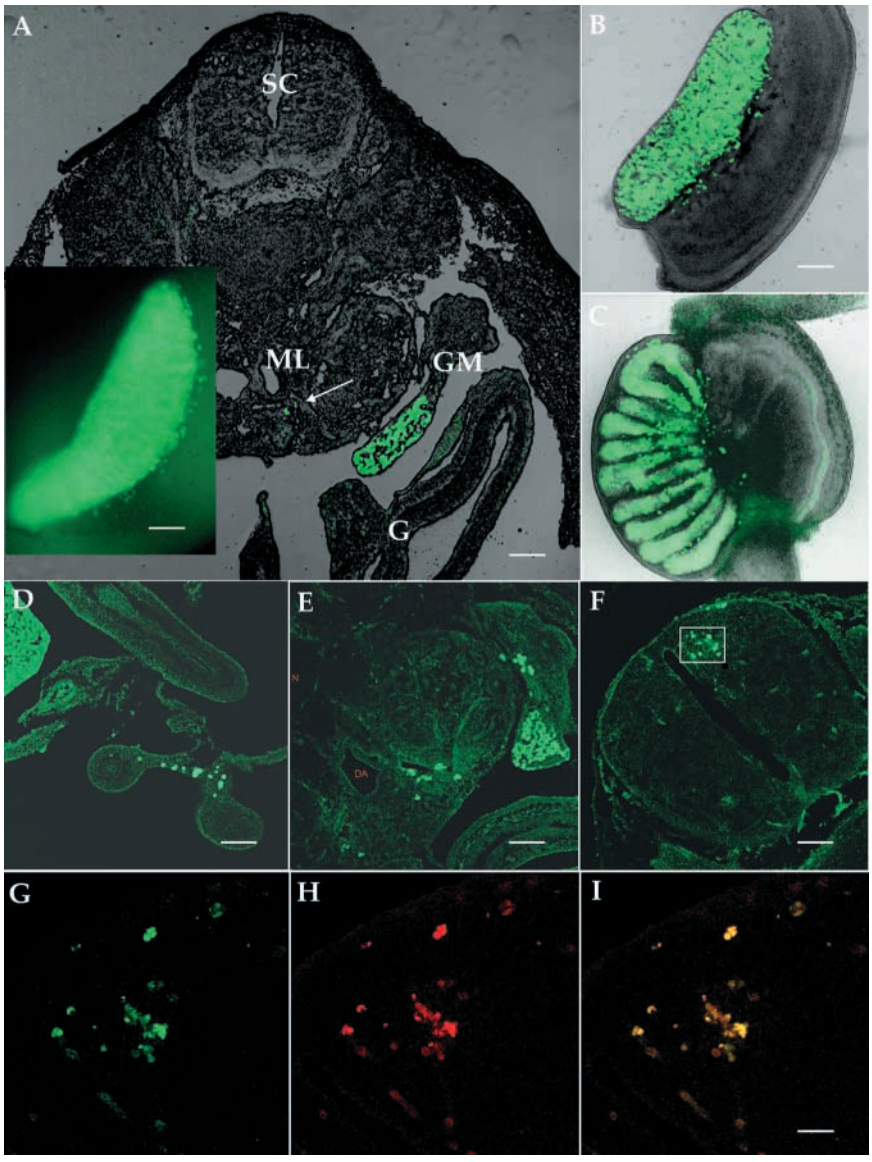

Fig. 3. Positions of ectopic PGCs in frozen sections or whole isolated gonads at E13.5. (A) Cryostat section through a *Bax*$^{+/+}$ embryo. SC, spinal cord; ML, midline mesenchyme; GM, gonad mesentery; G, gut. Almost all the germ cells are in the gonad, which can be seen as a GFP+ mass of cells at the end of the gonad mesentery. Occasional cells are found in the midline mesenchyme (arrow), and around the junction of the gonad and its mesentery (right hand side of whole gonad shown in the inset). (B,C) Whole isolated gonads from female (B) and male (C) *Bax*$^{-/-}$ embryos, showing ectopic PGCs at the hilus of the gonad, similar to the *Bax*$^{+/+}$ embryos. In addition, *Bax*$^{-/-}$ embryos have groups of ectopic PGCs at other locations, including the gut mesentery (D), gonad mesentery and midline mesenchyme (E) and spinal cord (F). In each location, PGCs were also positive for alkaline phosphatase staining, shown for a group of cells in the spinal cord (G-I; enlargement of boxed area in F; green, GFP; red, alkaline phosphatase; yellow, merged images). Scale bars: 160 μm in A (inset 174 μm); 215 μm in B,C; 115 μm in D-F; 163 μm in E; 53 μm in G-I.

