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Point mutation in Kit receptor tyrosine kinase reveals essential roles for Kit signaling in spermatogenesis and oogenesis without affecting other Kit responses

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The Kit receptor tyrosine kinase functions in hematopoiesis, melanogenesis and gametogenesis. Kit receptor-mediated cellular responses include proliferation, survival, adhesion, secretion and differentiation. In mast cells, Kit-mediated recruitment and activation of phosphatidylinositol 3′-kinase (PI 3-kinase) produces phosphatidylinositol 3′-phosphates, plays a critical role in mediating cell adhesion and secretion and has contributory roles in mediating cell survival and proliferation. To investigate the consequences *in vivo* of blocking Kit-mediated PI 3-kinase activation we have mutated the binding site for the p85 subunit of PI 3-kinase in the *Kit* gene, using a knock-in strategy. Mutant mice have no pigment deficiency or impairment of steady-state hematopoiesis. However, gametogenesis is affected in several ways and tissue mast cell numbers are affected differentially. While primordial germ cells during embryonic development are not affected, Kit^{Y719F}/Kit^{Y719F} males are sterile due to a block at the premeiotic stages in spermatogenesis. Furthermore, adult males develop Leydig cell hyperplasia. The Leydig cell hyperplasia implies a role for Kit in Leydig cell differentiation and/or steroidogenesis. In mutant females follicle development is impaired at the cuboidal stages resulting in reduced fertility. Also, adult mutant females develop ovarian cysts and ovarian tubular hyperplasia. Therefore, a block in Kit receptor-mediated PI 3-kinase signaling may be compensated for in hematopoiesis, melanogenesis and primordial germ cell development, but is critical in spermatogenesis and oogenesis.
Keywords: *Kit* receptor tyrosine kinase/Leydig cells/oogenesis/PI 3-kinase/spermatogenesis

Introduction

The receptor tyrosine kinase Kit and its only known ligand, Kit Ligand (KL), are encoded at the *White spotting* (*W*) and *Steel* (*Sl*) loci in the mouse, respectively (Chabot *et al*., 1988; Geissler *et al*., 1988; Copeland *et al*., 1990; Huang *et al*., 1990; Zsebo *et al*., 1990). Phenotypes of *W* and *Sl* mutations suggest roles for Kit signaling in melanogenesis, hematopoiesis and gametogenesis (Russell, 1979; Silvers, 1979). Many alleles of variable severity at both the *W* and *Sl* loci have been described and characterized (Besmer, 1997). *W* mutations either abolish or partially impair Kit receptor function. Whereas Kit receptor/*W* point mutations affect gametogenesis, melanogenesis and hematopoiesis during embryogenesis and in postnatal development to similar degrees, in contrast, mutations that affect *Kit* or *KL* expression may affect cellular targets of *W* and *Sl* mutations differentially. In hematopoiesis Kit function is critical in the stem cell hierarchy and in the erythroid and mast cell lineages; mutant animals have macrocytic anemia and lack tissue mast cells (Galli *et al*., 1994; Besmer, 1997). During embryonic development melanoblasts migrate from the neural crest to the periphery. They then enter the epidermal ectoderm and colonize hair follicles. Postnatally, amelanotic melanoblasts differentiate to become melanocytes. *W* and *Sl* mutations affect several aspects of melanogenesis, the early migratory phase in embryonic development, the time when melanoblasts reach the epidermal ectoderm as well as differentiated melanocytes in hair follicles causing differing degrees of white spotting (Silvers, 1979; Besmer *et al*., 1993).

The Kit receptor plays roles in Kit-mediated signaling events in primordial germ cells (PGCs), spermatogenesis and oogenesis (Bachvarova *et al*., 1993; Besmer *et al*., 1993). PGCs are derived from the posterior primitive streak and then migrate from the base of the allantois through the hindgut endoderm and the mesentery to the genital ridges; spermatogenesis and oogenesis then proceed following distinct, well studied developmental programs. During embryonic development the Kit receptor is expressed in PGCs from embryonic day 7.5 (E7.5) to E13.5 (Manova and Bachvarova, 1991). In the testis Kit expression starts at postnatal day 5 (P5) and is restricted to differentiating type A spermatogonia, type B spermatogonia, primary spermatocytes and Leydig cells (Manova *et al*., 1990; Yoshinaga *et al*., 1991). In the ovary the Kit receptor is expressed in primordial oocytes and growing oocytes throughout follicle development and in interstitial theca cells (Manova *et al*., 1990; Horie *et al*., 1991; Yoshinaga *et al*., 1991). KL is expressed in the microenvironment of Kit expressing cells along the migratory path of PGCs and in the gonads, in Sertoli cells of the testis and granulosa cells at all stages of follicle maturation. *W* and *Sl* mutations affect the survival, migration and proliferation of PGCs. The mutations also affect steps in spermatogenesis and oogenesis including the survival and proliferation of spermatogonia and oocyte growth, causing

impaired fertility (Bennett, 1956; Kuroda *et al*., 1988; Nakayama *et al*., 1988; Bachvarova *et al*., 1993). In *W* and *Sl* mutant mice that lack Kit receptor function PGCs fail to proliferate and migrate, and none or only a few reach the gonad. In weak *W* and *Sl* alleles, effects on spermatogenesis and oogenesis are observed as well. In W/W^v mice only a few germ cells reach the gonad, and subsequent spermatogenesis is more severely affected than oogenesis. The KL expression mutations Sl^{panda}, Sl^t and Sl^{con} impair female fertility, while males are fertile. In contrast, a cytoplasmic domain mutation of the membrane growth factor KL, Sl^{17H}, does not impair female fertility, but causes male sterility.

Taken together, the defects in *W* and *Sl* mutant mice are consistent with a role of the Kit receptor system in facilitating cell proliferation and survival of precursor cells as well as promoting cell migration, cell adhesion, secretion and other functions in differentiated cells (Galli *et al*., 1994; Besmer, 1997).

How does the Kit receptor mediate these diverse cellular responses in distinct cell populations during embryonic development and in the adult animal? Studies in bone marrow derived mast cells (BMMCs) have provided insight into the mechanism by which Kit mediates various cellular responses including cell proliferation, survival, adhesion, actin reorganization, membrane ruffling and secretion. Kit receptor activation leads to autophosphorylation, the phosphorylation of various substrates and the association with signaling molecules, thereby activating distinct signaling cascades. Molecules known to associate with the Kit receptor *in vivo* include the p85 subunit (p85α and p85β) of class IA phosphatidylinositol 3′-kinases (PI 3-kinases) (Blume-Jensen *et al*., 1994; Serve *et al*., 1994; Herbst *et al*., 1995), phospholipase Cγ-1 (Reith *et al*., 1991; Rottapel *et al*., 1991), the Grb2 adaptor protein, the Src kinase (Blume-Jensen *et al*., 1994) and the tyrosine phosphatases SHP1 and SHP2 (Yi and Ihle, 1993), but not the adaptor protein Shc and the exchange factor Nck (Blume-Jensen *et al*., 1994). In addition, Kit receptor activation causes phosphorylation and activation of the Shc adaptor protein (Cutler *et al*., 1993), Ras (Duronio *et al*., 1992) and the Vav GDP/GTP exchange factor (Alai *et al*., 1992). In BMMCs, mutation of the Kit receptor binding site for class IA PI 3-kinase adaptor proteins, KitY719, and for src, KitY567, was shown to affect cell proliferation, survival, adhesion and secretion to differing degrees (Serve *et al*., 1995; Vosseller *et al*., 1997; Timokhina *et al*., 1998). Whereas Kit-mediated PI 3-kinase activation contributes to the mitogenic and survival response in BMMCs, in the secretory response, cell adhesion response, actin polymerization and membrane ruffling responses, Kit-mediated PI 3-kinase activation is critical. Activation of PI 3-kinase results in the rapid accumulation of phosphatidylinositol-3,4-bisphosphate (PI 3,4-P_2) and phosphatidylinositol-3,4,5-trisphosphate (PI 3,4,5-P_3). PI 3,4-P_2 and PI 3,4,5-P_3 are important second messengers regulating the catalytic activity of downstream signaling molecules via binding to pleckstrin homology (PH) and FYVE domains, thereby mediating activation of a wide array of downstream targets including the protein kinases PDK1, Akt, PKCδ and the small GTPase Rac1 (Carpenter and Cantley, 1996; Toker and Cantley, 1997; Fruman *et al*., 1999a).

