Deficiency of Trp53 rescues the male fertility defects of Kit(W-v) mice but has no effect on the survival of melanocytes and mast cells

Citation for published version:
Jordan, SA, Speed, RM, Bernex, F & Jackson, IJ 1999, 'Deficiency of Trp53 rescues the male fertility defects of Kit(W-v) mice but has no effect on the survival of melanocytes and mast cells' Developmental Biology, vol. 215, no. 1, pp. 78-90. DOI: 10.1006/dbio.1999.9440

Digital Object Identifier (DOI):
10.1006/dbio.1999.9440

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Developmental Biology

Publisher Rights Statement:
elsevier's open archive article

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Deficiency of Trp53 Rescues the Male Fertility Defects of Kit<sup>W-v</sup> Mice but Has No Effect on the Survival of Melanocytes and Mast Cells

Siobhán A. Jordan, Robert M. Speed, Florence Bernex,* and Ian J. Jackson

MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, United Kingdom; and *URA-INRA de Génétique Moléculaire, Ecole Nationale Vétérinaire d’Alfort, Maisons-Alfort, France

Mutations of the receptor tyrosine kinase, Kit, or its ligand, mast growth factor (Mgf), affect three unrelated cell populations: melanocytes, germ cells, and mast cells. Kit signaling is required initially to prevent cell death in these lineages both in vitro and in vivo. Mgf appears to play a role in the survival of some hematopoietic cells in vitro by modulating the activity of p53. Signaling by Mgf inhibits p53-induced apoptosis of erythroleukemia cell lines and suppresses p53-dependent radiation-induced apoptosis of bone marrow cells. We tested the hypothesis that cell survival in Kit mutant mice would be enhanced by p53 deficiency in vivo. Double-mutant mice, which have greatly reduced Kit receptor tyrosine kinase activity and also lack Trp53, were generated and the affected cell lineages examined. Mast cell, melanoblast, and melanocyte survival in the double Kit<sup>W-v</sup>/Kit<sup>W-v</sup>:Trp53<sup>−/−</sup> mutants was not increased compared to the single Kit<sup>W-v</sup>/Kit<sup>W-v</sup>:Trp53<sup>+/−</sup> mutants. However, double-mutant males showed an increase in sperm viability and could father litters, in contrast to their homozygous Kit mutant, wild-type p53 littermates. This germ cell rescue appears to be male specific, as female ovaries were similar in mice homozygous for the Kit mutant allele with or without p53. We conclude that defective Kit signaling in vivo results in apoptosis by a p53-independent pathway in melanocyte and mast cell lineages but that in male germ cells apoptosis in the absence of Kit is p53-dependent. © 1999 Academic Press

Key Words: mice; melanocytes; germ cells; mast cells; Kit<sup>W-v</sup>; Trp53; Mgf.

INTRODUCTION

Mutations of the mouse receptor tyrosine kinase, Kit, or its ligand, mast cell growth factor (Mgf) (stem cell factor or steel factor) are also known as the classical dominant white spotting (Kit<sup>W</sup>) and steel (Mgf<sup>Sl</sup>) mutants. Numerous mutant alleles have been identified at both loci that profoundly affect the development of three apparently unrelated stem cell populations, namely melanoblasts, primordial germ cells, and hematopoietic stem cells. Mice homozygous for a loss of function at either the Kit or the Mgf locus show severe macrocytic anemia, have decreased numbers of mast cells, are infertile, and have almost a complete lack of coat pigmentation. Mgf binds to Kit as a dimer and induces homodimerization of the receptor and subsequent intracellular signaling (Philo et al., 1996).

Point mutations in the Kit receptor, such as Kit<sup>W-v</sup>, which affect tyrosine kinase activity, demonstrate an antimorphic or dominant negative phenotype when heterozygous. Half of the wild-type receptor monomers are inactivated by dimerization with mutant partners producing effectively 25% of normal active Kit receptors on the cell surface (Nocka et al., 1990). The protein encoded by Kit<sup>W-v</sup> harbors a point mutation which significantly reduces its tyrosine kinase activity compared to the wild-type receptor. Heterozygous Kit<sup>W-v</sup> animals display a characteristic pigmentation phenotype: lightening of the coat color, a white belly and head spot, and reduced pigmentation of the feet and tail. Although fertile, they are also slightly anemic. Homozygotes have the typical phenotype of severe loss of function of Kit. They have almost no coat pigmentation, although some animals have slightly pigmented skin on the pinna (external ear); they are also anemic, have a deficiency of mast cells, and are infertile.

Melanocytes originate from neural crest cells and migrate, as melanoblasts, to peripheral sites between days 10
and 16 of mouse embryonic development. We have previously shown that the survival of melanoblasts in vivo depends crucially on Kit signaling after embryonic day 11 (E11) (Steel et al., 1992, Cable et al., 1995, MacKenzie et al., 1997). We have also suggested that Kit signaling plays a vital role in melanoblast proliferation (MacKenzie et al., 1997). Others have shown a requirement of this signaling pathway for melanocyte survival, proliferation, and differentiation in culture (Murphy et al., 1992, Morrison-Graham and Weston, 1993, Lahav, 1994); for migration of melanoblasts from the neural crest (Wehler-Haller and Weston, 1995); and for the continued survival of melanocytes after their migration to the skin and hair follicles (Nishikawa et al., 1991).

