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Steel factor controls midline cell death of primordial germ cells and is essential for their normal proliferation and migration

Christopher Runyan[1], Kyle Schaible[1], Kathleen Molyneaux[2], Zhuoqiao Wang[3], Linda Levin[3] and Christopher Wylie[1,*]

During germ-cell migration in the mouse, the dynamics of embryo growth cause many germ cells to be left outside the range of chemoattractive signals from the gonad. At E10.5, movie analysis has shown that germ cells remaining in the midline no longer migrate directionally towards the genital ridges, but instead rapidly fragment and disappear. Extragonadal germ cell tumors of infancy, one of the most common neonatal tumors, are thought to arise from midline germ cells that failed to die. This paper addresses the mechanism of midline germ cell death in the mouse. We show that at E10.5, the rate of apoptosis is nearly four-times higher in midline germ cells than those more laterally. Gene expression profiling of purified germ cells suggests this is caused by activation of the intrinsic apoptotic pathway. We then show that germ cell apoptosis in the midline is activated by down-regulation of Steel factor (kit ligand) expression in the midline between E9.5 and E10.5. This is confirmed by the fact that removal of the intrinsic pro-apoptotic protein Bax rescues the germ-cell apoptosis seen in Steel null embryos. Two interesting things are revealed by this: first, germ-cell proliferation does not take place in these embryos after E9.0; second, migration of germ cells is highly abnormal. These data show first that changing expression of Steel factor is required for normal midline germ cell death, and second, that Steel factor is required for normal proliferation and migration of germ cells.

KEY WORDS: Germ cells, Steel factor (Kitl), Apoptosis, Migration, Mouse

INTRODUCTION

During early development of the mouse, the primordial germ cells (PGCs) occupy sequentially different tissues of the embryo. Movie analysis of whole embryos or embryo slices, using a strain expressing GFP in the germ cells (Anderson et al., 2000), has allowed visualization of PGC behavior during this process. At embryonic day (E)7.5, germ cells emerge from the posterior primitive streak region of the gastrula into the region of the embryonic endoderm fated to become the hindgut (Anderson et al., 2000). Although actively motile, they remain confined within the hindgut until E9.0-E9.5, when they migrate dorsally out of the hindgut, turn laterally, and migrate in the mesenchyme of the dorsal body wall towards the bilateral genital ridges (Molyneaux et al., 2001; Molyneaux et al., 2003). At E10.5, the germ cell population becomes divided into two groups. Those nearest the genital ridges continue to move directionally into the genital ridges, clustering into groups as they do so (Molyneaux et al., 2001). This clustering requires the expression by the germ cells of E-cadherin (Bendel-Stenzel et al., 1998). Those nearest the midline, either in the midline dorsal body wall, the mesentery of the gut (which forms between E9.5 and E10.5), or in the hindgut itself, rapidly fragment and disappear (Molyneaux et al., 2001), so that by E12.5 there are almost no PGCs left outside the genital ridges. These data suggest two important aspects of germ cell migration. First, targeted migration is a close-range phenomenon; only the germ cells nearest to the genital ridges at E10.5 can target them successfully. Second, there is a mechanism to remove the midline germ cells, which starts around E10.5, and eliminates most or all ectopic germ cells by E12.5. This mechanism is not present at E9.5, since all PGCs are still in midline structures at this stage. Midline germ cell death is important. Extragonadal germ cell tumors are common human pediatric tumors, and almost always form in the midline (Dehner, 1986). It is thought that they arise from germ cells that have escaped midline death (Ueno et al., 2004).

The signals that initiate midline germ cell death, and the details of its mechanism, are unknown. Previous reports have identified roles for individual signaling ligands in PGC survival. Both Steel factor [also known as kit ligand (Kitl) – Mouse Genome Informatics] and Leukaemia inhibitory factor increase survival in PGC cultures (Dolci et al., 1991; Matsui et al., 1991; Pesce et al., 1993). However, their roles in midline germ cell death in vivo are not known. In addition, individual signaling ligands may control more than one aspect of PGC behavior: a necessary homing signal for germ cells in both zebrafish and mice, *Sdf1* (Doitsidou et al., 2002; Molyneaux et al., 2003) has also been suggested to be a survival factor (Molyneaux et al., 2003), whereas a known survival factor for germ cells, Steel factor (Dolci et al., 1991) has also been suggested to be a guidance factor (McCoshen and McCallion, 1975).

There are a number of possible mechanisms for midline germ cell death (Fig. 1). First, activation of death signals by midline somatic cells, or receptors for these in midline germ cells, at E10.5, may activate the extrinsic cell death pathway. Second, switching off an essential survival factor at E10.5 by midline somatic cells, or its receptor in germ cells, may activate the intrinsic death pathway. There may also be combinations of these. To analyze this process, we first assayed the numbers of germ cells undergoing apoptosis in the midline and bilateral trunk regions of the mouse embryo at

[1]Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation, Cincinnati, OH 45229, USA. [2]Department of Genetics, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA. [3]Department of Environmental Health, College of Medicine, University of Cincinnati, Cincinnati, OH 45229, USA.

*Author for correspondence (e-mail: christopher.wylie@cchmc.org)

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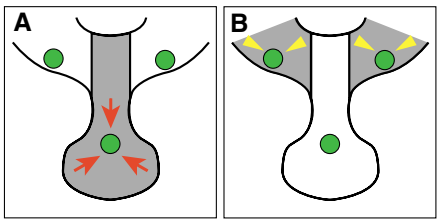

Fig. 1. Models of E10.5 midline germ cell death. (**A**) Midline expression of cell death factors (red arrows) and/or the activation of cell death factor receptors of midline germ cells may activate the extrinsic apoptotic pathway in PGCs (green). (**B**) Loss of survival signaling in the midline at E10.5 due to restricted expression of a survival factor (yellow arrowheads) or inactivation of a survival factor receptor in midline germ cells may activate the intrinsic apoptotic pathway.

E10.5, and found that apoptosis occurs in both populations during late migration, but is greatly enhanced in midline germ cells. Second, we screened for expression of components of both the extrinsic and intrinsic cell death pathways, in purified germ cells from both migratory and post-migratory germ cells. These data indicate that the extrinsic pathway is inactive in migratory germ cells, but that components of the intrinsic pathway are up-regulated during migration, suggesting that the latter is responsible for PGC death in the midline. These data are supported by previous data from our lab, wherein embryos lacking Bcl2-associated X protein (Bax), a pro-apoptotic member of the intrinsic pathway, have large numbers of ectopic germ cells in midline structures at E13.5 (Stallock et al., 2003).

The most likely candidate for a survival signal linked to Bax is Steel factor. Steel factor is a member of the short-chain helical cytokine family (Jiang et al., 2000; Zhang et al., 2000), and during development is expressed in primitive endoderm fated to become the hindgut (Motro et al., 1991), and later in somatic cells along the migratory pathway of PGCs (Keshet et al., 1991). The receptor for Steel is kit oncogene (c-Kit; also known as Kit – MGI), a tyrosine-kinase receptor of the PDGFRB superfamily that is expressed in PGCs throughout migration (reviewed by Loveland and Schlatt, 1997). Addition of Steel factor to PGCs in vitro is known to repress apoptosis (Pesce et al., 1993), and Bax protein levels in cultured PGCs have been suggested to be regulated by Steel (Felici et al., 1999). However, extreme Steel alleles and extreme alleles of the c-*Kit* gene result in severely reduced PGC numbers by E9.0 and few, if any, survive to the stage of midline germ cell death (Bennett, 1956; Mintz and Russell, 1957; Buehr et al., 1993; Mahakali Zama et al., 2005). So, a role for Steel factor in the removal of midline migratory germ cells has not been tested rigorously.