In order to investigate the mechanism of Kit receptor signaling and to elucidate the consequences of blocking signaling events mediated by the phosphorylation of Kit Tyr719 and the subsequent activation of PI 3-kinase *in vivo*, we mutated Tyr719 in the *Kit* receptor gene by substituting it with phenylalanine (Y719F) in the mouse genome by using a knock-in strategy. Analysis of homozygous mutant Kit^{Y719F}/Kit^{Y719F} mice reveals effects of the mutation on spermatogenesis, resulting in male sterility, on ovarian follicle development, severely impairing follicle maturation in females, and the development of hyperplastic changes in both the male and the female. This indicates an essential role for Kit Tyr719 and Kit-induced PI 3-kinase activity in mouse gametogenesis. Furthermore, an effect of the mutation is seen on peritoneal mast cell numbers but not on skin tissue mast cell numbers. Other Kit expressing lineages show no obvious phenotypes, suggesting that the cellular context is of great importance for the interpretation of the signal. Kit-induced PI 3-kinase activity in these tissues either is not required or can be compensated for most likely through synergy with other growth factor receptors.

Results

Point mutation in the Kit receptor (Kit^{Y719F}) abolishes PI 3-kinase signaling in vivo

To gain insight into the role of Kit-induced PI 3-kinase activity *in vivo* we substituted tyrosine residue 719 of the c-*kit* gene with phenylalanine (Y719F) by using knock-in gene targeting technology (Figure 1). The Y719F mutation prevents the binding of SH2 domain proteins including the p85 subunit of PI 3-kinase and abolishes subsequent signaling events (Serve *et al*., 1994, 1995; Vosseller *et al*., 1997; Timokhina *et al*., 1998). In the targeting construct shown in Figure 1A the Y719F mutation in exon 15 produced a restriction site for *Eco*RI, thereby allowing easy identification of the mutant allele (Figure 1A). The neomycin resistance gene in intron 14 was flanked by two loxP sites for subsequent removal *in vivo*. Homologous replacement in ES cells produced neomycin-resistant colonies and seven correctly targeted ES cell clones were identified by Southern blot analysis. Two of these clones produced chimeric animals after injection into C57BL/6J blastocysts and successfully contributed to the germline, resulting in heterozygous $Kit^{Y719F-neo}/+$ animals after mating with C57BL/6J mice (Figure 1B).

It was previously shown that the inclusion of a *neo* gene in intronic sequences may interfere with the expression of the associated gene (McDevitt *et al*., 1997). $Kit^{Y719F-neo}/+$ heterozygous mice displayed a pigmentation phenotype similar to the original *W* mutation, showing a white belly spot, white tail tips and white feet. Intercrossing of these mice produced $Kit^{Y719F-neo}/Kit^{Y719F-neo}$ homozygous mutant mice. These mice were black-eyed whites and in addition displayed other characteristics of *W* mutant mice, including mast cell deficiency and male and female infertility. Analysis of Kit expression levels by fluorescence-activated cell sorter (FACS) in BMMCs revealed a 75% reduction compared with Kit expression levels in BMMCs from wild-type litter mates (Figure 1C). Therefore, placement of the *neo* expression cassette in intron 14 of the c-*kit* gene produced a hypomorphic allele. In order to

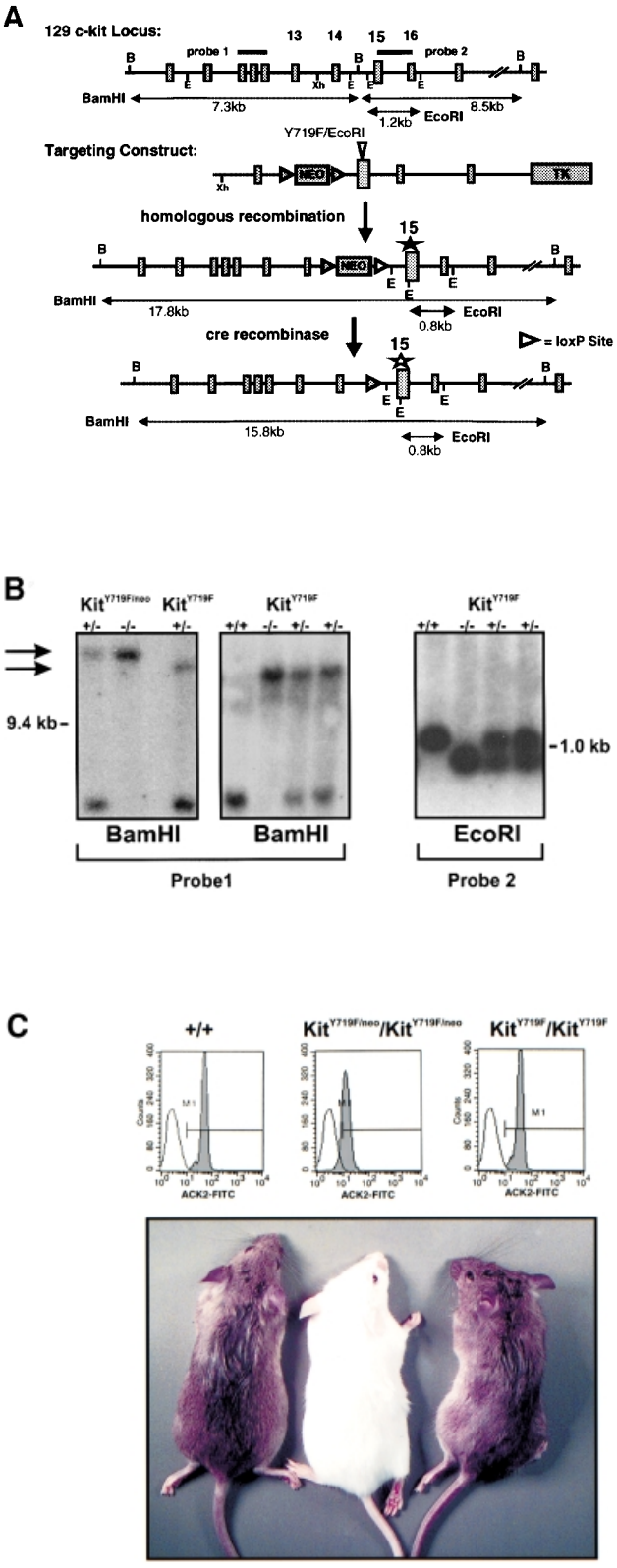

Fig. 1. Targeted mutation of Tyr719 in the 129/Sv *Kit* locus does not affect pigmentation in Kit^{Y719F}/Kit^{Y719F} mice. (**A**) Schematic representation of targeting strategy. B, *Bam*HI; E, *Eco*RI; Xh, *Xho*I. LoxP sites are indicated by triangles. (**B**) Southern blot analysis of tail tips using *Bam*HI and *Eco*RI digestion [see (A) for detail]. (**C**) Determination of cell surface expression of Kit in Kit^{Y719F}/Kit^{Y719F}, $Kit^{Y719F/neo}/Kit^{Y719F/neo}$ and control BMMCs using FACS. The pigmentation phenotype of +/+, $Kit^{Y719F/neo}/Kit^{Y719F/neo}$ and Kit^{Y719F}/Kit^{Y719F} mice is shown in the bottom panel.

obtain normal Kit expression levels *in vivo* we excised the *neo* expression cassette that is flanked by loxP sites by crossing the $Kit^{Y719F-neo}/+$ mice with mice expressing the Cre recombinase under the direction of a suitable promoter. Heterozygous $Kit^{Y719F-neo}/+$ mice were mated with transgenic mice in which expression of *Cre* was directed by the adenovirus EIIa promotor. In EIIa cre mice expression of the recombinase is restricted to the zygote stage of development (Lakso *et al*., 1996). Loss of the *neo* gene in the F_1 generation was confirmed by Southern blotting using a *Bam*HI restriction digest (probe 1; Figure 1B) and reprobing these filters with a neo-specific probe (not shown). Heterozygous $Kit^{Y719F}/+$ mice were then intercrossed to generate homozygous mutant Kit^{Y719F}/Kit^{Y719F} animals. The presence of the Y719F point mutation was confirmed by digestion with *Eco*RI and Southern blotting (probe 2; Figure 1B). Excision of the *neo* cassette restored Kit expression levels in Kit^{Y719F}/Kit^{Y719F} BMMCs to wild-type levels, as shown by FACS analysis (Figure 1C).