Germ cells require Kit signaling at several stages of their development. Kit is highly expressed in primordial germ cells (PGCs) during their proliferative phase from E7.5 to E13 (Manova and Bachvarova, 1991; Bachvarova et al., 1993), but is not required for their initial specification. Survival of PGCs in vitro requires stimulation of Kit by Mgf expressed on feeder cells (Dolci et al., 1991; Godin et al., 1991) as does proliferation of PGCs in similar systems (Matsui et al., 1991; Buehr et al., 1993). In addition, anti-Kit antibodies (Matsui et al., 1991; Pesce et al., 1997) block the adhesion of PGCs to somatic cells (Sertoli and Leydig cells).

Both male and female germ cells are dependent on active Kit for subsequent postnatal maturation. Kit expression has been detected in Leydig cells, spermatogonia, primary spermatocytes, and round spermatids of the testis (Manova et al., 1990, 1993; Rossi et al., 1993; Sandlow et al., 1997) and Kit signaling is necessary for the proliferation and postnatal maturation of spermatogonia (Yoshinaga et al., 1991). Follicle-stimulating hormone stimulates the expression of Mgf by Sertoli cells in the testes, which in turn activates DNA synthesis in spermatogonia (Rossi et al., 1993).

Kit is highly expressed at all stages of postnatal development in mouse oocytes, starting in the diplotene stage around the time of birth after which primordial follicles are formed (Manova et al., 1990, 1993; Rossi et al., 1993; Sandlow et al., 1997) and Kit signaling is necessary for the proliferation and postnatal maturation of spermatogonia (Yoshinaga et al., 1991). Follicle-stimulating hormone stimulates the expression of Mgf by Sertoli cells in the testes, which in turn activates DNA synthesis in spermatogonia (Rossi et al., 1993).

Kit expression is highest on the surface of oocytes arrested in the diplotene stage of meiosis but declines upon ovulation and the resumption of meiotic maturation (Horie et al., 1991). In contrast, Mgf expression is required to maintain the supporting granulosa cells surrounding the developing oocyte but also ceases upon the transition of the follicle to the antral stage (Kuroda et al., 1988; Motro and Bernstein, 1993). Several stages of postnatal oocyte development including the onset of primordial follicle development, primary follicle growth, and follicle maturation are disturbed in vivo using antibodies blocking Kit function (Yoshida et al., 1997). Kit is also required for growth of oocytes in culture (Packer et al., 1994). Thus Kit signaling appears to play a role not only in the development of primordial germ cells but also in postnatal ovarian folliculogenesis.

PGCs of mice with severe Kit and Mgf mutations are present in normal numbers at E8.5, but decline to about 2% of the normal level by E12.5 (Mintz and Russell, 1957; McCoshen and McCallion, 1975). Some germ cells populate the gonads in mice carrying weaker Kit or Mgf mutations, but defects are seen in later stages of germ cell development, often with more severe effects on either male or female gonads and fertility (Geissler et al., 1981; Copeland et al., 1990). Comparable numbers of β-gal-positive PGCs are detected in Kit<sup>W<sub>LacZ</sub></sup>/ and Kit<sup>W<sub>LacZ/W-LacZ</sub></sup> E9.5 embryos. However, the homozygous mutant cells show aberrant migration to the ventral half of the gut and have completely disappeared by E12.5 (Bernex et al., 1996).

Hematopoietic progenitor cells also express Kit (Ogawa et al., 1991). Mice which lack Mgf (Mgf<sup>W</sup> homozygotes) or which have Kit blocked by antibody have a severe reduction in hematopoietic stem cells (Ikuta and Weissman, 1992; Ogawa et al., 1993). The hematopoietic defect in Kit<sup>W</sup> mice is first apparent in the blood islands of the embryonic yolk sac and then persists as the stem cells migrate to the fetal liver and to the sites of neonatal and adult hematopoiesis. In addition to depletion of hematopoietic precursors, Kit- or Mgf-deficient mice show a specific deficit of mast cells. Mast cell precursors are present in these mutant mice in apparently normal numbers and will, through action of IL-3, differentiate into bone marrow-derived mast cells (BMDC). However, absence of Kit signaling results in failure of these precursors to proliferate, adhere, and migrate (Reith et al., 1990).

Thus, stimulation of the Kit receptor pathway is essential for several functions in cells of diverse lineages. The initial requirement for Kit activation, in most if not all lineages, is survival of the progenitor cells. We wished to address the question of how the progenitor cell survival is mediated by examining the mechanism by which the cell is lost in the absence of signaling.