In this investigation we have quantified apoptosis and proliferation of germ cells in normal embryos and embryos mutant for Steel factor and/or Bax, at different stages. We show first that the massive loss of PGCs with null alleles of *Steel* is caused by apoptosis of germ cells beginning on or before E9.0, prior to emigration from the hindgut. Second, we show that Steel factor expression changes significantly between E9.5 and E10.5, becoming restricted to the lateral domains of germ cell migration, and lost in the midline, during this period. Third, we use both gain- and loss-of-function assays of Steel function to show that the loss of Steel factor in the midline at E10.5 causes midline germ cell death. Fourth, we show that Bax is required for germ cell apoptosis downstream of Steel factor in vivo, by rescuing the apoptosis phenotype in null mutants of Steel at E9.0 by removing Bax. Rescue of germ cell apoptosis in the absence of Steel factor revealed the fact that Steel factor has other functions in germ cells. Germ cells in Bax/Steel double null embryos failed to emigrate from the gut and failed to proliferate. This establishes novel roles for Steel factor in germ cell migration and proliferation.

MATERIALS AND METHODS

Mouse breeding, embryo preparation and genotyping

All animals were treated according to protocols approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Hospital. Mouse embryos were obtained by crossing males homozygous for the Oct4ΔPE:GFP transgene (Anderson et al., 2000) on an FVB background, with CD1 females (Charles River). Embryonic day 0.5 (E0.5) was assumed to be noon of the morning a vaginal plug was observed. *Oct4ΔPE:GFP*$^{+}$ mice were bred with *Bax* heterozgotes as described previously (Stallock et al., 2003), and offspring were crossed with *Kitl*^{Sl} heterozygotes (Jackson Laboratories, Stock number 000693) to obtain mice that were *Oct4ΔPE:GFP*$^{+/+}$, *Steel*$^{+/-}$, and *Bax*$^{+/-}$ or *Bax*$^{-/-}$. These were interbred to yield *Oct4ΔPE:GFP*$^{+/+}$, *Steel*$^{-/-}$, *Bax*$^{-/-}$ embryos. Genomic DNA was isolated from tails (adults) and heads (embryos), and genotypes were determined by PCR. Primers used were: *Oct4ΔPE:GFP* (Yeom et al., 2001) F-5′GGAGAGGTGAAACCGTCCCTAGG-3′, R-5′GCATCGCCCTCG-CCCTCGC-3′; *Bax* (Deckwerth et al., 1996) EX5-F-5′GAGCTG-ATCAGAACCATCATG-3′, IN5-R-5′GTTGACCAGAGTGGCGTAGG-3′, NeoPGK-R-5′CCGCTTCCATTGCTCAGCGG-3′; *Kitl*^{Sl} Common-F-5′CGGGGTTTATGAGGGTAGGA-3′, WT-R-5′TTGGGCCTGTG-TGACAAACT-3′, DEL-R-5′-ACTTCCTAGGGCTGGAGAGATG-3′. *Oct4ΔPE:GFP* transgene expression was determined by the presence of a 250 bp fragment. For genotyping of *Bax*, wild-type and mutant alleles were determined by the presence of 304 and 507 bp fragments, respectively. For *Kitl*^{Sl}, wild-type and deleted alleles were determined by 294 and 646 bp fragments, respectively.

Flow cytometry, RT-PCR and chip analysis

Genital ridge regions (E10.5-11.5) or gonads from males or females (E12.5-16.5) were dissected from embryos obtained from Oct4ΔPE:GFP × CD1 matings. GFP^{+} germ cells were FACS-purified and RNA was extracted as previously described (Molyneaux et al., 2004). cDNA were generated from 10 (n=1) or 25 (n=2) ng RNA using Superscript II or III First-Strand Synthesis Systems (Invitrogen). Real-time RT-PCR was performed on an Opticon Cycler (MJ Research, Waltham, MA, USA) using QuantiTect SYBR Green mix (Qiagen). Primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Predicted fragment lengths and primer sequences are as follows: *Bad* (174 bp, F: AGGATCGCTGTGTCCCTTTA R: GGCAGTCCAGAACAGGAGAG); *Bak* (147 bp, F: AATCCTGTGTCCTTCCTGACCC R: ATAGTGGC-TGAGTCTGAGGCTCTG); *Bax* (166 bp, F: ATGCGTCCACCAAG-AAGCTGAG R: CCCCAGTTGAAGTTGCCATCAG); *Bcl2* (183 bp, F: CTGGCATCTTCTCCTTCCAG R: GACGGTAGCGACGAGAGAAG); *Bcl-w* (*Bcl2l2* – MGI; 240 bp, F: TAGCAGACCTTCAGCCAGGT R: GCCATACTCAGCCTTCTTGG); *Bcl-x*$_L$ (*Bcl2l1* – MGI; 249 bp, F: ACTGACCGTCCACTCACCTC R: AGGAGCTGGTTTAGGGGAAA); *Bid* (159 bp, F: AAGGCATCCACAAACCTGTC R: AGTGTGCATGT-GGGAGTCAG); *Bim* (*Bcl2l11* – MGI; 200 bp, F: CGACAGTC-TCAGGAGGAACC R: CAATGCCTTCTCCATACCAGA); *Casp3* (169 bp, F: GTGTCCATGCTCACGAAAGA R: TGCTAGGCAGTGGTAG-CGTA); *Casp6* (235 bp, F: CCAGACAGACAAGCTGGACA R: GCGCTGAGAGACCTTTCTGT); *Casp7* (246 bp, F: GAGGAGGA-CCACAGCAACTC R: GGGATTAGCGTCAATGTCGT); *Casp8* (186 bp, F: GGAATGGCTACGGTGAAGAA R: CATCTGCTTTCCCTTGTTCC); *Fas* (163 bp, F: CAACCCCAGACACTGGAAAT R: TGCTGGCA-AAGAGAACACAC); *p53* (213 bp, F: CCAGTCTACTTCCCGCCATA R: GGCCAGGAACCACTACTCAG); *Actb* (138 bp, F: AGAGGGAA-ATCGTGCGTGAC R: CAATAGTGATGACCTGGCCGT); *Gapdh* (452 bp, F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGC-TGTA); *Odc1* (179 bp, F: TTGCCACTGATGATTCCAAAGC R: ATCCGACACTGCCTGAACGAAG); *Tbp* (199 bp, F: CTTCGTGC-AAGAAATGCTGA R: AGAACTTAGCTGGGAAGCCC); *Ubc* (118 bp,

F: AGCCCAGTGTTACCACCAAG R: CTAAGACACCTCCCCCATCA); *18S rRNA* (217 bp, F: ATGGCCGTTCTTAGTTGGTG R: CGCTGAGCCAGTCAGTGTAG). Gel electrophoresis confirmed predicted sizes for each PCR product.

Expression levels of apoptotic genes were analyzed separately to estimate the effects of embryonic day and gender. Apoptotic expression levels were normalized by each of the six best housekeeping genes in mouse embryos [beta-actin (*Actb*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), ornithine decarboxylase (*Odc1*), TATA box binding protein (*Tbp*), ubiquitin C (*Ubc*) and 18S rRNA (Willems et al., 2006)] similar to previous reports (Vandesompele et al., 2002; Pfaffl et al., 2004), and normalized data were combined and analyzed together in an analysis of variance (ANOVA) model. Prior to analysis, normalized apoptotic gene expression levels were standardized by setting the levels on E10.5 as 1 (except when the data on E10.5 were not available, when the levels on E11.5 were set as 1), and dividing the data on all other days by E10.5 (or E11.5). An array was constructed from the data for each apoptotic gene by cross-classifying days and genders. Standardized data were analyzed after a logarithmic transformation ($\log_e$) was applied to approximate homogeneity of experimental variance, which was estimated by averaging the variances of data within the array. The ANOVA model for testing differences between mean values of days and genders was expanded a priori to test differences between all combinations of day and gender. Inferences were based on an overall 1% alpha level. Paired comparisons between individual levels of day-gender groups were evaluated at the 0.1% alpha level to adjust for multiple comparisons, as an increase in the number of tests will inflate the type 1 error rate even if differences are due to chance alone.