cryosections of embryos of this size, but numbers were estimated at 30-500 ectopic germ cells per embryo. In cryosections from E18.5 embryos (two *Bax*$^{+/+}$, three *Bax*$^{+/-}$, three *Bax*$^{-/-}$, from two litters), we found only one cluster of ectopic germ cells in one *Bax*$^{-/-}$ embryo, near the dorsal aorta, in the lower thoracic/upper lumbar region of the embryo. Two 2-day-old *Bax*$^{-/-}$ mice were fixed, dissected and cleared. No ectopic germ cells were found.

These results suggest that Bax is required for the normal mechanism that kills off ectopic germ cells at E13.5-14.5, but ectopic germ cells die of a Bax-independent mechanism later in development.

Loss of Bax causes temporary, but not permanent, protection from cell death of germ cells when explanted into culture

Primordial germ cells explanted into culture are extremely sensitive to the absence of survival signals, and will only survive for short periods in culture, and require specific types of feeder layer to do so (Donovan et al., 1986). As Bax is required for the death of germ cells seen in the mesentery and midline during migration at E10.5, we hypothesized that *Bax*$^{-/-}$ germ cells explanted into culture, and thus removed from their normal signals, might be rescued from the high levels of cell death normally seen. Germ cells were isolated from E10.5 and E12.5 embryos, and cultured on either STO cells, or primary cultures of mouse embryonic fibroblasts (MEFs), both of which have been shown to promote germ cell survival. In each experiment, each embryo from a single litter was taken, the region containing the genital ridges dissected as described previously (Cooke et al., 1993) and the rest of the embryo used for genotyping. The genital ridges were disaggregated, and the number of germ cells in each separate embryo determined by hemocytometer counting. Harvested cells from each embryo were then cultured separately on either STO of MEFs and the germ cells counted at different time intervals under the fluorescent microscope. Because the germ cells express GFP, the same living cultures can be scored for germ cell number at different time-points. In order to formally exclude the possibility that other cell types express the Oct4/GFP transgene in culture, we co-stained one culture with alkaline phosphatase to confirm that the GFP positive cells were germ cells (data not shown). Fig. 5 and Table 1 show data from two E10.5 litters and one E12.5 litter. First, the numbers of germ cells present in the cultures at 48 hours (which we have defined as 'survival' in Fig. 5 and Table 1, although the numbers will reflect cell division as well as failure to die) was variable from experiment to experiment; a greater percentage of germ cells survived in the second batch of embryos. We routinely find this in culture experiments, and it is the reason for the experimental design used here: to make an embryo-by-embryo comparison within single litters. The variability from experiment to experiment may be due to the health of the MEFs, or the stringency with which embryo dissections from different experiments are trypsinized. Second, it shows that large numbers of germ cells died in culture, even when seeded onto feeder layers that promote their survival. This is also a common observation. Third, it is clear that there is a significant difference between the percentage survival of *Bax*$^{-/-}$ germ cells, compared with both *Bax*$^{+/+}$ and heterozygous germ cells. There is also a significant difference between germ cell survival between *Bax*$^{+/+}$ and heterozygous embryos, indicating a gene dosage effect of Bax. The same result was seen with E12.5 embryos. It has previously been reported that fewer germs cells survive from E12.5 embryos than from E10.5 embryos (Donovan et al., 1986), and this is seen again in Fig. 5. However, as at E10.5, loss of Bax function has a rescuing effect on the germ cells.

Although loss of Bax function significantly increased short-term survival, long-term survival was not rescued. In all the experiments shown above, all germ cells had died 7 days after culture, and attempts to passage them onto new feeder layers did not increase survival (data not shown). This supports the conclusion from the in vivo results above, that there is a later-occurring Bax-independent mechanism of germ cell death.