The p53 tumor suppressor protein plays a critical role in regulating apoptosis and G1/S cell cycle arrest by acting as an integral part of the cellular response to DNA damage. Cells lacking p53 fail to arrest in G1 following exposure to ionizing radiation and are resistant to apoptosis induced in response to DNA damage (Lowe et al., 1993; Clarke et al., 1993). Cell death in absence of growth factor stimulation is mediated by p53 for a number of cell lines. Expression of p53 in a myeloid leukemia cell line results in the cells becoming dependent on interleukin 6 (IL-6) for survival. In its absence the cells die by apoptosis, unless the p53 protein is inactivated (Yonish-Rouach et al., 1991, 1993). Furthermore, murine hematopoietic cell lines will become apoptotic after irradiation unless stimulated by IL-3 and functional p53 is required to initiate death (Canman et al., 1995).
Transformed Friend erythroleukemia cells do not normally express p53 and can grow independent of erythropoietin (Epo). Expression of p53 in these cells leads to a rapid loss in viability unless exogenous Epo is added; withdrawal of Epo results in apoptosis (Johnson et al., 1993).

Trp53 mutant mice have a complete absence of p53 protein, yet develop normally. They are, however, predisposed to spontaneous tumor formation and develop predominantly lymphomas. The majority of animals develop tumors or are dead by 10 months of age (Donehower et al., 1992).

Significantly, several studies have shown that defective Kit/Mgf signaling leads to cell death in vitro and this death is mediated by a p53-dependent pathway. Addition of Mgf to BMMC suppressed the induction of apoptosis by γ-irradiation or growth factor deprivation (Yee et al., 1994). Interestingly, p53-deficient BMMC continued to undergo apoptosis upon withdrawal of Mgf, but at a lower rate than normal BMMC.

Lotem and Sachs (1993) concluded that bone marrow myeloid progenitor cells and irradiated thymocytes from p53-deficient mice are more resistant to induction of apoptosis in culture when the concentration of any of several viability factors, GM-CSF, IL-1α, IL-3, IL-6, or Mgf, is low. The loss of one allele of wild-type p53 (cells from heterozygous mice) is sufficient for increased resistance. Finally, the addition of exogenous Mgf can attenuate p53-mediated apoptosis and terminal differentiation in Friend erythroleukemia cells (Abrahamson et al., 1995). These are normally p53-deficient and independent of Mgf, but after the introduction of temperature-sensitive p53 the cells become Mgf-dependent at the permissive temperature and respond to its withdrawal by apoptosis.

All of the studies described above used cells in culture as model systems to examine the relationship between Kit/Mgf signaling and p53. We wished to determine whether germ cell, mast cell, and melanoblast cell death due to defective Kit/Mgf signaling was mediated via a p53-dependent pathway in vivo. We produced mice that completely lacked p53 protein and which also were defective in Kit/Mgf signaling and p53. We wished to determine whether these animals showed a reduction in apoptosis and thus increased survival of Kit signaling and examined whether these animals showed a decrease in apoptosis in vivo, and which also were defective in Kit/Mgf signaling and p53. We wished to determine whether these animals showed a reduction in apoptosis and thus increased survival of Kit signaling and examined whether these animals showed a decrease in apoptosis in vivo.

**Materials and Methods**

**Mice**

Mice with a targeted null allele of the gene encoding p53 (Trp53), designated Trp53 “−” in this paper (Clarke et al., 1993), were crossed to mice heterozygous for the Kit“−” mutation, as homozygous Kit“−” mice are infertile. The resultant Kit“−” :Trp53“−” mice were intercrossed and the melanocyte, mast, and germ cell contribution was assessed in the offspring. To specifically analyze the melanoblast population homozygous Dct-lacZ transgenic animals (MacKenzie et al., 1997) were also crossed to the heterozygotes. Compound heterozygotes (Kit“−” :Trp53“−”) with one parent carrying the Dct-lacZ transgene were crossed to generate embryos with one of the nine possible genotypic combinations.

**Analysis of Embryos**

Embryos were generated from intercrosses of compound heterozygous (Kit“−” :Trp53“−”), one of which also contributed the Dct-lacZ transgene. The time of gestation was calculated by taking noon of the day of detection of a vaginal plug as E0.5 and also noting the external appearance of the embryo (according to Kaufman, 1992). Expression of the transgene was detected by XGal staining as outlined previously (MacKenzie et al., 1997).

**Genotyping**

DNA isolated from tail biopsy or extraembryonic membranes was used for PCR assays to identify Kit“−” mutants (Cable et al., 1995), Trp53 mutants (Malcomson et al., 1997), and mice carrying the Dct-lacZ transgene (MacKenzie et al., 1997).

**Histology**

To ensure that the study was unbiased, all histological analyses were performed blind to genotype. For analysis of mast cells in skin. Whole skin was peeled from tail biopsies, pinned out flat, fixed overnight in 4% paraformaldehyde at 4°C, and processed for paraffin embedding. Seven-micrometer sections were stained for 1 min in Azur blue solution. The number of mast cells was quantified by counting the number of stained cells in five 0.142-mm² fields (the microscope field of an ×20 objective (Zeiss Axioplan 2)). The mean number of mast cells in these fields was calculated for each genotype.