Immunofluorescence analysis on whole-mount embryos or frozen sections

Embryos were fixed in 4% PFA. Whole-mount embryos were washed in 0.5% NP-40, blocked in PBSST (PBS/0.3% Triton X-100 with 5% goat or donkey sera) and incubated overnight at 4°C with primary antibody. The following day, the embryos were washed in PBST (PBS/0.3% Triton X-100). FITC- or Cy5-conjugated secondary antibodies (Jackson Immuno Research) were used for detection, and embryos were cleared in glycerol for imaging. Embryos to be sectioned were dehydrated in sucrose (except c-Kit-stained samples) and mounted in OCT compound (Tissue-Tek) for cryosectioning. Bax/Steel embryos were sectioned serially onto eight glass slides. At least three of eight slides from each embryo were analyzed, and from these the total PGC numbers were calculated. Sectioned embryos were rehydrated with PBST, blocked with PBSST, and incubated with primary antibody overnight at 4°C or room temperature. The next day, slides were washed with PBST, bound with secondary antibody, and mounted with DABCO (Sigma) for imaging.

Primary antibodies were used at the following dilutions: Steel factor (sc-1303), 1:200; c-Kit (sc-1494), 1:100; and SSEA-1 (sc-21702), 1:200 (all from Santa Cruz Biotechnology). Anti-cleaved-PARP (#9544 Cell Signaling),1:200. Anti-phospho-histone H3 (#05-806 Upstate), 1:2000. Secondary antibodies (Jackson Immuno Research) were used at the following dilutions: Cy5 donkey anti-mouse, 1:200; Cy5 goat anti-rabbit, 1:200; Cy5 donkey anti-goat, 1:300; FITC donkey anti-mouse IgM, 1:200. Imaging was performed on an Axiovert 100M microscope equipped with a Zeiss LSM 510 confocal scanhead. Unpaired, two-tailed Student's *t*-tests with equal variances were used to compare counts from embryos of Bax/Steel crosses.

Embryo slice culture

Embryo slices were prepared as previously described (Takeuchi et al., 2005). To examine the role of Steel/c-Kit signaling in migratory PGCs, soluble recombinant Steel factor (#455-MC, R&D Systems) or c-Kit blocking antibody, Ack2 (a kind gift from Dr Fred Finkelman, CCHMC) were added to the slice cultures. The slices were immediately imaged on the confocal microscope, then returned for overnight incubation into a humidified 37°C incubator. The following day, the slices were again imaged and scored for PGC number and location. Germ cells located in the midline (ventral to the entire diameter of the dorsal aorta) were scored as ectopic. Experiments were repeated twice, with four to six slices per dose group. For statistical comparisons, the data were analyzed using unpaired, two-tailed Student's *t*-tests with equal variances.

Steel^{kitl} deletion mapping

Using genomic DNA from *Kitl*$^{Sl+/+}$ adults and *Kitl*$^{Sl-/-}$ embryos, we performed PCR on increasingly narrowed regions toward the 5′ breakpoint. Primers were designed to bind to sequences 50, 150, 250, 350 and 450 kb upstream of *Kitl*. DNA from *Kitl*$^{Sl+/+}$ mice generated products with all primer sets, however, the *Kitl*$^{Sl-/-}$ DNA yielded a product only with the –450 kb primer set, mapping the 5′ breakpoint between 350 and 450 kb upstream of steel. Three new primer sets were then designed at regular intervals between –350 and –450 and another round of PCR was performed to further narrow the 5′ breakpoint. After five total rounds of PCR the 5′ breakpoint was mapped to a 350 bp region. The 3′ breakpoint was similarly mapped to a 380 bp region. We then performed PCR on *Kitl*$^{Sl-/-}$ DNA using primers designed to span the deletion, and then sequenced the products to define the precise boundaries of the deletion.

RESULTS

Germ cells die by apoptosis in the midline at E10.5

First, we confirmed that midline germ cell death at E10.5 is due to apoptosis. Although suggested by previous data, in which removal of the pro-apoptotic protein Bax caused increased germ cell survival in the midline (Stallock et al., 2003), this has not been quantified, or confirmed by molecular markers of apoptosis. We fixed whole dorsal body walls of *Oct4ΔPE:GFP* embryos at E10.5, and immunostained for the cleaved form of the caspase substrate, PARP (Fig. 2). Germ cells (GFP+) in the 'midline' region (between the coelomic angles) and 'bilateral' regions (between the coelomic angles and the genital ridges on each side) were counted and scored as apoptotic when they also showed red fluorescence (cleaved-PARP). We found a 3.7-fold increase in the percentage of germ cells undergoing apoptosis in the midline, compared with those in the lateral regions (Fig. 2C, 1.55±0.55% in midline versus 0.42±0.18% in lateral region). This is a conservative estimate of the difference, because the hindgut was removed in order to image the dorsal body wall from the ventral aspect (Fig. 2A), and germ cells that fail to exit the hindgut also fragment and die (Molyneaux et al., 2001). These data show that

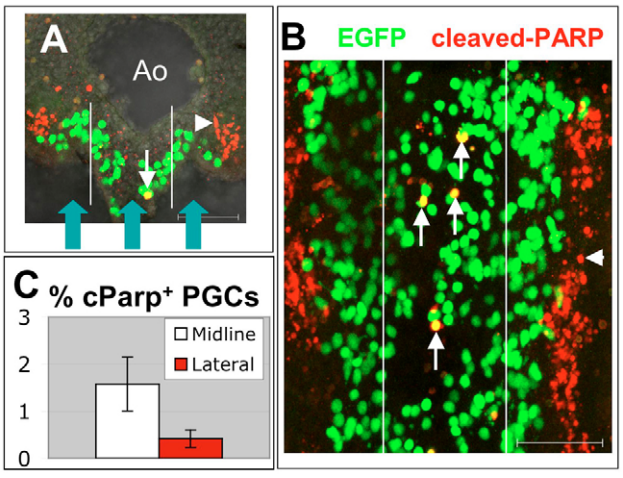

Fig. 2. Spatial distribution of apoptosis among migratory germ cells. (**A**,**B**) Cleaved-PARP immunostaining (red) labels apoptotic cells, including PGCs (white arrows) and the mesonephroi (arrowheads), which are known to degenerate during this time period (Pole et al., 2002). (A) A transverse section showing the direction of imaging (blue arrows) of whole-mount embryos in B. (B) Apoptotic PGCs (yellow) occurred in greater numbers in the midline (area between white lines). (**C**) Quantification of PGC apoptosis in B, showing enhanced death amongst midline PGCs (*n*=8). Scale bars: 100 μm.

germ cell death occurs by apoptosis in both midline and lateral populations, but that the incidence dramatically increases in the midline at E10.5.