In order to establish that association of the p85 subunit of PI 3-kinase with the activated Kit receptor is abolished in BMMCs obtained from Kit^{Y719F}/Kit^{Y719F} mice, mutant and normal BMMCs were stimulated with KL/Mgf, cell extracts were prepared, and the Kit receptor was immunoprecipitated, fractionated by SDS–PAGE and immunoblotted using anti-p85 antibody. As shown in Figure 2A, no association of p85 with the activated Kit^{Y719F} receptor was detected. In addition, blots were reprobed with anti-phosphotyrosine antibody. While both mutant and wild-type receptors were autophosphorylated in response to KL/Mgf (Figure 2A), phosphorylation levels in Kit^{Y719F}/Kit^{Y719F} BMMCs were slightly reduced, in agreement with published results (Timokhina *et al*., 1998).

Next, KL/Mgf-stimulated activation of Akt and JNK in Kit^{Y719F}/Kit^{Y719F} BMMCs was analyzed. Kit-induced activation of Akt and JNK was determined using *in vitro* kinase assays with histone H2B and glutathione *S*-transferase (GST)–Jun proteins as substrates, respectively. Similarly to the results obtained with Kit negative W^{sh}/W^{sh} BMMCs reconstituted with mutant Kit^{Y719F} receptor (our unpublished results), the PI 3-kinase binding site mutation abolished KL/Mgf-stimulated Akt activation (Figure 2B). However, reduction of KL/Mgf-stimulated JNK activity by the PI 3-kinase binding site mutation was somewhat stronger in BMMCs derived from the Kit^{Y719F}/Kit^{Y719F} mice (~90% inhibition) (Figure 2C) compared with that observed previously in reconstituted BMMCs (Timokhina *et al*., 1998). Finally, KL/Mgf-induced proliferation and suppression of both irradiation- and deprivation-induced apoptosis in Kit^{Y719F}/Kit^{Y719F} BMMCs was evaluated (Figure 3). In all of the cellular assays, i.e. cell cycle progression determined by thymidine incorporation, protection from apoptosis by growth factor deprivation and irradiation, Kit^{Y719F}/Kit^{Y719F} BMMCs exhibited a 40–60% reduced response to KL/Mgf, in agreement with previous observations made in Kit negative BMMCs transduced to express Kit^{Y719F} (Timokhina *et al*., 1998). Therefore, our results indicate critical roles for *Kit*-induced PI 3-kinase activity in mediating these mast cell responses, presumably by activating the Akt and Rac–JNK signaling pathways.

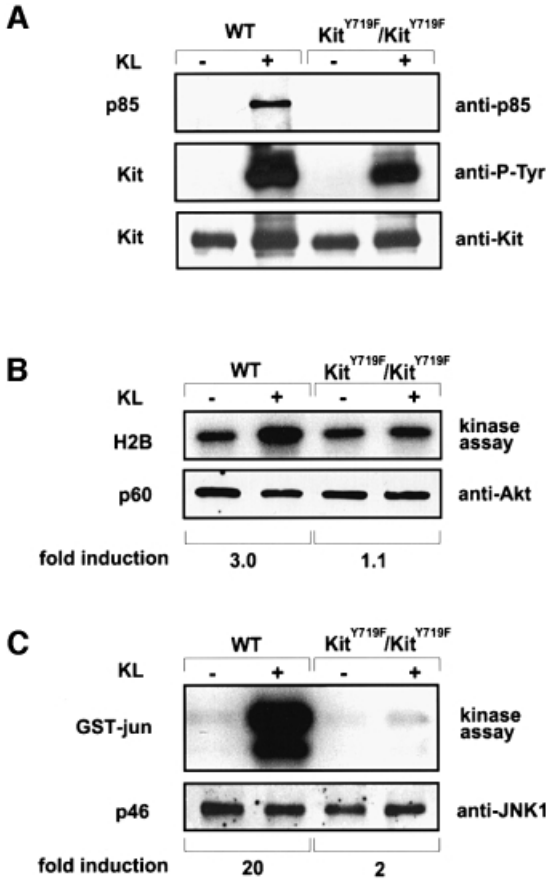

Fig. 2. KL/Mgf-stimulated activation of Akt and JNK in Kit^{Y719F}/Kit^{Y719F} BMMCs. (**A**) Phosphorylation of Kit receptors in Kit^{Y719F}/Kit^{Y719F} BMMCs in response to KL/Mgf and association with the p85 subunit of PI 3-kinase. Cells were starved for 12 h in serum-free medium and then stimulated with KL/Mgf for 5 min. Middle panel: Kit proteins were immunoprecipitated, fractionated by SDS–PAGE and blotted with anti-phosphotyrosine antibody. Upper panel: membranes were stripped and reblotted with the anti-p85 antibody. Lower panel: Kit protein levels are shown. (**B**) Kit^{Y719F}/Kit^{Y719F} and wild-type BMMCs were starved in a serum-free medium for 12 h, followed by stimulation with KL/Mgf for 5 min. Cells were lysed and an Akt *in vitro* kinase assay was performed using histone H2B as a substrate. Lower panel: Akt protein (p60) levels are shown. (**C**) Kit^{Y719F}/Kit^{Y719F} and wild-type BMMCs were starved in a serum-free medium for 12 h and subsequently stimulated with KL/Mgf for 15 min and a JNK *in vitro* kinase assay was performed using GST–Jun fusion protein as a substrate. Lower panel: JNK1 protein (p46) levels are shown. The relative phosphorylation was quantitated using a phosphoimage analyzer (Fuji Mac Bas).

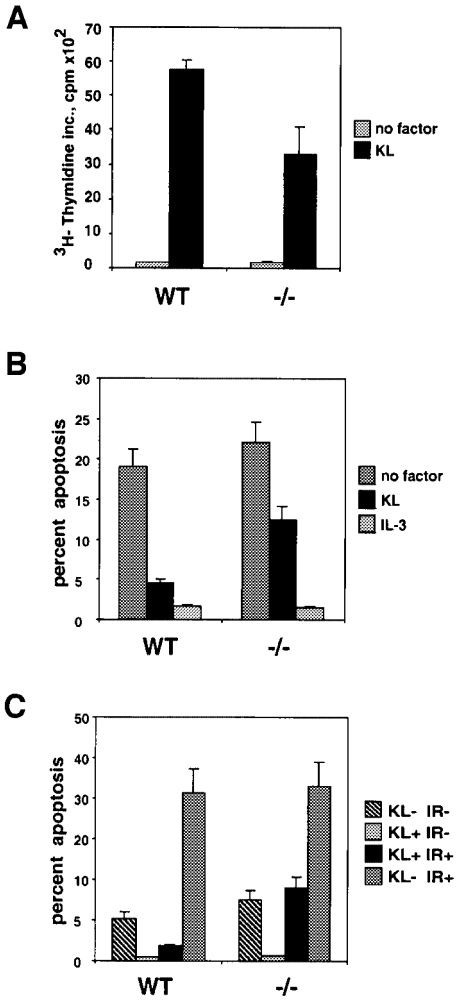

Fig. 3. Kit-mediated proliferation and suppression of apoptosis are affected in Kit^{Y719F}/Kit^{Y719F} BMMCs. (**A**) Proliferation. Kit^{Y719F}/Kit^{Y719F} and wild-type BMMCs were pretreated in a serum-free medium containing IL-3 (20 ng/ml) for 12 h, then starved for 1 h without factors; KL/Mgf (200 ng/ml) or IL-3 (20 ng/ml) was added and after 24 h [^{3}H]thymidine incorporation was determined as described. (**B**) Deprivation-induced apoptosis. Kit^{Y719F}/Kit^{Y719F} and wild-type BMMCs were pretreated as in (A), deprived of growth factors for 50 h and analyzed as described. (**C**) Irradiation-induced apoptosis. Kit^{Y719F}/Kit^{Y719F} and wild-type BMMCs were pretreated as in (A), subjected to γ-irradiation (25 Gy) or left untreated in the presence or absence of KL/Mgf (200 ng/ml). After 24 h cells were harvested and analyzed for apoptosis.