Analysis of germ cells from ovaries and testes. Testes and ovaries from newborn and adult mice were fixed overnight in Bouin’s fixative and processed for paraffin embedding. Five-micrometer sections were stained with hematoxylin and eosin. Sperm cell counts were carried out as outlined previously (Searle and Beechey, 1974). Gonocyte counts were recorded from adult tissues and testes sections.

**RESULTS**

To study interactions between the Kit signaling pathway and p53 in vivo, we used mice carrying the spontaneous Kit mutation, Kit“−”, and mice with a targeted null allele of the gene encoding p53 (Trp53). We generated mice defective for both genes by intercrossing compound heterozygous (Kit“−” :Trp53“−”) mice to generate all nine possible genotypes. The offspring from these crosses were used to study melanocytes and their precursor cells mast cells and germ cells.

**Melanocytes**

Over 30 adult Kit“−” :Trp53“−” mice were generated from the compound heterozygote intercrosses and they all
showed a lack of coat pigmentation identical to their Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} and Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} littermates. Some of the double mutants showed pigmentation of their pinna that is also occasionally seen in Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} mice. Absence of p53 therefore does not increase melanocyte survival in adult Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} mutant mice. Similarly, heterozygous Kit\textsuperscript{W\textsuperscript{v}} mice that lacked p53 showed reduced coat color pigmentation and spotting identical to their Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} and Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} littermates.

Our previous analyses, using a Dct-lacZ reporter transgene, showed that early melanoblasts can be detected emerging from the neural crest from day E10.5, well before they begin to produce pigment. A comparison of wild-type and homozygous Kit\textsuperscript{W\textsuperscript{v}} embryos shows that at E10.5 mutant embryos contain fewer melanoblasts, although their spatial distribution over the head and rostral trunk is similar to wild type (MacKenzie et al., 1997). By E11.5, the number of melanoblasts in mutant embryos has greatly decreased so that very few remain at E12.5 and none are present at later stages.

To examine whether doubly mutant Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} embryos show prolonged survival of melanoblasts during this critical period (E10.5–13.5), the Dct-lacZ transgene was crossed onto the compound heterozygote Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} animals. Embryos retrieved from matings between intercrosses of the compound heterozygotes, one of which contributed the Dct-lacZ transgene, were stained using XGal to assay the effects of both mutations on melanoblast development. Figures 1A–1C show the nine possible combinations of genotypes for Kit and Trp53 obtained for embryonic stages E11.5 and Fig. 1D shows the transgene staining at E12.5 for Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} embryos with all three Trp53 genotypes. No increase in melanoblast survival was noted for the doubly mutant Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} embryos compared to their Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} and Kit\textsuperscript{W\textsuperscript{v}}:Trp53\textsuperscript{1/1} littermates for the stages examined (E10.5–13.5). By E13.5, no stained melanoblasts were detected in any Kit\textsuperscript{W\textsuperscript{v}} embryo regardless of their p53 status. Thus the signal provided by Kit for melanoblast survival appears to act independent of p53 at both embryonic and adult stages.
Mast Cell Counts

![Graph showing mast cell counts](image)

**FIG. 2.** Analysis of mast cell numbers present in the skin of Kit<sup>W/v</sup> and Trp53 double-mutant mice. Tail skin was fixed and stained with Azur blue (see Materials and Methods). Eight mice from each genotype were analyzed and the mean number of Azur blue-positive cells from five microscope fields (×20 objective) was determined. The relative number of mast cells present in sections of skin from +/+:+/+ , Kit<sup>W/v</sup>:Trp53<sup>−/−</sup>, Kit<sup>W/v</sup>:Trp53<sup>−/−</sup>, and Kit<sup>W/v</sup>:Trp53<sup>−/−</sup> is expressed as a percentage of the number of mast cells present in +/+:+/+ mice.

**Mast Cells**

Kit homozygous mutant mice exhibit the hematopoietic phenotype of severe macrocytic anemia and mast cell deficiency. We analyzed tail skin from the offspring of compound heterozygotic intercrosses for the presence of mast cells using Azur blue staining. Eight mice were examined for each genotype. The number of mast cells in the skin of homozygous Kit<sup>W/v</sup> mice is significantly reduced to 37% (±2) relative to wild-type littermates (P < 0.1, α = 0.01) (Fig. 2). Kit<sup>W/v</sup>:Trp53<sup>−/−</sup> mice had a further decrease in mast cell number to 16% (±1.4) compared to wild-type littermates. Absence of Trp53 function does not lead to increased survival of these cells and may actually enhance mast cell death, although this data set does not provide statistical support for this hypothesis.