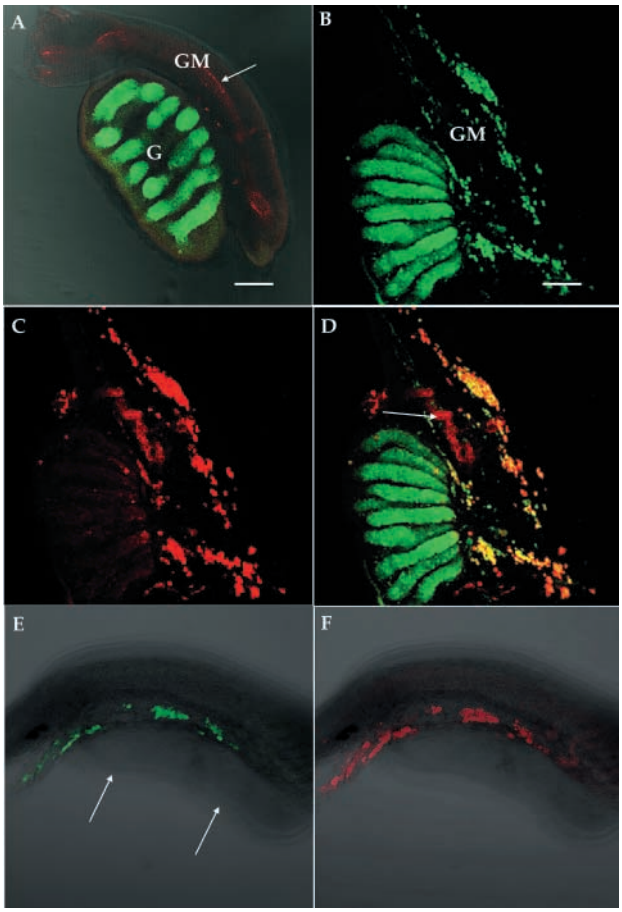

Fig. 4. Whole gonads (G), together with their mesenteries (GM) dissected from E14.5 embryos. In the *Bax*$^{+/+}$ embryos (A), no ectopic germ cells were found in the gonad mesenteries. Alkaline phosphatase staining was seen in blood vessels in the gonad mesentery (arrow), but not in the PGCs. In *Bax*$^{-/-}$ embryos (B-F), large numbers of ectopic PGCs were found in the gonad mesenteries. These retain alkaline phosphatase staining (C, overlaid in D) and SSEA1 staining (E,F; green, GFP; red, anti-SSEA1 stain). In D, the arrow indicates alkaline phosphatase staining in blood vessels. In E and F, the plane of section is through the hilus of the gonad. The body of the gonad is out of focus (arrow in E). Scale bars: 145 μm in A; 163 μm in B-F.

Interaction between Bax and Steel/Kit signaling

As Steel factor is a known survival factor for early mouse germ cells, we tested whether cell death following inhibition of Steel

