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

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During germ-cell migration in the mouse, the dynamics of embryo growth cause many germ cells to be left outside the range of chemotactic 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
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 (Pesces 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., 2003). 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 heterozygotes as described previously (Stallock et al., 2003), and offspring were crossed with KitR® 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’ GGAGAGGTGAAACCCCTTACGG-3’; R-5’ GTACGCGCTCCTCGGAC-3’; Bax (Deckwerth et al., 1996) EX5-F-5’ ATCGTGCGTGAC-3’; IN5-R-5’ GTTGACGAGTGCTTACGGG-3’, NeoPGK-R-5’ CGGCTTCAATGCGATTAGG-3’, KitR® Common-F-5’ CCGGTTATATAGCGGGTAA-3’, WT-R-5’ TTTGGCGCTTCTGTAAC-3’, DEL-R-5’ ACTTCTAGGCGCAGAGTATG-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 KitR®, wild-type and deleted alleles were determined by 294 and 466 bp fragments, respectively.

**Flow cytometry, RT-PCR and chip analysis**

Genital ridge regions (E10.5-11.5) or gonads from males or females (E12.5-13.5) 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) mg RNA using Superscript II or III First-Strand Synthesis System (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://troto.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Predicted fragment lengths and primer sequences are as follows: Bad (174 bp, F: AGGATCTGCTGTGTCCTCTTTTA R: GGCAGTCCGAAAACGGAGAGA); Bak (147 bp, F: AACGCCTGGTCTACTCCACCC R: ATATGTTACG TGAAGCTTACTGCTCT); Bax (166 bp, F: ATGCCCTGCAAAGA GTCTGAG R: CCCAGGTGAAATGTTGCACTAG); Bcl-xL (183 bp, F: CTGGCATCTTCTCTTCCAG R: GACGGTGATGGGAGAGA); Bcl-w (122 bp, MGI); Bcl-2 (240 bp, R: GACGCATCTGGCAGCCACGTG; GCCATACCTCAGCTCTTGTG); Bcl-xL (1211 – MGI); 249 bp, F: ACTGACCGTCGACATCCT R: AGGGACTGTGGTTAGGAAA); Bim (159 bp, F: AAGGATCGTCACAGAAAAGA R: GTGTGACTGTTGGGACGCTA); Bid (166 bp, F: ATGCCCTGCAAAGACTGCTCT); Casp3 (169 bp, F: GTCTCCATGCTCCTCTACAGAAG R: TGTTAAGCTGTTGCTGAGA); Casp6 (235 bp, F: CCAGCACGACAGAGGTTCGA R: GCGCTGAGAGCTCTGCTCT); Casp7 (105 bp, F: GAAACACTGATGCGATGCGAA R: CAATGCTGATGAAAGAAC); Odc1 (179 bp, F: TTGCCACACTGTGATTTCCCAAGC R: ATCCGACACTGGTTAGGGAACG); Ptp1b (199 bp, F: CTGGCGTCGAGAAATGCTGA R: AGAACCTTAGGAGAGACG).

**Development**

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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 (Octd1), 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_{10}) was applied to approximate homogeneity of variances among midline PGCs (white arrows) and the mesonephroi (arrowheads), including PGCs (white 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, Bim 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...
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 (Felic 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 (Kit<sup>−/−</sup>) 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 to identify.
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 KitlR2 mutant, for which only the Steel coding region is deleted (Bedell et al., 1996), phenocopies Kitl, 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 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 Bax–/–, Steel–/– embryos compared with E9.0 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.

Table 1. Known and predicted (italicized) genes contained within the Kitl deletion

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Gene name</th>
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<tbody>
<tr>
<td>XM_893239</td>
<td>Hypothetical protein LOC628488</td>
</tr>
<tr>
<td>NM_027945</td>
<td>Citrate synthase like (Gsi)</td>
</tr>
<tr>
<td>NM_013598</td>
<td>Kit ligand (Kit)</td>
</tr>
<tr>
<td>XM_893302</td>
<td>Hypothetical protein LOC628546</td>
</tr>
<tr>
<td>XM_128231</td>
<td>Similar to THO complex subunit 4 (Tho4) RNA and export factor binding protein 1 (REF1-I) (All of AML-1 and LIF-1) (Alv/REF)</td>
</tr>
<tr>
<td>XM_893313</td>
<td>Similar to zinc finger protein 277 isoform 1</td>
</tr>
<tr>
<td>NM_001033332</td>
<td>Transmembrane and tetrapeptide repeat containing 3 (Tmtr3)</td>
</tr>
<tr>
<td>NM_175128</td>
<td>RIKEN cDNA 4930430F08 gene</td>
</tr>
<tr>
<td>XM_487140</td>
<td>RIKEN cDNA 1700017N19 gene</td>
</tr>
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(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...
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), germ cell mitoses were found to
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 pH3-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+/+ 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 (\( P=3.6×10^{-2} \); 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 (Cocouvanis 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 chemorepellent, 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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References