**Germ Cells**

We examined the gonads of the mutant animals for changes in germ cell numbers. Testes and ovaries were removed from adult mice (>6 weeks) generated from the compound heterozygotic intercrosses. Histological sections of the gonads from at least three age-matched animals (both male and female) were examined for each of the nine different genotypes. The results of the histological analyses of control and Kit<sup>W/v</sup> mice (carrying varying numbers of mutant p53 alleles) are outlined in Table 1.

The ovaries and testes from Kit<sup>W/v</sup> mice regardless of their p53 status were reduced by approximately 85% in size compared to Kit<sup>W/v</sup> and Kit<sup>−/−</sup> mice. Only traces of Graafian follicles and no ova were present in Kit<sup>W/v</sup>:Trp53<sup>−/−</sup>, Kit<sup>W/v</sup>:Trp53<sup>−/−</sup>, and Kit<sup>W/v</sup>:Trp53<sup>−/−</sup> ovaries compared to controls wild type at Kit with the three Trp53 genotypes (Figs. 3A–3C). Similar numbers of follicles and mature ova were detected in Kit<sup>W/v</sup> ovaries, regardless of Trp53 genotype. Homozygous Kit<sup>W/v</sup> ovaries, regardless of p53 status, show a densification of the fibrous stromal structure with ovarian stromal cells frequently arranged in thick swirling cords, consistent with what was previously observed for Kit<sup>W/v</sup> mutants (Coloumbre and Russell, 1954).

The mean testes masses from Kit<sup>W/v</sup>:Trp53<sup>−/−</sup> and Kit<sup>W/v</sup>:Trp53<sup>−/−</sup> males were 15.9% (±1.4) and 26.6% (±3.3) of wild type, respectively. Histological examinations revealed abnormalities in many regions of the seminiferous tubules of Kit<sup>W/v</sup>:Trp53<sup>−/−</sup> and Kit<sup>W/v</sup>:Trp53<sup>−/−</sup> male testes. Vacuolization of the Sertoli cell cytoplasm and degenera-
Histological Data from Adult Testes and Ovaries

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Description of gonads</th>
<th>Sperm count × 10⁶ (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-; +/-</td>
<td>Male</td>
<td>All stages normal</td>
<td>354 ± 27</td>
</tr>
<tr>
<td>KitW-v/W-v;Trp53 +/-</td>
<td>Male</td>
<td>33% tubes have Sertoli and spermatogonia cells only</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Some pachytene cell arrest</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increase in interstitial cells between tubules</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No sperm present in epididymis</td>
<td></td>
</tr>
<tr>
<td>KitW-v/W-v;Trp53 +/-</td>
<td>Male</td>
<td>10-38% tubes have Sertoli and spermatogonia cells only</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Partial pachytene arrest</td>
<td></td>
</tr>
<tr>
<td>KitW-v/W-v;Trp53 +/-</td>
<td>Male</td>
<td>Most normal cells present</td>
<td>24 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~1% tubes have Sertoli and spermatogonia cells only</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lumen of some tubes packed with cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Majority of tubules show germ cell activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sperm seen in most segments of epididymis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sperm seen in some interstitial segments</td>
<td></td>
</tr>
<tr>
<td>+/-; +/-</td>
<td>Female</td>
<td>Normal histology; Graafian follicles and developing oocytes</td>
<td></td>
</tr>
<tr>
<td>KitW-v/W-v;Trp53 +/-</td>
<td>Female</td>
<td>Traces of Graafian follicles</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Densification of the ovary, ovarian stromal cells in thick swirling cords</td>
<td></td>
</tr>
<tr>
<td>KitW-v/W-v;Trp53 +/-</td>
<td>Female</td>
<td>Traces of Graafian follicles</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Densification of the ovary, ovarian stromal cells in thick swirling cords</td>
<td></td>
</tr>
<tr>
<td>KitW-v/W-v;Trp53 +/-</td>
<td>Female</td>
<td>Traces of Graafian follicles</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Densification of the ovary, ovarian stromal cells in thick swirling cords</td>
<td></td>
</tr>
</tbody>
</table>

* Histological sections were examined from three mice of each sex.

* Sperm counts were taken from three males.

Interestingly, KitW-v/W-v;Trp53 +/- mice had a mean sperm count of 3.5 × 10⁶, which increased to 24 × 10⁶ for the KitW-v/W-v;Trp53 +/- mice. Thus in agreement with the histological data there is an increased survival of sperm in the double mutants.

In order to determine whether the rescue to approximately 10% of normal adult sperm counts in the KitW-v/W-v;Trp53 +/- mice could bring about a reversal of the sterility seen in KitW-v/W-v homozygotes, paired matings were set up between mutant mice of either sex and control proven breeder F1 mice. Three mice each, of both sexes, with the following genotypes were tested: KitW-v/W-v;Trp53 +/-, KitW-v/W-v;Trp53 +/-, and KitW-v/W-v;Trp53 +/- Copulatory plugs were evident in the females when caged with the males, indicating that mating had occurred. All of the KitW-v/W-v;Trp53 +/- and KitW-v/W-v;Trp53 +/- mice appeared sterile and did not yield any offspring. The three KitW-v/W-v;Trp53 +/- male mice each mated with three females as evidenced by copulatory plugs. Eight of these nine females gave birth to normal-sized litters (6–12 pups), further confirming the survival of mature sperm in the double male mutants. One of the KitW-v/W-v;Trp53 +/- males gave rise to three successive generations of offspring from the same female over a period of 3 months prior to being sacrificed for histological analysis. However, double-mutant females were infertile and failed to produce any offspring from matings with wild-type males.