Melanogenesis and steady-state hematopoiesis are normal in mutant mice

Intercrossing of $Kit^{Y719F}/+$ heterozygous mice resulted in the expected distribution of genotypes (+/+, 28.9%, $n = 124$; $Kit^{Y719F}/+$, 46.2%, $n = 198$; Kit^{Y719F}/Kit^{Y719F}, 24.9%, $n = 107$), indicating that all mice were equally viable. The sex ratio of Kit^{Y719F}/Kit^{Y719F} mutants was not affected (50.5% male, $n = 52$; 49.5% female, $n = 51$). All mice developed normally and appeared healthy. No pigmentation phenotype is seen in Kit^{Y719F}/Kit^{Y719F} mutant mice. In contrast, homozygous mutant $Kit^{Y719F-neo}/Kit^{Y719F-neo}$ mice are depigmented. The depigmentation phenotype in the $Kit^{Y719F-neo}/Kit^{Y719F-neo}$ mice is comparable to that seen in mice heterozygous for the dominant-negative Kit^{W42} allele (Tan *et al*., 1990) and this is in agreement with the notion that the $Kit^{Y719F-neo}$ allele is a hypomorph (Figure 1C).

In hematopoiesis, KL/Mgf and Kit have multiple roles. In mice carrying *W* mutations, effects are seen in erythropoiesis, the stem cell compartment and in mast cells (Galli *et al*., 1994; Besmer, 1997). Interestingly, hematocrit values and red blood cell, white blood cell, granulocyte and platelet numbers in mutant mice did not deviate from normal (Table I). These results suggest that Kit-induced PI 3-kinase signaling does not have an apparent role in melanogenesis and steady-state hematopoiesis.

Tissue mast cell numbers in mutant mice are differentially affected

W mutations affect the mast cell lineage (Kitamura *et al*., 1978; Galli *et al*., 1994; Besmer, 1997). Mutation of the

Table I. Peripheral blood cell counts in +/+, Kit^{Y719F}/+ and Kit^{Y719F}/Kit^{Y719F} mice

Genotype	Hematocrit (%)	RBC × 10^8/mm^3	WBC × 10^3/mm^3	ANC × 10^3/mm^3	PLT × 10^6/mm^3
+/+ (n = 5)	51.2 ± 0.47	254 ± 19.5 (n = 2)	6940 ± 2467	1390 ± 592	1.36 ± 025
Kit^{Y719F}/+ (n = 6)	51.7 ± 5.08	291 (n = 1)	6466 ± 700	1000 ± 357	1.55 ± 0.31
Kit^{Y719F}/Kit^{Y719F} (n = 10)	52.2 ± 2.2	275 ± 5.5 (n = 3)	7190 ± 2777	1180 ± 940	1.4 ± 0.26

RBC, red blood cells; WBC, white blood cells; ANC, absolute neutrophil count; PLT, platelets.

Table II. Mast cell numbers in the skin and peritoneum of +/+ and Kit^{Y719F}/Kit^{Y719F} mice

Genotype	Age (weeks)	Skin mast cells (CTMC/cm)	Peritoneal mast cells (% of total peritoneal cells)
+/+	10–16	325 ± 56 (n = 4)	3.58 ± 1.69 (n = 6)
Kit^{Y719F}/Kit^{Y719F}	10–16	300 ± 46 (n = 3)	0.98 ± 0.64 (n = 5)
+/+	32	199 ± 18 (n = 2)	
Kit^{Y719F}/Kit^{Y719F}	32	223 ± 13 (n = 2)	

Kit PI 3-kinase binding site, Kit^{Y719F}, in BMMCs has been shown to affect several cellular responses that are mediated by stimulation of BMMCs with KL/Mgf, including adhesion to fibronectin, enhancement of IgE-triggered secretion, cell survival and proliferation (Serve *et al*., 1995; Vosseller *et al*., 1997; Timokhina *et al*., 1998). Interestingly, in Kit^{Y719F}/Kit^{Y719F} mice, mast cell numbers in dorsal skin sections were not reduced (Table II). In contrast, analysis of peritoneal mast cell numbers showed a 3.5-fold reduction in the mutant mice. Therefore, the Kit^{Y719F} mutation exerts differential effects on mast cell development and/or survival in the peritoneum and the skin.

Primordial germ cells are not affected by the mutation

Typically, *W* mutations affect male and female fertility (Besmer *et al*., 1993). The number of PGCs reaching the gonadal ridges is affected by *W* mutations, indicating an important role for Kit in survival, proliferation and/or migration of PGCs (Buehr *et al*., 1993). In the fetal gonad, *Kit* expression correlates with the proliferative state of both female and male germ cells (Manova and Bachvarova, 1991). At ~E13.5 *Kit* expression ceases. Cessation of *Kit* expression coincides with germ cell entry into meiosis for the female and with entry into a quiescent phase for the male. To examine a possible effect of the Kit^{Y719F} mutation on PGCs we compared the number of germ cells in the gonadal ridges of normal and homozygous mutant embryos at E12.5, a time when the majority of PGCs have completed the migratory phase. No differences were observed in Kit^{Y719F}/Kit^{Y719F} and wild-type mice when hematoxylin and eosin (H&E)-stained sections were compared (McLaren and Southee, 1997) (data not shown).

As a second approach to evaluate the number of PGCs, newborn ovaries were examined (Manova *et al*., 1990; Huang *et al*., 1993). Female germ cells undergo the last round of DNA replication at E13.5 before entering the first meiotic division. They pass through the leptotene, zygotene and pachytene stages and then arrest at the diplotene stage around the time of birth. Therefore, reduced numbers of germ cells at the time of birth may reflect reduced numbers of PGCs reaching the gonadal ridge. Again, examination of H&E-stained sections of three Kit^{Y719F}/Kit^{Y719F} and four heterozygous control ovaries at P0 did not reveal any differences (not shown). In contrast to other *W* mutations then, the Kit^{Y719F} mutation does not affect the survival, proliferation and migration of PGCs.

Spermatogenesis is blocked at premeiotic stages in mutant mice

In the postnatal murine testis Kit expression is observed starting at P5 and is restricted to differentiating type A (A_1–A_4) and type B spermatogonia, preleptotene spermatocytes and the interstitial Leydig cells (Manova *et al*., 1990; Yoshinaga *et al*., 1991). Detailed histological analysis of P6 and P7 Kit^{Y719F}/Kit^{Y719F} mutant testis did not show any noticeable difference between mutant and control animals (Figure 4a–d). The seminiferous tubules have similar numbers of germ cells and the majority of spermatogonia have migrated to the periphery. Comparable frequencies of mitotic figures reflecting germ cell and Sertoli cell proliferation in normal and mutant tubules were observed. However, a striking difference between mutant (n = 6) and control testis [wild type (wt) n = 4 and heterozygotes n = 4] was evident at P10 (Figure 4e–h). In wild-type testis the cellularity of tubules is increased as a result of the proliferation of spermatogonia and the onset of meiosis, as shown by H&E staining and Ki-67 immunohistochemistry (Figures 4e and i and 5c). Ki-67 is a nuclear antigen that is expressed exclusively in the S, G_2 and M phases of the cell cycle (Schluter *et al*., 1993). In contrast, we failed to observe germ cells that had entered meiosis in mutant testis at P10 (Figure 4g and h). Overall, the number of proliferating cells was significantly reduced and the thickness of the wall of the seminiferous epithelium of the mutant testis resembled that of immature tubules of wt animals at P6–P7. In conjunction with reduced proliferation of spermatogonia, an increase in the frequency of apoptotic germ cells was observed by the TUNEL method (Figure 5a and b). As a consequence, mutant tubules were rapidly depleted and their diameter did not increase compared with wild type. At P21 only a few germ cells remained at the basal membrane of the seminiferous tubules (Figure 4i–l). This phenotype was quite uniform in all mutant animals.