Expression of components of the intrinsic and extrinsic cell death pathway members in purified germ cells

To determine apoptotic pathway utilization by germ cells in the midline at E10.5, we examined expression levels of members of the different apoptotic pathways in germ cells at different stages. Germ cells were purified from *Oct4ΔPE:GFP* embryos by flow cytometry each day between E10.5 and E16.5. Germ cells from males and females were isolated separately from E12.5 onwards. After E15.5, expression of the EGFP transgene is lost in females, consistent with previous reports of female-specific *Oct4* downregulation (Menke et al., 2003), so the analysis was not performed in E16.5 females. Real-time RT-PCR was used to analyze cDNA transcripts of a selection of pro- and anti-apoptotic pathway components. In each case, predicted mRNA levels were normalized to five or six different housekeeping genes, to avoid the effects of changing expression patterns of any individual housekeeping gene. This method of normalization was developed by members of the Statistics Dept at the University of Cincinnati (see Materials and Methods). Expression patterns are shown in Fig. 3, and are separated into those without (Fig. 3A) and with (Fig. 3B) gender-specific differences in expression.

The mRNAs for pro-apoptotic Bcl2 proteins found in the intrinsic pathway (Bax, Bak, Bad and Bim), and Casp3 (caspase 3), were all upregulated at E10.5, and downregulated in PGCs after colonization of the gonad. This implicates the intrinsic pathway in midline germ cell death at E10.5. By contrast, mRNAs encoding the extrinsic pathway receptor Fas, and Casp8 (not shown), were not expressed at E10.5, ruling out the possible involvement of the extrinsic apoptotic pathway. mRNA encoding the anti-apoptotic Bcl2 family member, Bcl-x, was upregulated specifically in male PGCs at E15.5 and E16.5 (Fig. 3B) consistent with previous reports (Rucker et al., 2000). Other genes with gender-specific regulation (Fig. 3B) were *Fas*, which was present only in males at the latest stages examined, consistent with previous reports (Wang et al., 1998), and *Casp6*, which was briefly but consistently upregulated in female PGCs at E13.5 and E14.5, suggesting a possible role in the removal of defective germ cells during meiosis. The following genes were also examined, but did not have significant differential regulation: *Bcl2*, *Bcl-w*, *Bid* and *Casp7*. These data were independently confirmed by microarray analysis of purified PGCs (M. Ramalho-Santos, personal communication). In summary, we observed that migratory germ cells have increased expression of intrinsic pro-apoptotic genes compared with later stages, and that they also lack expression of key extrinsic apoptotic factors.

Expression of Steel factor and c-Kit

To test the hypothesis that Steel factor plays a role in midline germ cell death at E10.5, we analyzed the expression patterns of both Steel factor and c-Kit at E9.5 (when germ cells in the midline survive), and E10.5 (when germ cells in the midline die). First, we stained sections of frozen *Oct4ΔPE:GFP*$^{+}$ E10.5 embryos with anti-c-Kit antibody. Plasma membranes of both midline and lateral PGCs were labelled by the antibody, and were enhanced in the former (Fig. 4A), ruling out the possibility that midline germ cell death is mediated by switching off *c-Kit*. A faint signal was detected in somatic cells of anti-c-Kit-stained samples (Fig. 4A, top). Although this wasn't detected in isotype-matched controls (Fig. 4C), it is probably due to non-specific binding of the goat polyclonal antibody, as every cell in the embryo had the same level of background staining. To assay the expression of Steel factor, we first measured the expression levels of *Steel* mRNA. Dorsal body walls, including the genital ridges, from E10.5 embryos were dissected into midline and lateral regions (dotted lines in Fig. 4B), and *Steel* mRNA levels, relative to the housekeeping gene, *Odc1*, were assayed by real-time RT-PCR. Fig. 4B shows that *Steel* mRNA levels are 36.5±3.8% lower in the midline relative to expression in lateral regions at this stage. To test for Steel factor protein expression, we stained E9.5 and E10.75

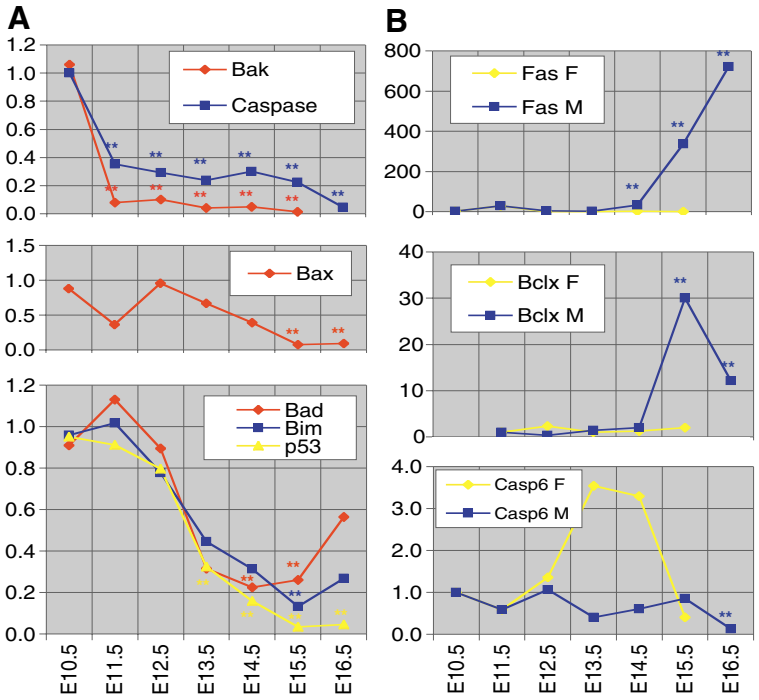

Fig. 3. Germ cell expression of apoptosis-related genes. (**A**) Real-time RT-PCR shows that pro-apoptotic genes, including Bcl2 superfamily members *Bax*, *Bak*, *Bad* and *Bim*, were downregulated in postmigratory PGCs. (**B**) Genes with gender-specific differential regulation include *Fas* and *Bcl-x*, which increased in postmigratory germ cells in the male, and *Casp6*, which was upregulated in the female from E13.5-14.5. E10.5 was considered baseline for statistical comparisons, except for *Bcl-x*, for which E11.5 was considered baseline. F, female; M, male. ***P*<0.001.

embryo slices with anti-Steel factor antibody. Steel-null embryos did not stain positively for the protein (Fig. 4D). At E9.5, Steel protein was expressed in the mesentery of the hindgut and the midline dorsal body wall, the ventral aspect of the aorta and at the coelomic angles and genital ridges (Fig. 4E). At E10.75, Steel staining was reduced or absent in the midline dorsal body wall and gut mesentery, and enriched in the coelomic epithelium and genital ridges (Fig. 4F). This shows that expression patterns of Steel factor change between E9.5 and E10.5, supporting the hypothesis that a change in the expression pattern of Steel factor concentration causes midline germ cell death at E10.5.

Two experiments were carried out to test this hypothesis further. First, we cultured E10.5 embryo slices in the presence of Steel factor. If the hypothesis is correct, then addition of Steel factor to the medium should allow midline germ cells to survive. Second, we cultured embryo slices in the presence of a blocking antibody against c-Kit, Ack2. If the hypothesis is correct, then all germ cells, whether located in the midline or laterally, should die. Fig. 5 shows that both of these predictions were true. Compared with untreated controls, PGCs in the midline of Steel factor-treated slices did not fragment and disappear (Fig. 5A,C), whereas blockade of Steel factor/c-Kit signaling using the Ack2 antibody caused germ cells to fragment and disappear everywhere in the slice (Fig. 5B,D). These data show that the pattern of Steel factor expression controls survival laterally, and death of midline migrating primordial germ cells.