We next examined testes from newborn males to compare numbers of germ cells at this stage between the...
Kit<sup>W-v</sup>/W-v:Trp53<sup>-/-</sup> mice and control, Kit<sup>W-v</sup>/W-v:Trp53<sup>+/+</sup>, and Kit<sup>W-v</sup>/W-v:Trp53<sup>+/+</sup> animals. Mean gonocyte counts were taken from 50 testes cross sections for each genotype (Table 2). Interestingly, the Kit<sup>W-v</sup>/W-v:Trp53<sup>-/-</sup> mice had 33.8% (+4.5) of the wild-type counts compared to 12.3% (+5.3) and 8.6% (+1.5), respectively, for the Kit<sup>W-v</sup>/W-v:Trp53<sup>+/+</sup> and Kit<sup>W-v</sup>/W-v:Trp53<sup>+/+</sup> animals.

To determine whether prenatal germ cells have an increased ability to survive we have stained mutant gonads from 129/Ola (129P/Ola) mice, and have been maintained on a CBA background. Male mice homozygous for the Kit<sup>W-v</sup>-<sup>LacZ</sup> mutation, which was made by homologous recombination in ES cells, were used (Bernex et al., 1996). As these mice die at birth or a few days later, usually before they are 3 days of age, newborn testes were examined. No gonocyte cells could be found in the Kit<sup>W-v</sup>-<sup>LacZ</sup> testes, while wild-type littermates gave numbers similar to those obtained from a CBA background (Table 2). Thus, this 129 strain background (129/ SvPas now called 129S2/SvPas) does not rescue the male germ cell defect of Kit mutation. Although there are some genetic differences between the Ola and the Sv substrains of 129 mice, simple sequence length polymorphic markers do not show differences between the substrains in the region of chromosome 11 around the Kit locus, in contrast to other regions of the genome (Threadgill et al., 1997; Simpson et al., 1997). It appears that the increased germ cell survival and reversal of sterility observed in the Kit<sup>W-v</sup>-<sup>LacZ</sup> males are due solely to the lack of the p53 apoptotic-inducing signal.

### TABLE 2
Mean Genocyte Counts from Newborn Testes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Genetic background</th>
<th>No. examined</th>
<th>Gonocyte count&lt;sup&gt;a&lt;/sup&gt; (mean ± SD)</th>
<th>% of wild-type count</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+;+/-</td>
<td>CBA, 129P/Ola</td>
<td>3</td>
<td>2.42 ± 0.39</td>
<td>129 ± 5.3</td>
</tr>
<tr>
<td>Kit&lt;sup&gt;W-v&lt;/sup&gt;/W-v:Trp53&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>CBA, 129P/Ola</td>
<td>5</td>
<td>0.30 ± 0.13</td>
<td>6 ± 1.5</td>
</tr>
<tr>
<td>Kit&lt;sup&gt;W-v&lt;/sup&gt;/W-v:Trp53&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>CBA, 129P/Ola</td>
<td>2</td>
<td>0.21 ± 0.04</td>
<td>129 ± 5.3</td>
</tr>
<tr>
<td>Kit&lt;sup&gt;W-v&lt;/sup&gt;/W-v:Trp53&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>CBA, 129P/Ola</td>
<td>3</td>
<td>0.82 ± 0.11</td>
<td>33.8 ± 4.5</td>
</tr>
<tr>
<td>+/+;+/-</td>
<td>129S2/SvPas</td>
<td>3</td>
<td>3.59 ± 1.06</td>
<td>129 ± 5.3</td>
</tr>
<tr>
<td>Kit&lt;sup&gt;W-v&lt;/sup&gt;/W-v:Trp53&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>129S2/SvPas</td>
<td>2</td>
<td>0.00</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean gonocyte cell count taken per 50 cross sections.
DISCUSSION

Activation of the signaling pathway controlled by the Kit receptor promotes cell division and the growth of three distinct lineages, melanocytes, mast cells, and germ cells. Activation of p53 leads to apoptosis in several cell types. Several stimuli, including withdrawal of growth factors, can cause p53-dependent apoptosis. Previous cell studies using bone marrow-derived mast cells (Yee et al., 1994), bone marrow myeloid progenitor cells, irradiated thymocytes (Lotem and Sachs, 1993), and erythroleukemia cells (Abrahamson et al., 1995) suggest that the cell death induced by p53 can be inhibited the action of Mgf.