The rapid depletion of the juvenile testis suggests an effect of the Kit^{Y719F} mutation during the spermatogonial

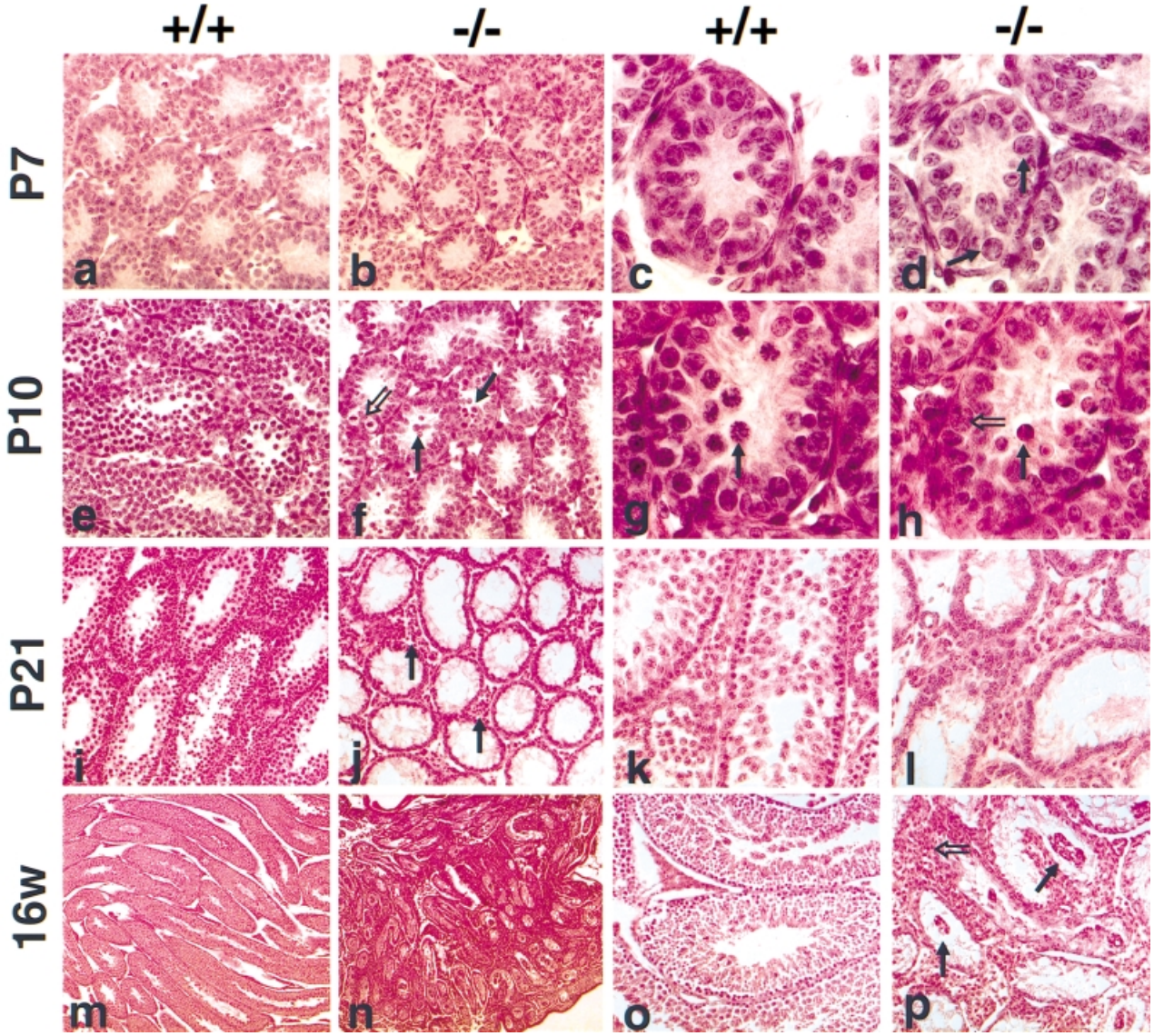

Fig. 4. Histological analysis of postnatal testis in Kit^{Y719F}/Kit^{Y719F} mice. Paraffin sections obtained from mutant (b, d, f, h, j, l, n and p) and wt (a, c, e, g, i, k, m and o) testis were stained with H&E. P7 (**a** and **b**) and (**c** and **d**); closed arrows identify spermatogonia at the basal membrane of tubules (d). Six mutant and four control animals were analyzed at P6/P7. At P10 no meiotic cells are found in mutant testis (**e**–**h**). The closed arrow in (g) identifies a primary spermatocyte. In mutant testis, spermatogonia at different stages of development are present. An open arrow identifies dividing spermatogonia in (f) and type A spermatogonia in (h). Mutant testis shows an increased frequency of apoptotic cells (closed arrows in f and h). At P10/P12 four mutant and three control animals were analyzed. At P21 Kit^{Y719F}/Kit^{Y719F} seminiferous tubules are empty and only a few germ cells are at the base of tubules (**i**–**l**). An increase in the interstitial space in mutant testis is detectable (closed arrows in j). At P21 two mutants and one control were analyzed. Adult Kit^{Y719F}/Kit^{Y719F} testis (16w) are significantly smaller than controls (**m** and **n**). Clusters of cells in the center of seminiferous tubules (closed arrows in p) are prominent and the interstitial space is enlarged by hyperplastic Leydig cells (open arrow in p). Five mutant adults and three controls were analyzed. Magnifications are 5× (m and n), 20× (i, j, o and p), 40× (a, b, e, f, k and l) and 100× (c, d, g and h).

stages. However, at P10 some differentiating type A_1–A_4 spermatogonia were still observed (Figure 4h). The absence of meiotic cells and the localization of germ cells containing apoptotic bodies toward the lumen of the tubules might indicate either a block in the differentiation from type A to type B spermatogonia or failure of type B spermatogonia to initiate meiosis and form primary spermatocytes. Thus, it seems possible that the Kit^{Y719F} mutation might affect more than one cellular response during the spermatogonial stages to cause the observed block in germ cell development. These observations indicate an absolute requirement for Kit-induced PI 3-kinase signaling during the early stages of spermatogenesis.

In the adult mutant testis we observed clusters of cells in the center of the seminiferous tubules (Figure 4p). Some of the cells in the clusters show germ cell characteristics. High magnification microscopy revealed intercellular bridges between cells within the clusters. In addition, individual cells in clusters were found to express Kit mRNA, a hallmark of spermatogonia (not shown). However, they do not express either KL/Mgf or AMH as determined by *in situ* hybridization and they do not express the mitosis-specific Ki-67 epitope (Figures 5f and 6c and