Rescue of germ cell apoptosis by a null mutation in *Steel* by removal of Bax

In animals with severe alleles of *Steel* and *c-Kit*, reduced numbers of germ cells are first seen at E9.0 (Bennett, 1956; Buehr et al., 1993), when germ cells are in the hindgut. This effect was originally ascribed to a loss of proliferation (Bennett, 1956; Matsui et al., 1991; Buehr et al., 1993). More recent observations suggest that Steel signaling may be necessary to prevent apoptosis. For example, cultured PGCs exposed to Steel factor decreased expression of Bax protein (Felici et al., 1999), and Bax-null germ cells survived longer in culture when treated with a c-Kit blocking antibody (Stallock et al., 2003). Also, germ cells with abnormal morphology were recently identified in Steel mutant embryos (Mahakali Zama et al., 2005). To test more stringently whether cell survival is the primary target of Steel factor signaling, and whether Bax is downstream of this in vivo, we systematically scored cleaved-PARP+ germ cells, germ cells stained with the proliferation marker phospho-histone H3, and germ cell position, in embryos lacking both Steel factor and Bax at E9.0 and E10.5.

The Steel ($Kitl^{Sl}$) mutation was first identified as a naturally occurring dominant allele resulting in anemia, coat color anomalies and infertility (Sarvella and Russell, 1956). Steel homozygotes are embryonic lethal near E15.5 due to anemia, whereas heterozygotes are viable and are identifiable by a typical coat color phenotype (Silvers, 1979). However, early heterozygous embryos are difficult

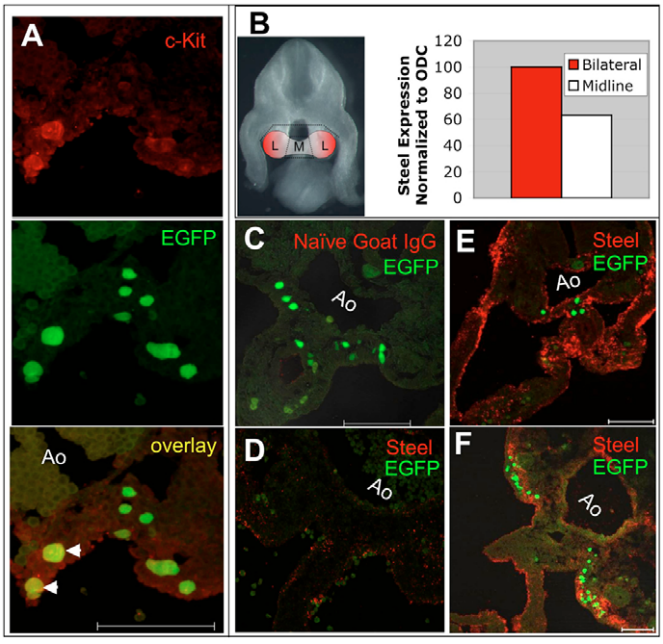

Fig. 4. Expression of c-Kit and Steel Factor. (**A**) Anti-c-Kit staining of E10.5 embryo slices shows that both midline (arrowheads) and lateral germ cells express c-Kit protein (observe colocalization in overlay). (**B**) Dissected midlines (M) have less than two-thirds *steel* expression compared with bilateral (L) regions as determined by real-time RT-PCR. (**C**) Isotype-matched primary antibody serves as a negative control for c-Kit and Steel antibodies. (**D**) Immunostaining of a $Steel^{-/-}$ embryo with an anti-Steel antibody demonstrates the antibody's specificity (note the lack of red signal). (**E**) A *Steel* wild-type embryo at E9.5 has Steel protein in the genital ridges and midline structures, including the coelomic angles and tissues ventral to the dorsal aorta. (**F**) At E10.75, Steel protein becomes restricted bilaterally to the genital ridges. Ao, aorta. Scale bars: 100 μm.

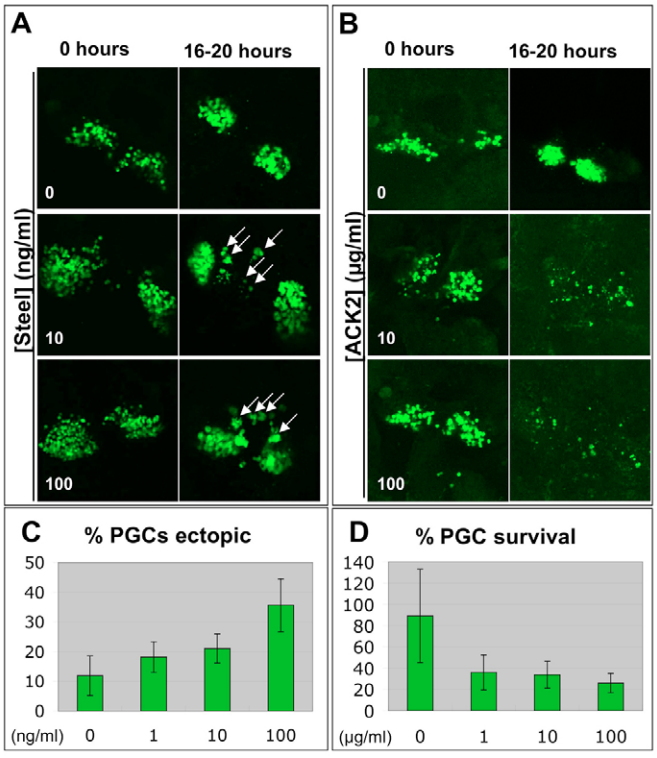

Fig. 5. Slice culture experiments. (**A**,**C**) Germ cells in E10.5 embryo slices survive in the midline in the presence of Steel factor in a dose-dependent manner. Midline PGCs scored as ectopic in (C) are marked with arrows in A. No significant change in total PGC numbers was observed in the different treatment groups in A. (**B**,**D**) Conversely, PGCs cultured with Ack2 died in a dose-dependent manner. Error bars represent means ± s.e.m. (*n*=8-12).

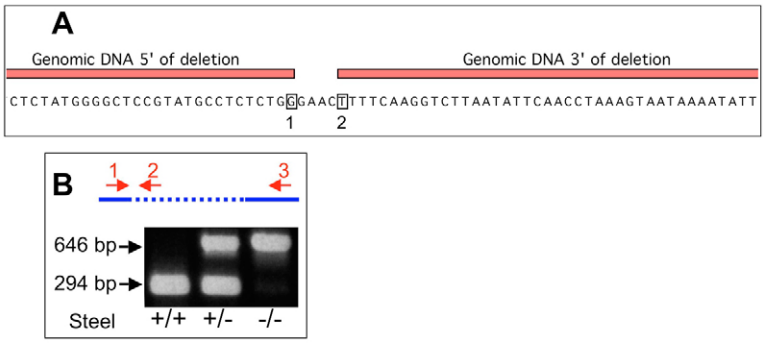

Fig. 6. Mapping the *Steel* (*Kitl*^{Sl}) deletion. (**A**) The exact positions of the 5′- and 3′-breakpoints of *Kitl*^{Sl} are indicated as boxed nucleotides, 1 (position 99,177,807) and 2 (position 100,151,173). The total deletion is 973,366 bp, not including an insert of four nucleotides. Nucleotide positions correspond to NCBI Map Viewer numbers. (**B**) A genotyping strategy was designed to identify wild-type and deleted alleles of *Steel*. (Top) The blue line represents DNA containing the Steel gene, and the dotted region represents the portion that is missing in the deleted allele. The red arrows represent primers (see Materials and methods). (Bottom) A gel showing a genotyping reaction using a mix of all three primers. Heterozygotes are identifiable by bands generated from both 1+2 and 1+3.

to identify, and the steel phenotype is difficult to identify in *Bax*$^{-/-}$ adults (Knudson et al., 1995), which have a grey or light chinchilla coat color. For ease in setting up breeding pairs and in order to be able to assess whether Steel has a heterozygous gene dosage effect, we mapped the Steel deletion and designed a simple genotyping strategy based on standard PCR of genomic DNA (Fig. 6B). We used a novel strategy to map the deletion (see Materials and methods), which consists of 973,366 bp (Fig. 6A) on chromosome 10 and contains three known and six predicted genes (Table 1). The deletion of genes flanking *Steel* is not likely to influence the embryonic steel phenotype, as the *Kitl*^{gb} mutant, for which only the *Steel* coding region is deleted (Bedell et al., 1996), phenocopies *Kitl*^{Sl}, which is referred to hereafter simply as '*Steel*'.