We have taken advantage of mice mutant in Kit and p53 to dissect the role of p53 in regulating cell death in the absence of Kit signaling in vivo. In contrast to the cultured cell studies we have found no evidence that deficiency of p53 prevents cell death due to the absence of Kit signaling in melanoblasts, melanocytes, or mast cells. It appears that cell death in these lineages occurs by a mechanism independent of p53. However, homozygosity for a mutation in Trp53 ameliorated one aspect of the Kit\(^{W-v}\) phenotype, namely male sterility.

**No Increased Survival of Melanocytes or Mast Cells**

Death of melanoblasts occurs in Kit\(^{W-v}\) mutants from E11.5 and at later gestational stages no cells can be detected. Cell death of melanoblasts still occurs in embryonic double, Kit\(^{W-v}\):Trp53\(^{-/-}\), mutants and the adult double mutants are devoid of coat pigmentation. Melanocyte survival in the absence of Kit/Mgf interactions is not ameliorated by the absence of Trp53.

Exogenous Mgf acts as a survival factor for hematopoietic cells in culture (Brandt et al., 1994) and mast cells derived from W mutant mice exhibit reduced or no survival in the presence of Mgf, depending on the severity of the mutation on Kit kinase activity (Reth et al., 1990; Nocka et al., 1990; Fujita et al., 1988). We have shown that the loss of mast cells in vivo is not mediated by p53. The mast cell deficiency associated with the Kit mutation is ameliorated in mice doubly homozygous for mutations in both the Kit receptor (Kit\(^{W^{+}}\)) and the hematopoietic cell phosphatase (Hcph\(^{mm}\)) genes (Paulson et al., 1996). They show that the double Kit\(^{W^{+}}\)/Kit\(^{W^{+}}\):Hcph\(^{mm}\)/Hcph\(^{mm}\) mutant mice have approximately 75% of wild-type levels of mast cells, indicating that the absence of hematopoietic cell phosphatase function largely compensates for the almost complete loss of Kit receptor tyrosine kinase activity.

**Rescue of the Male Kit\(^{W-v}\) Sterility Defect in the Absence of p53**

A two-step requirement for active Kit signaling in germ cells has been well documented. Initially, Kit is required for the migration of the germ cells from the yolk sac to the genital ridges in the embryo and after birth for the maturation of the spermatogonia (McLaren, 1980; Mintz and Russell, 1957; Coloumbre and Russell, 1954; Sorrentino et al., 1991). From our analyses it would appear that the initial Kit requirement for migration of the germ cells to the seminal ridges and survival at this early stage during development is rescued by the lack of p53 in the double mutants. The Kit\(^{W-v}\):Trp53\(^{-/-}\) mutants show ~30% of wild-type gonocyte cell numbers at birth, but this decreases to ~10% of sperm counts by adulthood. In contrast, the Kit\(^{W-v}\) males have 12% of wild-type germ cells at birth and no sperm by adulthood. Therefore, the increased numbers of cells that survive to birth in the absence of Kit and Trp53 function are sufficient to allow maturation to spermatogonia and to support fertility in mutant males. This sperm maturation process can occur even in the presence of only low levels of Kit signaling. All the tested adult Kit\(^{W-v}\):Trp53\(^{-/-}\) males fathered offspring although their actual sperm levels were only approximately 10% of wild-type levels.

The panda mutation at the Mgf locus (Mgf\(^{S-gan}\)), which results in a fivefold reduction in Mgf, allows normal production of sperm (Huang et al., 1993; Bedell et al., 1995). Interestingly, germ cell numbers in panda males at birth were reduced to 30% of controls similar to the levels observed in our Kit\(^{W-v}\):Trp53\(^{-/-}\) neonates. Meiotic spermatocytes were produced on schedule in juvenile panda males and there was no appreciable effect on male fertility. It would appear that levels of 10–30% of wild-type gonocytes and approximately 10% of adult sperm numbers are also sufficient to ensure fertility in these mutant males.

**Absence of p53 Sperm Defects**

Defects in sperm production or tubule histology have been previously reported for p53-deficient mice on varying genetic backgrounds. An increased level of multinucleate giant cells, spreading from the periphery into the lumen of the seminiferous tubules, was observed in p53-null mice from an inbred 129/Sv background. Homozygous tubules of C57BL/6j × 129/Sv mixed genetic background exhibited apparently normal tubules (Rotter et al., 1993). Our analyses of single Trp53\(^{-/-}\) or double-mutant Kit\(^{W-v}\):Trp53\(^{-/-}\) mice do not show any giant cell defects. Neither have we observed any increase in sperm head or tail defects in the Trp53\(^{-/-}\) mice, in contrast to the higher numbers of abnormal spermatocytes and reduced fertility of Trp53-null mice on a C57BL/6j background (Yin et al., 1998). The numbers of spermatocytes obtained from the Trp53\(^{-/-}\) and control mice were comparable on our mixed CBA × 129/Ola genetic background.