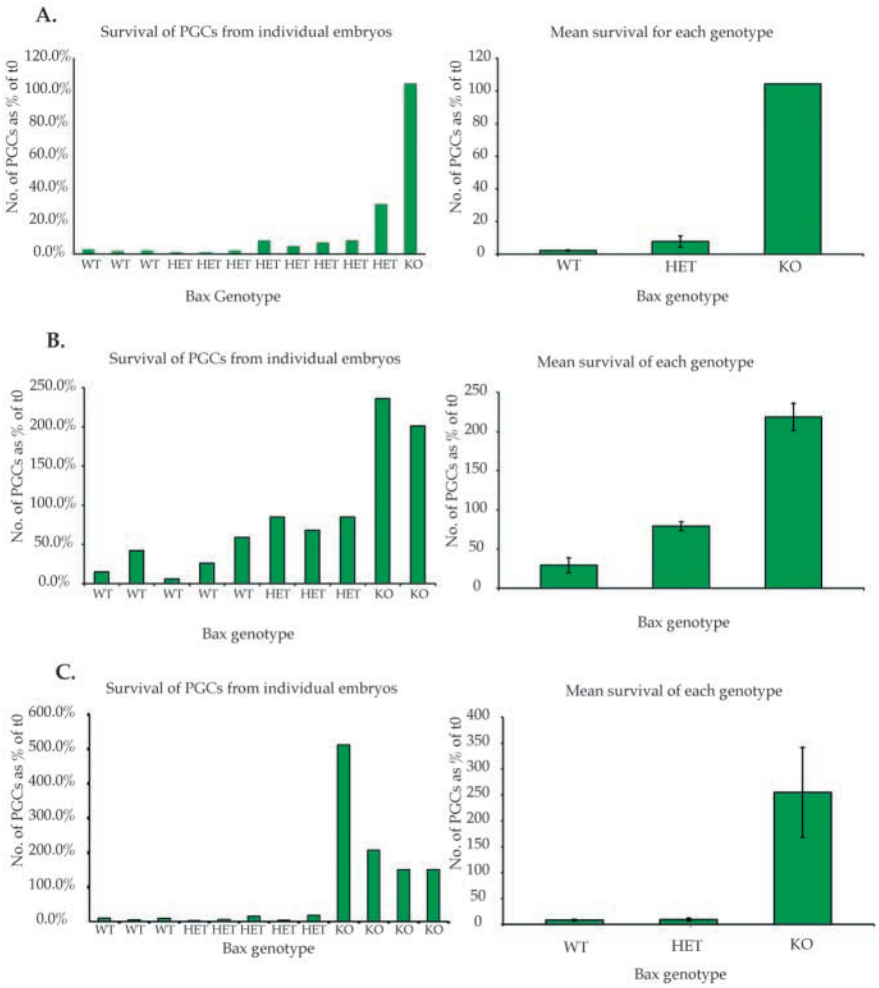

Fig. 5. Forty-eight hour survival of PGCs isolated from E10.5 (A,B), and E12.5 (C) embryos, and cultured on MEFs. The data are shown as 48 hour survival of PGCs from each embryo of one litter (left hand side) and the means (±s.e.m.) for embryos of the same genotypes in the litter (right hand panels). There is a significant increase in 48 hour survival of PGCs that are *Bax*$^{-/-}$ (KO), compared with *Bax*$^{+/+}$ (WT) and *Bax*$^{+/-}$ (HET) PGCs at 48 hours. Survival is measured as the % of germ cells counted in each sample at T0. See text for details.

factor is mediated by Bax. The germ cell-containing regions of individual E10.5 embryos from Bax$^{+/-}$,Oct4DPE:GFP$^{+/+}$ crosses were dissected as described previously (Cooke et al., 1993). The germ cell number in each cell suspension was counted on a hemocytometer, and the cell suspensions were then split and seeded onto MEF or STO cell feeder layers in the presence of either the Kit blocking antibody Ack-2 (Okada et al., 1991) or an isotype-matched control immunoglobulin J1.2. The Ack-2 antibody has been previously shown to block the survival of cultured germ cells (Matsui et al., 1991). Fig. 6 and Table 2 show that the absence of Bax protected germ cells against cell death by of the Steel/Kit interaction, and therefore that Bax is an essential downstream component of germ cell death following loss of the Steel/Kit signaling interaction.

Discussion

The pro-apoptotic Bcl2 family member Bax is already known to mediate the normal apoptosis of both female and male gonadal germ cells (Knudson et al., 1995; Perez et al., 1999). It is also thought that a steady state number of germ cells in the gonads may be maintained by a balance of pro-and anti-apoptotic Bcl2 family members (Rucker et al., 2000). However, the death of early germ line cells that do not migrate correctly to the genital ridges is not understood. Their apoptosis is assumed to be due to the limited range of survival factors released by surrounding tissues, but how this results in their death in not known. Bax has not previously been identified in primordial germ cells before gonad

Table 1. Forty-eight-hour survival of PGCs cultured on MEFs

A E10.5 Litter 1

Genotype	Number of PGCs at 0 minutes	% still present at 48 hours	Mean (±s.e.m.)
Bax$^{+/+}$	900, 351, 401,	2.8, 1.7, 2.0	2.2% (±0.3)
Bax$^{+/-}$	702, 495, 198, 250, 302, 351, 99, 250	1.0, 1.0, 2.0, 8.0, 4.6, 6.8, 8.1, 30.4	7.7% (±3.4)
Bax$^{-/-}$	302	104.3	104.3

B E10.5 Litter 2

Genotype	Number of PGCs at 0 minutes	% still present at 48 hours	Mean (±s.e.m.)
Bax$^{+/+}$	198, 198, 630, 252, 252	14.6, 41.9, 5.6, 25.8, 58.7	29.3%(±9.5)
Bax$^{+/-}$	198, 149, 252	84.8, 67.8, 84.9	79.2% (±5.7)
Bax$^{-/-}$	495, 302	236, 201	218.5% (±17.5)