We first examined embryos from Steel/Bax crosses at E9.0. Whole-mount and serially sectioned frozen embryos were stained using the cleaved-PARP antibody, and representative images from whole-mount embryos are shown in Fig. 7A-D. *Bax*$^{+/-}$, *Steel*$^{-/-}$ embryos (Fig. 7C) had frequent Parp^{+} PGCs, whereas in *Bax/Steel* compound heterozygotes (Fig. 7A) and *Bax* null embryos (Fig. 7B,D), Parp^{+} figures were less commonly observed. *Bax*$^{+/-}$, *Steel*$^{-/-}$ embryos also had an obvious reduction in PGC number compared with embryos with an intact allele of *Steel* (Fig. 7E). Embryos containing one or more intact allele of *Bax* and *Steel* had 177±64 PGCs, and this was used as a baseline for comparison with littermate embryos. *Bax* null embryos did not have significantly different numbers of PGCs, with 207±66 for *Bax*$^{-/-}$, *Steel*$^{+/-}$ or *Bax*$^{-/-}$, *Steel*$^{+/+}$ (P=0.47) and 132±38 for *Bax*$^{-/-}$, *Steel*$^{-/-}$ (P=0.28) embryos. By contrast, *Steel*$^{-/-}$ embryos with an intact allele of *Bax* had a significant decrease in PGCs, with 43±40 per embryo (P=0.005). This corresponded with the PARP staining data (Fig. 7F): 6.70±4.77% of PGCs in *Bax*$^{+/-}$ *Steel*$^{-/-}$ embryos were apoptotic, compared with 2.37±1.42% in *Bax/Steel* compound heterozygotes (P=0.036). By contrast, there was no significant difference between *Bax*$^{-/-}$, *Steel*$^{+/-}$ or *Bax*$^{-/-}$, *Steel*$^{+/+}$ (0.66±0.46%, P=0.12) and *Bax*$^{-/-}$, *Steel*$^{-/-}$ (1.94±2.48%, P=0.75) embryos. These data show that in vivo, PGCs die by apoptosis in the absence of Steel factor. This requirement for Steel presumably occurs between E8.5 and E9.0, as PGC numbers are already significantly reduced by E9.0. Furthermore, the loss of Bax on the *Steel*$^{-/-}$ background is sufficient to rescue both PGC number (P=0.006) and apoptosis (P=0.058) compared with *Steel* null embryos with an intact allele of *Bax*. These

Table 1. Known and predicted (italicized) genes contained within the *Kitl*^{Sl} deletion

Accession number	Gene name
XM_893239	Hypothetical protein LOC628488
NM_027945	Citrate synthase like (Csl)
NM_013598	Kit ligand (Kitl)
XM_893302	Hypothetical protein LOC628546
XM_128231	Similar to THO complex subunit 4 (Tho4) (RNA and export factor binding protein 1) (REF1-I) (Ally of AML-1 and LEF-1) (Aly/REF)
XM_893313	Similar to zinc finger protein 277 isoform 1
NM_001033332	Transmembrane and tetratricopeptide repeat containing 3 (Tmtc3)
NM_175128	*RIKEN cDNA 4930430F08 gene*
XM_487140	*RIKEN cDNA 1700017N19 gene*

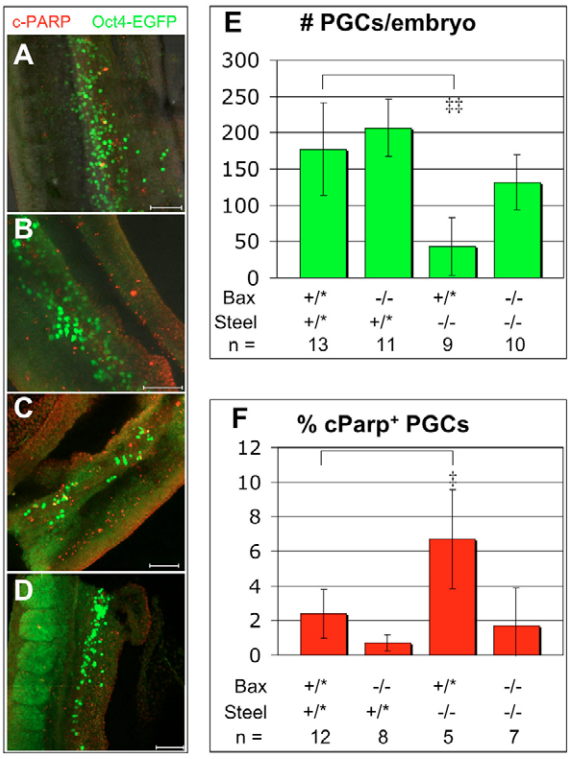

Fig. 7. Bax is required for E9.0 germ cell death in the absence of Steel. (**A-D**) Immunostaining of *Bax/Steel* E9.0 embryos for cleaved PARP shows that apoptosis occurs at a higher frequency in *Steel*$^{-/-}$ embryos, and is reduced by the absence of Bax. (A) *Bax*$^{+/-}$ *Steel*$^{+/-}$, (B) *Bax*$^{-/-}$ *Steel*$^{+/-}$, (C) *Bax*$^{+/-}$ *Steel*$^{-/-}$, (D) *Bax*$^{-/-}$ *Steel*$^{-/-}$. Scale bars: 100 μm. (**E**) Germ cell numbers were significantly reduced in *Bax*$^{+/*}$ *Steel*$^{-/-}$ embryos compared with *Bax*$^{+/*}$ *Steel*$^{+/*}$ littermates (* denotes + or –), but are rescued by the loss of Bax. (**F**) The percentage of cleaved-Parp+ PGCs is significantly increased in *Bax*$^{+/*}$ *Steel*$^{-/-}$ embryos compared with *Bax*$^{+/*}$ *Steel*$^{+/*}$ littermates, but is rescued by the loss of Bax. $^{‡}P$<0.05, $^{‡‡}P$<0.01. Error bars represent means ± s.e.m.

data confirm in vivo that Bax is downstream of Steel/c-Kit signaling in the hindgut at E9.0, and is required for the loss of germ cell survival caused by the absence of Steel factor.

The loss of Bax blocks germ cell apoptosis caused by loss of Steel factor at E9.0; this allowed us to test for other potential roles of Steel factor. If survival is its primary function, then other aspects of germ cell behaviour should proceed normally in *Steel*$^{-/-}$, *Bax*$^{-/-}$ embryos.

To test this, we serially sectioned E10.5 embryos from *Bax/Steel* crosses, and scored them for germ cell numbers, position of germ cells in the embryo, apoptosis and mitosis (Fig. 8). Representative images from serially sectioned embryos are shown in Fig. 8A-I. At least three out of eight of the slides in a serial series were analyzed, and from these the total PGCs were calculated in each embryo. As expected, germ cells in E10.5 *Bax*$^{+/-}$, *Steel*$^{-/-}$ embryos (not shown) were nearly absent, with only 17±14 per embryo compared with 1,145±252 for *Bax*$^{+/-}$, *Steel*$^{+/+}$ embryos, $P<0.001$, see Fig. 8L. *Steel*$^{+/-}$ embryos also had fewer germ cells than *Steel*$^{+/+}$ embryos (730±288 vs 1145±252 for *Bax*$^{+/-}$, $P=0.067$; 860±303 vs 1226±218 for *Bax*$^{-/-}$, $P=0.112$, Fig. 8L); although not quite significant, this suggests a gene dosage effect of Steel factor on PGC number at E10.5.