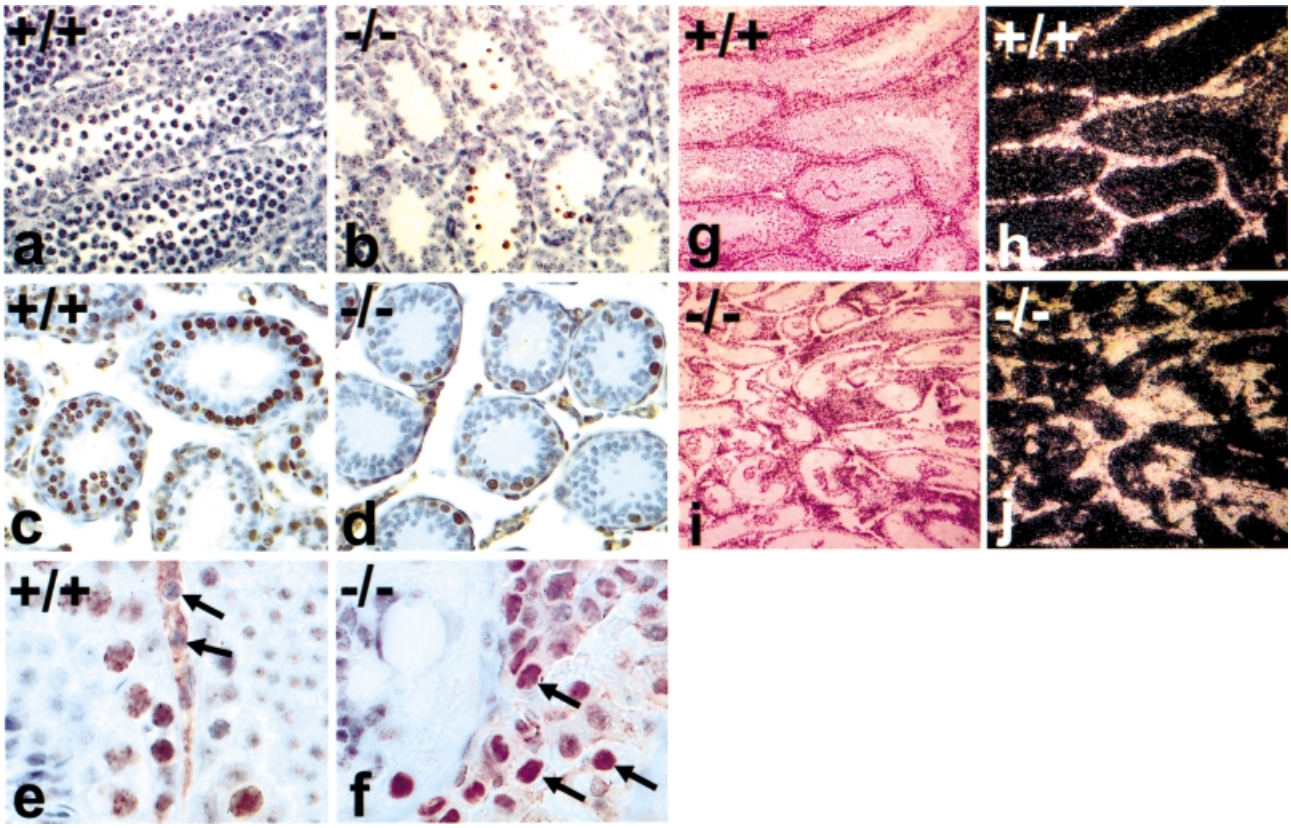

Fig. 5. Identification of mitotically active and apoptotic germ cells in Kit^{Y719F}/Kit^{Y719F} and wt testis. TUNEL analysis was performed on paraffin sections of P10 wt (**a**) and mutant (**b**) testis. Ki-67 staining specific for proliferating cells was performed on paraffin sections of wt (**c** and **e**) and mutant (**d** and **f**) testis at P12 (c and d) and 16 weeks (e and f). Closed arrows in (e) and (f) show nuclei of Leydig cells in wt (e) and Kit^{Y719F}/Kit^{Y719F} (f) testis. Expression of *Kit* mRNA in 16 week wt and Kit^{Y719F}/Kit^{Y719F} testis examined by *in situ* hybridization (**g–j**). Dark field images of wt and Kit^{Y719F}/Kit^{Y719F} testis (g and i) show uniform Kit expression in the interstitial space (white grains). Corresponding bright field images are shown (h and j). Magnifications are 20× (a, b, c and d), 100× (e and f) and 10× (g, h, i and j).

g). It is possible that disruption of spermatogenesis in the mutant causes shedding of germ cells into the seminiferous lumen.

Leydig cell hyperplasia in adult mutant mice

Adult mutant testes (20–24 weeks) are markedly reduced in size [0.0235 ± 0.003 g ($n = 6$) for Kit^{Y719F}/Kit^{Y719F} compared with 0.118 ± 0.03 g ($n = 4$) for controls], whereas the overall morphology of the reproductive tract appears normal and the size of the seminal vesicles is comparable to wild type (not shown). The seminiferous tubules in mutant adults have an aberrant structure, only a few germ cells remain at the basal membrane and spermatogonia could be identified occasionally (Figure 4m–p). The interstitial space in adult Kit^{Y719F}/Kit^{Y719F} testis is disproportionately increased and filled out with Leydig cells (Figure 4p). A first sign of the expansion of Leydig cells in the interstitial space is evident at P21. This increase of Leydig cell numbers does not appear to be caused by a significant reduction in the size of the seminiferous tubules but rather by mitotically active Leydig cells. In agreement with this hypothesis, Leydig cells in mutant testis were stained uniformly with the mitosis-specific antibody Ki-67 (Figure 5e and f). This phenotype was observed in all adult males ($n = 5$) examined. Despite the Leydig cell hyperplasia no tumor development in mutant males has been observed.

In situ hybridization of adult wild-type and Kit^{Y719F}/Kit^{Y719F} testis using a Kit-specific hybridization probe revealed significant *Kit* expression in interstitial Leydig cells but no *Kit* expression was detected in seminiferous tubules of mutant testis (Figure 5g–j). Examination of KL/Mgf expression by *in situ* hybridization at 16 weeks showed increased levels of KL/Mgf mRNA in Sertoli cells of mutant seminiferous tubules when compared with wild type (not shown). It has been reported previously that KL/Mgf mRNA expression in germ cell-depleted testis of Sl^d/Sl^d mice is elevated (Motro *et al.*, 1991).

To analyze the functional state of the hyperplastic Leydig cells we determined serum testosterone and luteinizing hormone (LH) levels using radioimmunoassay (RIA) in adult mutant and control animals (Table III). While no significant differences in the testosterone levels in Kit^{Y719F}/Kit^{Y719F} and wild-type animals were found, LH levels

Table III. Serum levels of testosterone and luteinizing hormone (LH) in +/+ and Kit^{Y719F}/Kit^{Y719F} males

Genotype	Testosterone (ng/ml)	LH (ng/ml)
+/+	3.283 ± 1.703 ($n = 5$)	0.137 ± 0.087 ($n = 4$)
Kit^{Y719F}/Kit^{Y719F}	4.208 ± 1.586 ($n = 6$)	1.022 ± 0.264 ($n = 10$)

Values represent the mean ± SE. Statistical significance compared with +/+ controls was determined using Mann–Whitney U values with $p \leqslant 0.05$. Values were $p = 0.357$ for testosterone and $p = 0.008$ for LH.