C E12.5 Litter

Genotype	Number of PGCs at 0 minutes	% still present at 48 hours	Mean (±s.e.m.)
Bax$^{+/+}$	2473, 1493, 2707	10, 5, 9	8%(±1.5)
Bax$^{+/-}$	3262, 4200, 560, 2380, 2753	2.2, 6, 15, 4, 18	9%(±3.1)
Bax$^{-/-}$	793, 1680, 2147, 3267	512, 207, 150, 150	254.8%(±86.8)

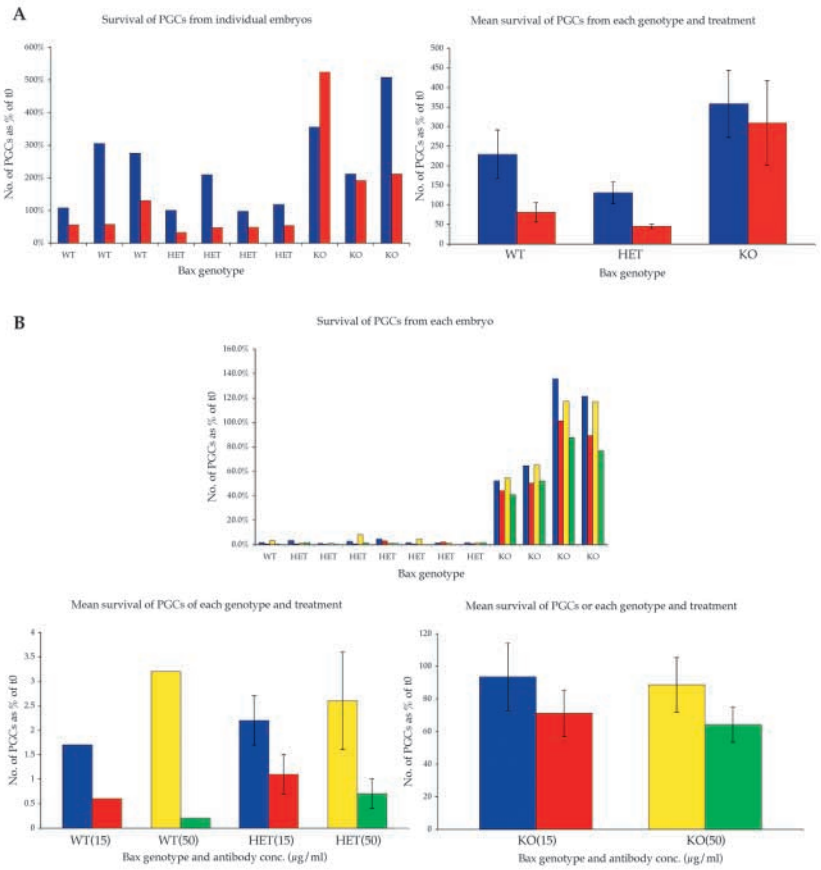

Fig. 6. Shows the effect of an anti-Kit antibody or an isotype matched control antibody (J1.2) on 48 hour survival of either E10.5 (A) or E12.5 (B) germ cells. Survival is shown first as % of the number of PGCs counted in each embryo sample from a single litter at T0, and second, as means (±s.e.m.) for all embryos of the same genotype in the litter. In the E10.5 sample (A), 15 μg/ml of control (blue bars) and anti-Kit (red bars) were used. In the E12.5 sample (B), two concentrations of antibody were used; 15 μg/ml and 50 μg/ml. The effects of each dose are shown separately on the charts. Antibody concentrations of 15 μg/ml are represented by blue (control antibody) and red (anti-Kit antibody) bars (as in A), while the effects of 50 μg/ml of control or anti-Kit are shown as yellow and green bars, respectively. The anti-Kit antibody causes a significant reduction in germ cell survival at 48 hours in both E10.5 and E12.5 *Bax*$^{+/+}$ and *Bax*$^{+/-}$ embryos, but not in *Bax*$^{-/-}$ embryos. In the E12.5 litter, there was only one *Bax*$^{+/+}$ embryo, so there is no s.e.m.

colonization, and in embryos carrying targeted mutations in Bcl2l, normal numbers of germ cells arrive in the genital ridges (Rucker et al., 2000). Apoptosis resulting from the limited range of survival factors released by the genital ridges is theoretically a mechanism for removing potential tumorigenic cells in the embryos. This is supported by data from cultured germ cells, which rapidly die in the absence of survival factors from feeder layers (Donovan et al., 1986), and can be seen to die at sites distant from the genital ridges in slice cultures of embryos (Molyneaux et al., 2001). The current work lends additional support to this notion, and provides some additional insights into the mechanism of ectopic germ cell death.