Loss of Bax rescued the loss of germ cells in *Steel*$^{-/-}$ E10.5 embryos (140±39, $P=0.002$), however, germ cell numbers were restored only to the level seen in E9.0 Steel/Bax double knockout (DKO) embryos (132±38, $P=0.782$). This interesting result suggested either that there is an additional, Bax-independent pathway of apoptosis downstream of Steel factor at E10.5, or that Steel factor is also required for germ cell proliferation between E9.0 and E10.5 (or both). Fig. 8J,K shows that Steel factor is required for germ cell proliferation. In Fig. 8J, the percentage of apoptotic (cleaved-PARP$^+$) PGCs from *Bax*$^{+/-}$ *Steel*$^{+/-}$ (see Fig. 8D) and *Bax*$^{-/-}$ *Steel*$^{-/-}$ (Fig. 8G) embryos are compared. Apoptosis was completely abrogated in DKO embryos (0±0% vs 2.44±1.83% for compound heterozygotes, $P=0.021$), showing that there is no alternative pathway of germ cell apoptosis downstream of Steel factor. By contrast, in embryo sections stained for the marker of mitosis, phospho-histone H3 (Fig. 8C,F,I), germ cell mitoses were found to

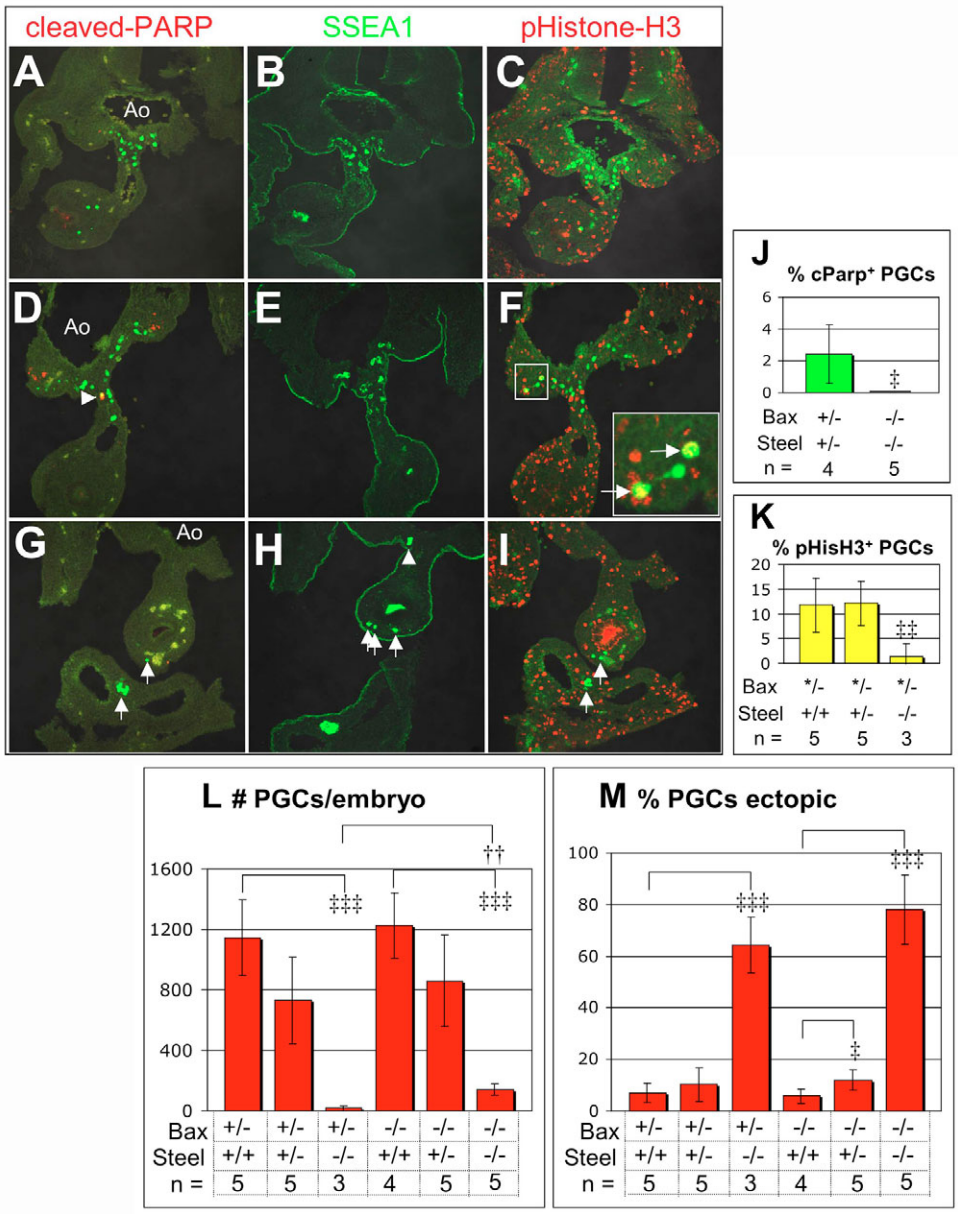

Fig. 8. Steel is required for proper germ cell localization and for proliferation at E10.5. (A-I) *Bax/Steel* embryos were immunostained for phospho-histone H3, cleaved Parp, and the PGC marker SSEA1. (**A-C**) *Bax*$^{-/-}$ *Steel*$^{+/+}$, (**D-F**) *Bax*$^{+/-}$ *Steel*$^{+/-}$, and (**G-I**) *Bax*$^{-/-}$ *Steel*$^{-/-}$. Apoptotic PGCs (arrowheads) were enriched in *Bax*$^{+/-}$ embryos that lacked at least one allele of *Steel* (D). Proliferating germ cells (arrows) were enriched in embryos with an intact allele of *Steel* (C,F). Germ cells in *Bax/Steel* DKO embryos were mislocalized (G-I), and were found in the ventral aspect of the hindgut and further ventral structures (arrows), with few near the genital ridges (arrowhead). SSEA1 staining (B,E,H) confirmed the presence of PGCs, independent of Oct4. No differences in PGC numbers or locations were observed between SSEA1 stained and unstained slices. (**J**) Cleaved-PARP$^+$ PGCs from *Bax*$^{+/-}$ *Steel*$^{+/-}$ (D) and *Bax*$^{-/-}$ *Steel*$^{-/-}$ (G) embryos were compared and found to be significantly reduced in the absence of Bax. (**K**) Phospho-histone H3-positive PGCs were significantly reduced in *Steel*$^{-/-}$ embryos (I) compared with *Steel* heterozygous (F) and wild-type (C) littermates. (**L**) PGCs were reduced in *Steel* heterozygous compared with wild-type embryos, and nearly absent in *Steel*$^{-/-}$ embryos. The additional loss of Bax rescues PGC numbers to E9.0 levels. $^{††}P<0.01$ for *Bax*$^{+/-}$ *Steel*$^{-/-}$ vs *Bax*$^{-/-}$ *Steel*$^{-/-}$ embryos. **(M)** The percentage of ectopic germ cells in *Bax/Steel* embryos is markedly higher in Steel-null embryos. Horizontal brackets indicate samples that were compared for statistical significance measurements. $^{‡}P<0.05$, $^{‡‡}P<0.01$, $^{‡‡‡}P<0.001$. Error bars represent means ± s.e.m.

be dramatically reduced (Fig. 8K) in *Bax*$^{-/-}$, *Steel*$^{-/-}$ embryos (1.3±2.6%, compared with 11.8±5.5% and 12.1±4.4% in *Steel*$^{+/+}$ and *Steel*$^{+/-}$ embryos, respectively, P=0.006). As the presence of Bax had no effect upon pHis-H3 staining, Bax$^{+/-}$ and Bax$^{-/-}$ genotypes were combined in Fig. 8K. These data show that the loss of Bax rescues apoptosis caused by the loss of Steel factor, but that germ cells also require Steel factor for proliferation between E9.0 and E10.5.