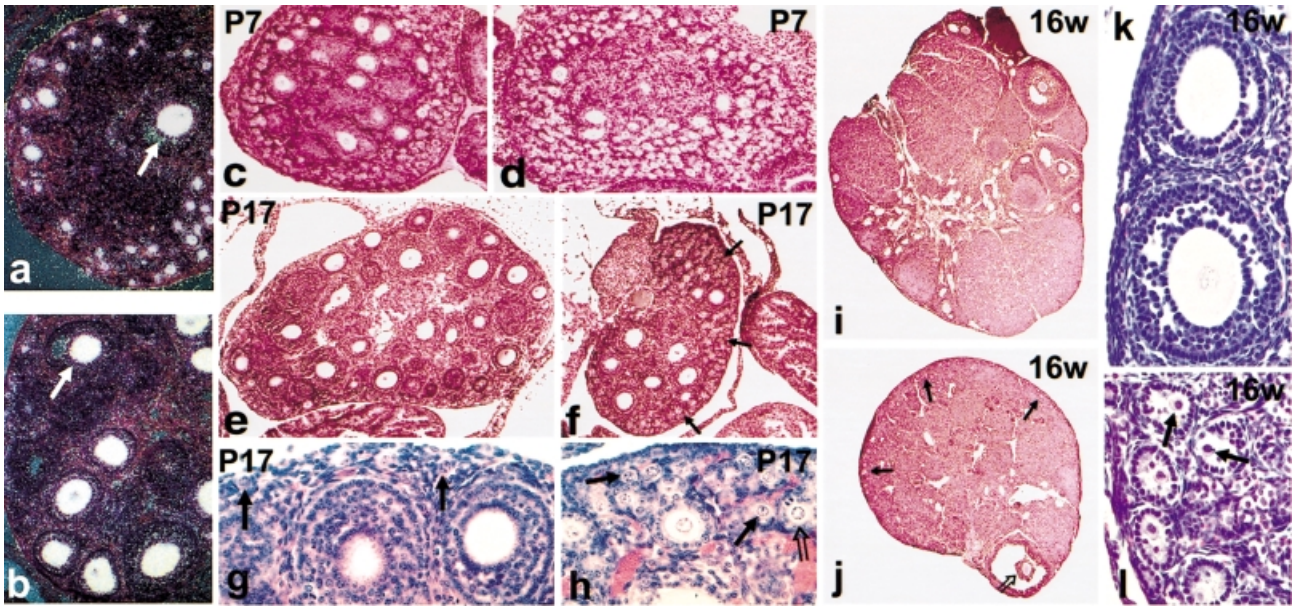

Fig. 6. Histological analysis of postnatal ovaries in Kit^{Y719F}/Kit^{Y719F} mice. Kit mRNA expression in wt and mutant ovaries was determined by *in situ* hybridization. The mutation does not affect Kit mRNA expression in oocytes of P17 ovaries and comparable levels of Kit are observed in oocytes of Kit^{Y719F}/Kit^{Y719F} (**a**) and wt (**b**) ovaries. In the dark field images (a and b) closed arrows show similar hybridization in antral follicles. Paraffin-embedded sections obtained from Kit^{Y719F}/Kit^{Y719F} (d, f, h, j and l) and wt (c, e, g, i and k) ovaries were stained with H&E. Follicle development in Kit^{Y719F}/Kit^{Y719F} mutant females is delayed at the cuboidal stages and the number of mature follicles is greatly reduced. At P7 there are fewer growing follicles in mutant ovaries (**d**) compared with wt (**c**). At P17 (**e**, **f**, **g** and **h**) an increase in the number of primordial/primary type 2 (closed arrow) and type 3a (open arrow) follicles is seen in the cortex of the ovary of mutant mice compared with wt. Growing follicles in Kit^{Y719F}/Kit^{Y719F} ovaries are typically surrounded by one or two layers of granulosa cells (f and h). A few follicles escape the defect and mature to antral stages (open arrow in j), as seen in the adult ovary (16 weeks) (**i**, **j**, **k** and **l**). Oocytes in the cortex of mutant ovaries are degenerated, acentric and follicle development is arrested at the type 3b stage (l). Follicles in the cortex of wt ovary are shown in (k). Solid arrows identify primordial and primary follicles in (j) and (l). The sections shown are representative of eight mutant and eight control animals at the juvenile stages and of six adult mutant and three adult control animals. Magnifications are 5× (i and j), 10× (e and f), 20× (a, b, c and d) and 40× (g, h, k and l).

were increased >7-fold in mutant animals. These results may be explained as a disruption in the negative feedback regulation of the hypothalamus and pituitary by androgen.

Follicle development in mutant mice is impaired at the cuboidal stages

In the ovary, Kit expression is observed in oocytes from the time of birth until ovulation as well as in theca cells (Manova *et al*., 1990; Horie *et al*., 1991; Yoshinaga *et al*., 1991). Interaction of Kit with KL/Mgf expressed by granulosa cells is known to be essential for follicle development (Kuroda *et al*., 1988; Huang *et al*., 1993). We confirmed expression of *Kit* and *KL/Mgf* in mutant ovaries by *in situ* hybridization. Comparable levels of *Kit* or *KL/Mgf* expression were observed in Kit^{Y719F}/Kit^{Y719F} and wild-type ovaries (Figure 6a and b and not shown).

In juvenile Kit^{Y719F}/Kit^{Y719F} ovaries, follicle development is impaired and appears to be delayed when compared with wild-type ovaries (Figure 6). Analysis of H&E-stained paraffin sections at P7 (Figure 6c and d), P10 (not shown) and P17 (Figure 6e–h) showed greater numbers of small follicles in the cortex of the ovary than in wild-type controls. Some of these follicles are surrounded by a single layer of follicle cells [types 2 and 3a, respectively, according to the nomenclature of Peters and Pedersen (1967)], others contain a bigger ring of follicle cells (type 3b), and only very few follicles with two or more layers of follicle cells (types 4, 5a and 5b) are present in the ovarian medulla of the mutants. As a result of limited recruitment of follicles for growth, the number of pre-antral and antral follicles is greatly reduced (Figure 6e–h), as is the size of the entire mutant ovary. Adult mutant ovaries contain only occasional large and Graafian follicles (types 6–8, respectively) (Figure 6i and j), whereas the cortex is enriched with abnormal small follicles (types 2–3a) (Figure 6k and l). The follicle cells have lost their concentric orderly arrangement and the oocytes are not centrally placed and show signs of degeneration (arrow in Figure 6l). In contrast to the complete arrest in spermatogonial development in mutant mice, ovarian follicle development is not completely blocked, indicating that Kit^{Y719F} mutation does not affect the later stages in follicle development. Superovulation experiments using adult females produced ova, although in low numbers, thus confirming the notion that later stages in oogenesis are not affected by the mutation (not shown). However, fertility of Kit^{Y719F}/Kit^{Y719F} females is greatly reduced.

Ovarian hyperplasia in adult Kit^{Y719F}/Kit^{Y719F} females

With increasing age there is a gradual depletion of follicles in Kit^{Y719F}/Kit^{Y719F} ovaries and pronounced abnormal development. At 6 weeks very few follicles continue to grow and develop into Graafian follicles in mutant ovaries. Pathological changes are evident with groups of small follicles showing irregularly shaped oocytes and the emergence of cyst-containing structures, possibly the remains of Graafian follicles (not shown). By 16–18 weeks interstitial tissue composed mostly of luteal-like cells occupies almost the entire ovary (Figure 7b). Only portions of the ovarian