Apoptotic cells were visible in the tubules from the Kit\(^{W-v}\):Trp53\(^{-/-}\) mice (Fig. 3F), despite the loss of Trp53. Thus cell death still occurs in the mutant tubules by a p53-independent pathway.
Cell Death in Female Germ Cells Is p53 Independent

Histological analysis indicates that lack of p53 in female germ cells cannot rescue death due to a reduction of Kit signaling. The mutant ovaries from the Kit$^{+/\text{W-v}}$:Trp53$^{+/\text{f}}$, Kit$^{+/-}\text{W-v}}$:Trp53$^{+/-}$, and Kit$^{+/-}\text{W-v}}$:Trp53$^{+/-}$ mice all show similar defects. Oocyte growth and follicle cell growth and proliferation advance only to the earliest stages with no further progression for all three genotypes. In addition the double-mutant females failed to produce any progeny when mated with wild-type males. Germ cell death in the absence of Kit signaling appears to be independent of p53 in females.

Only 20% of normal oocytes survive at birth in Sl$^{+\text{on}}$ female mutants (Huang et al., 1993). The initiation and maintenance of ovary follicle development in juvenile and adult Sl$^{+\text{on}}$ mice were affected and subsequent follicle development arrested at the one-layered cuboidal stage (Huang et al., 1993; Bedell et al., 1995). This phenotype is very similar to our observations of the ovarian defects in the Kit$^{+\text{v}}$ mutants regardless of p53 genotype. Hence, a higher threshold level of germ cells is required at birth to enable complete oocyte maturation compared with that required for spermatogonia development.

Are p53-Independent Pathways Involved in Apoptosis Due to a Lack of Kit/Mgf?

If the melanocytes, mast cells, and female germ cells lacking p53 die in the absence of Kit what mechanism is involved? Cell death by p53-independent pathways has been previously documented both in vivo and in vitro. The remodeling of mammary gland tissue during involution proceeds through p53-independent pathways in vivo (Li et al., 1996; Shibata et al., 1996). Furthermore, in the developing mouse nervous system, cell death is p53-dependent in the central nervous system but p53-independent in the peripheral nervous system (Macleod et al., 1996). More recently, it has also been shown that primary mast cell cultures derived from p53-null mice are still dependent on IL-3 for survival (Silva et al., 1997). In contrast, another study (Gottlieb and Oren, 1998) showed that functional p53 is required for efficient apoptosis in response to IL-3 withdrawal in lymphoid cell lines. The pathways determining survival or death appear tissue and sex specific as from our analyses different mechanisms operate in both male and female germ cells.

The intracellular signaling pathways by which growth factors promote survival are poorly understood and much of the work has been carried out in disparate cell types and may not reflect the true situation in vivo. In recent years a number of secondary messengers including protein kinase C, mitogen-activated kinases, and phosphatidylinositol (PI)-3 kinases have been shown to be induced by Kit/Mgf signaling (Blume-Jensen et al., 1993; Timokhina et al., 1998; Hemesath et al., 1998; Blume-Jensen et al., 1994). Functional signaling presumably directs downstream signal transducers to block cell death. In the absence of Kit/Mgf signaling and subsequent secondary messenger activation, the apoptotic response can be engaged without a specific requirement for p53. Mgf appears unlikely to interfere directly with the apoptotic machinery, because the expression of the p53-responsive genes bax and bcl-2 is not directly affected by its action (Digiuseppe et al., 1999).

PI-3 kinase is required for Mgf-induced mitogenesis and cell survival and is itself activated by Kit/Mgf signaling (Blume-Jensen et al., 1994; Serve et al., 1995). A cascade of events occurs, mediated by PI-3 kinase, including activation of the serine/threonine protein kinase Akt, which in turn can activate and phosphorylate Bad, the proapoptotic molecule (Blume-Jensen et al., 1998). Upon phosphorylation, Bad in turn is sequestered, preventing an apoptotic response (Zha et al., 1996). Lack of Kit signaling in the Kit$^{+/\text{W-v}}$:Trp53$^{+/-}$ mutant melanocytes and germ cells may prevent the phosphorylation and subsequent removal of Bad, thereby inducing cell death even in the absence of Trp53.

In summary, we have shown that in the absence of Kit and p53 signaling, melanocyte and mast cell death still occurs; however, sufficient male germ cells can survive to support fertility. Different mechanisms appear to operate in different cell types in vivo in order to bring about life or death.

ACKNOWLEDGMENTS

We thank the staff of the MRC Transgenic Unit and the Biomedical Research Facility for technical assistance and the Photography Department for their efficient service. We also thank Dr. Alan Clarke for providing the Trp53-null mice, Dr. Katerina Manolakou and Dr. Peter Teague for advice on statistical analyses, and Professor Nick Hastie and Dr. Pen Rashbass for useful discussions. This work was funded by the UK Medical Research Council and was initiated while S.A.J. was a Human Frontier Science Program Fellow.

REFERENCES


Copyright © 1999 by Academic Press. All rights of reproduction in any form reserved.


Received for publication June 14, 1999
Revised July 20, 1999
Accepted July 27, 1999