The rescue of germ cell apoptosis by the loss of Bax also revealed a requirement for Steel factor in germ cell emigration from the gut. Fig. 8G-I shows that most of the germ cells in E10.5 *Bax*$^{-/-}$, *Steel*$^{-/-}$ embryos failed to migrate out of the hindgut, and occupied the same positions as at E9.0. To quantify this, the positions of individual germ cells were scored in E10.5 serial sections. Germ cells ventral to the dorsal body wall (including the hindgut, the ventral half of the hindgut mesentery and structures ventral to the gut) were scored as 'ectopic'. Nearly 78% of germ cells in *Bax*$^{-/-}$, *Steel*$^{-/-}$ E10.5 embryos were ectopic compared with 5.8% in *Steel*$^{+/+}$, *Bax*$^{-/-}$ embryos (P=3.6×10^{-5}; Fig. 8M). Loss of one allele of Steel on a *Bax* null background (*Bax*$^{-/-}$, *Steel*$^{+/-}$) also increased the number of ectopic germ cells compared with *Steel*$^{+/+}$ embryos (12.1%, P=0.019; Fig. 8M), revealing a gene dosage effect of Steel factor in germ cell migration that may only be seen when apoptosis of ectopic PGCs is prevented by the loss of Bax. These data show that Steel factor is essential for many aspects of germ cell behaviour during migration, a fact that is usually masked by its requirement for germ cell survival.

DISCUSSION

When germ cells emerge from the hindgut between E9.0 and E9.5, the distance to the genital ridges is small (about 100 μm), and there is no hindgut mesentery. During the next 24 hours, the embryo doubles in size, and a mesentery forms separating hindgut from dorsal body wall. Both of these events make the migratory route longer for germ cells, many of which are still leaving the hindgut during this period. This results in PGCs being distributed over a wide region at E10.5. They are found in the hindgut, the hindgut mesentery, the dorsal midline, and lateral to the midline near the genital ridges. Movie analysis of germ cells leaving the hindgut at E9.5, and migrating towards the genital ridges at E10.5, shows a dramatic difference in germ cell behaviour in the midline. At E9.5, germ cells migrate from the gut into the midline dorsal body wall. They survive there, and migrate actively towards the genital ridges (Molyneaux et al., 2003). At E10.5, germ cells still in the midline do not migrate directionally. Instead, they move randomly, then fragment and disappear (Stallock et al., 2003). In this paper, we define the mechanism for this process.

First, we show that regulation of apoptosis during migration (E10.5) causes removal of midline germ cells. Apoptosis plays an important role in elimination of germ cells throughout development, and seems to be tightly regulated, as there are gender- and stage-specific variations in apoptotic frequency (Coucouvanis et al., 1993; Koji, 2001) and gene expression (Rucker et al., 2000). We show that pro-apoptotic genes of the intrinsic pathway are upregulated in migratory germ cells. Further, we show that most PGC apoptosis at E10.5 occurs within the midline. These data support a model (Fig. 1) in which migratory germ cells are eliminated by reduction of a survival signal in the midline.

Second, we show that the expression of Steel factor changes significantly between E9.5 and E10.5. When germ cells are in the midline at E9.5, Steel factor is expressed in midline cells around the hindgut. By E10.5, midline expression of Steel factor has become downregulated, but remains high bilaterally, as shown by both mRNA levels and protein distribution. Conversely, the receptor for Steel, c-Kit, is maintained in midline PGCs. Manipulation of the system, by addition of Steel factor everywhere, or abrogation of signal reception everywhere, confirms that the change in expression of Steel factor is the cause of the pattern of germ cell survival at E10.5 to E11.5. These data show that a combination of targeted migration and controlled cell death are required for the eventual localization of germ cells in the genital ridges. This is likely to be a general mechanism for cell migrations that occur during the period of rapid growth and organogenesis.

Steel factor has been known for many years to be a necessary survival signal for germ cells (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991). In null mutants for *Steel* or its receptor, *c-Kit*, germ cell numbers are normal at E8.0, but are drastically reduced compared with control embryos from E9.0 onward, purportedly due to a defect in PGC proliferation, although the presence of morphologically dying cells were also reported (Mintz, 1957; Mintz and Russell, 1957; Buehr et al., 1993; Mahakali Zama et al., 2005). We provide the first molecular evidence (cleaved-PARP) that the loss of Steel factor leads to germ cell apoptosis in vivo. We show that PGC apoptosis is already well underway by E9.0, and continues until most germ cells are eliminated by E10.5. Because most germ cells in *Steel* mutants die prior to or early in the normal period of PGC migration, and because no germ cells survive to E11.5, the PGCs observed at E9.5 and E10.5 are presumably dying. This must be considered when interpreting observed abnormalities in proliferation and/or migration, particularly in null mutations.

Previous work has implicated the pro-apoptotic protein Bax in midline germ cell death. In *Bax*$^{-/-}$ embryos, midline germ cells fail to die in the E10.5 to E11.5 period. Instead, they colonize midline structures (Stallock et al., 2003). To confirm that Bax is a necessary downstream component of the Steel/c-Kit signaling pathway in activating midline germ cell death, we examined double mutants. The results of this throw light upon several aspects of Steel signaling. First, the absence of Bax in vivo did rescue germ cell apoptosis caused by the loss of Steel factor. Germ cells continued to survive throughout the migratory stages. However, they did not behave normally, indicating that Steel factor plays other roles. First, germ cell proliferation stopped at approximately E9.0. Thus, loss of Bax restored germ cell numbers in *Steel*$^{-/-}$ embryos to approximately wild-type levels at E9.0, but these numbers did not change over the next few days, and germ cells in double knockout embryos did not stain with antibodies against phospho-histone H3, a marker of mitotic cells. Furthermore, germ cells failed to migrate from the hindgut, and clumps of germ cells were also found in structures ventral to the gut. These data show that Steel factor is required for both proliferation and migration of germ cells, in addition to its essential role in their survival. Observations on embryos with severe alleles of *c-Kit*, in which the few germ cells left at E9.0 were found only in the ventral regions of the gut (Buehr et al., 1993), and more recently, observations on embryos with hypomorphic alleles of *Steel* that had some germ cell survival past E9.0 (Mahakali Zama et al., 2005), have also suggested that this ligand-receptor interaction does more than just control germ cell survival. It will be interesting to establish whether the downregulation of Steel factor in the midline is also responsible for loss of directional germ cell migration in the midline at E10.5, or whether germ cells are out of range of a different homing signal. For example, midline PGCs may be unable to respond to the chemoattractant, SDF1, either by lack of proximity to the source of the ligand, or possibly by downregulation of its receptor, Cxcr4.

The incidence and distribution of human germ cell tumors illustrates the importance of regulated cell death on the midline. Germ cell tumors are one of the most common human neonatal and infantile neoplasms. More than 50% of these are found outside the gonads, and the vast majority of these are in midline structures. These midline germ cell tumors are thought to arise from germ cells that fail to undergo midline cell death (Ueno et al., 2004). It will be interesting to find out whether blockade of midline germ cell death will, by itself, cause them to become tumorigenic, as does their transplantation under the kidney capsule for example, or whether other genetic factors are also required.

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