The visible survival of ectopic germ cells in the midline of the embryo slices suggests that there is a defined range of survival signals being released by the genital ridges, that the mesentery and midline body wall are outside of this range, and that the absence of such signals activates a Bax-mediated cell death event. Such a mechanism is elegant, because as the

Table 2. Effect of anti-Kit blocking antibody ACK2 on germ cell survival compared with an isotype-matched control antibody J1.2

A Effect on germ cells from each embryo of one litter

	Number of PGCs at 0 minutes	J1.2 (15 μg/ml)	ACK (15 μg/ml)	J1.2 (50 μg/ml)	ACK (50 μg/ml)
Wild type	467	1.7%	0.6%	3.2%	0.2%
Bax$^{+/-}$	336	3.3%	0.6%	1.2%	1.5%
Bax$^{+/-}$	798	0.8%	0.4%	0.9%	0.1%
Bax$^{+/-}$	467	2.6%	0.6%	8.1%	1.3%
Bax$^{+/-}$	264	4.5%	3.0%	1.1%	0.8%
Bax$^{+/-}$	198	1.5%	0.5%	4.5%	0.0%
Bax$^{+/-}$	336	1.2%	2.1%	1.2%	0.0%
Bax$^{+/-}$	534	1.5%	0.7%	1.5%	1.5%
Bax$^{-/-}$	864	52.1%	44.1%	54.6%	40.6%
Bax$^{-/-}$	534	64.4%	50.2%	65.4%	51.9%
Bax$^{-/-}$	402	135.8%	101.2%	117.2%	87.3%
Bax$^{-/-}$	264	121.2%	89.0%	117.0%	76.5%

B Mean (±s.e.m.) of % survival from each genotype and treatment

	Number of PGCs at 0 minutes	J1.2 (15 μg/ml)	ACK (15 μg/ml)	J1.2 (50 μg/ml)	ACK (50 μg/ml)
Wild type	467	1.7%	0.6%	3.2%	0.2%
Bax$^{+/-}$	419	2.2% (±0.5)	1.1% (±0.4)	2.6% (±1.0)	0.7% (±0.3)
Bax$^{-/-}$	516	93.4% (±20.7)	71.1% (±14.1)	88.6% (±16.7)	64.1% (±10.8)

embryo grows (it grows twofold in size between E9.5 and 11.5), and if the range of survival signals stays constant, it progressively restricts the survival of germ cells to a smaller and smaller component of the embryo, and acts as a back-up mechanism to that of directed cell migration. There is strong evidence that the survival signal is Steel factor. It has been previously shown to be essential to germ cell survival in vivo, and in vitro. In this study, we show that withdrawal of Steel signaling using a blocking antibody against Kit, which has been previously shown to rapidly kill cultured germ cells, is not as effective in killing $Bax^{-/-}$ germ cells, showing that Bax acts downstream of the Kit receptor. This observation fits nicely with those of Felici et al. (Felici et al., 1999), who showed that Bax protein is upregulated in germ cells undergoing apoptosis in culture, and that addition of Steel factor prevented this rise.

Although we cannot exclude the possibility that the later disappearance of ectopic germ cells is due to loss of expression of the GFP marker, perhaps by differentiation, the failure of $Bax^{-/-}$ germ cells to survive for long periods in culture suggests that there is an alternative mechanism for their cell death. This could be due to the replacement later in development of the Steel/Kit/Bax mechanism by different survival factors and/or proapoptotic proteins. It also suggests that Bax functions specifically to remove germ cells that are out of range of early survival signals that support the mechanism of directional migration of germ cells to the genital ridges. If germ cells survive this early mechanism of elimination, it is replaced by another one later in development. The fact that the $Bax^{-/-}$ mouse does not have an increased incidence of germline tumors supports this idea. In order to gain insights into the etiology of extragonadal germline tumors, it will be necessary to identify this later cell death mechanism.

An interesting finding in this study is that the large number of ectopic PGCs in the $Bax^{-/-}$ embryos continue to be motile, and continue to express surface markers of their earlier migratory stage. These data suggest that contact with the somatic cells of the gonad, rather than an inherent timing mechanism, is required to switch off the early migratory phenotype of the germ cells. It is likely that germ line tumors arise from ectopic germ cells that have escaped these signals.

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