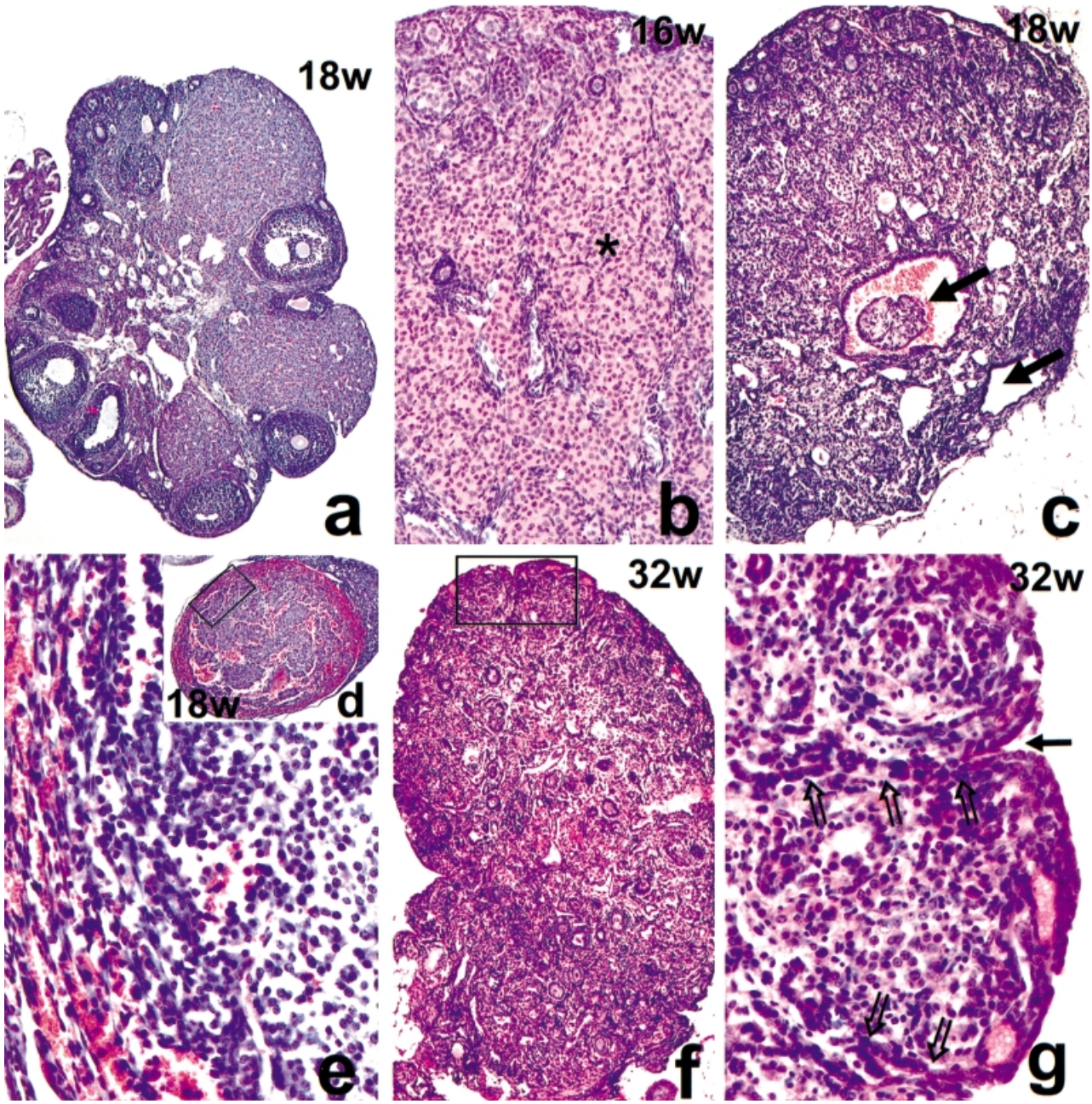

Fig. 7. Identification of ovarian cysts and tubular hyperplasia in adult Kit^{Y719F}/Kit^{Y719F} ovaries. H&E staining of paraffin sections of 18-week-old wt (**a**) and mutant ovaries at 16 (**b**), 18 (**c**–**e**) and 32 weeks (**f** and **g**) are shown. Only portions of the ovarian cortex at 16–18 weeks contained follicles and follicle-like structures (b and c). Large regions of the ovary are occupied by luteinized interstitial cells (asterisk in b). The oocyte-depleted ovaries have a high incidence in the development of ovarian cysts (closed arrows in c) and in one case an ovarian tumor has been observed (d); detail of the tumor (rectangle) is shown at high magnification in (e). At 32 weeks the ovary represents a mixture of follicle-like structures and somatic cells surrounding disorganized groups of interstitial and luteal-like cells (f). Invagination of the germinal epithelium (g, closed arrow) and tubular hyperplasia can be observed (f and g). Open arrows in (g) show invading epithelial cells. Magnifications are 5× (a), 10× (c, d and f), 20× (b) and 40× (e and g).

cortex contain follicles and follicle-like structures (Figure 7b and c). Anovular small follicles with disarranged granulosa cells are predominant. They represent a mixture of granulosa and interstitial-like cells. Gradually these follicle-like structures are fused into large masses by invasion of surrounding luteinized tissue. In addition, ovarian cysts develop in mutant females and in one case an encapsulated, hemorrhagic ovarian tumor was identified (Figure 7c–e). Analysis of *Kit* and *KL/Mgf* expression profiles by *in situ* hybridization showed regions of strong staining for both *Kit* and *KL/Mgf* in the tumor (not shown). This may suggest that the tumor originates from *Kit* expressing interstitial thecal or *KL/Mgf* expressing granulosa cells, and that Kit/KL might play an active role in ovarian tumor development. By 32 weeks mutant ovaries consist of a mixture of somatic cells surrounding disorganized groups of interstitial and luteal-like cells (Figure 7f). Invading tubules of the germinal epithelium penetrated the disorganized ovarian tissue (Figure 7g). Finally, by 16 months, complex tubular adenomas, similar to the

ones described by Murphy in W^x/W^v, W^j/W^v and W^v/W^v mice were observed (data not shown) (Murphy, 1972). This phenotype was observed in all samples ($n = 7$) analyzed. These data imply that Kit-mediated PI 3-kinase signaling is indispensable for proper oocyte growth and development and its absence presumably leads to an abnormal relationship between oocyte and granulosa cell.

Discussion

In an attempt to understand the biological roles of PI 3-kinase signaling mediated by the Kit receptor we have characterized the phenotypes of mice expressing a mutant Kit receptor (Kit^{Y719F}, obtained by a knock-in strategy) that fails to interact with PI 3-kinase. Whereas the Y719F mutation blocks the direct binding by Kit of the p85 regulatory subunit of PI 3-kinase and its activation, we cannot exclude the possibility that this mutation blocks other activating or inactivating interactions as well. The Kit^{Y719F} mutation affects Kit function only in specific developmental processes and this is in contrast to other mutations in the Kit receptor gene that broadly affect Kit function in hematopoiesis, gametogenesis and melanogenesis. Thus, the mutation fails to affect steady-state hematopoiesis and tissue mast cell numbers, but substantially reduces peritoneal mast cell numbers. Furthermore, although Kit has important functions at multiple stages in embryonic and postnatal gametogenesis, our results show that PI 3-kinase signaling is critical only in a specific subset of the postnatal stages in the ovary and testis. We conclude, therefore, that in most cells that require Kit, there are redundant signaling pathways, but that in certain cell types the PI 3-kinase pathway is critical.

In vivo, in hematopoietic and melanogenic cell types and in skin mast cells the mutant defect may be compensated for by parallel Kit signaling pathways and/or other cytokine- or growth factor-mediated signaling involving PI 3-kinase and/or other mechanisms. Because of the intricate intracellular signaling networks, absence of a signal to activate or produce a second messenger may be redundantly produced by another cytokine- or growth factor-mediated signaling cascade. It is possible that the diversity of class IA PI 3-kinases contributes to this redundancy. For instance, there are at least three alternative catalytic subunits: p110α, β and δ, and an equal number of p85 adapter subunits: p85α, p85β and p85γ (Domin and Waterfield, 1997). Expression of some PI 3-kinase subunit isoforms is cell type specific and this may contribute to cell type-specific dependence on Kit activation, whereas others are expressed more ubiquitously. Consequently, mutations of p85 subunit and PI 3-kinase isoforms are expected to produce rather broad mutant phenotypes. In fact, recently it was shown that mutation of the p85α subunit of PI 3-kinase produced a perinatal phenotype in mutant mice (Fruman *et al*., 1999b; Suzuki *et al*., 1999). In contrast, our analysis of the phenotype of Kit^{Y719F}/Kit^{Y719F} mice was designed to investigate a specific subset of PI 3-kinase functions, those mediated by *Kit* receptor activation.

Another question concerns the downstream Kit/PI 3-kinase signaling pathways, which are crucial in different cell types affected by the Kit^{Y719F} mutation. The phospholipid products of PI 3-kinase, PI 3,4-P_2 and PI 3,4,5-P_3, have pleiotropic roles in distinct signaling processes mediated primarily through the interaction of the phospholipids with PH and FYVE domain-containing proteins (Toker and Cantley, 1997; Rameh and Cantley, 1999). Our previous *in vitro* analysis of Kit signaling in BMMCs showed that downstream targets of phospholipid products produced by Kit-activated PI 3-kinase included Akt, Rac, JNK and PKCδ (Vosseller *et al*., 1997; Timokhina *et al*., 1998; I.Timokhina, unpublished; K.Vosseller, unpublished). Importantly our characterization of signaling in BMMCs obtained from Kit^{Y719F}/Kit^{Y719F} mice confirmed our previous findings. In contrast, downstream signaling mediated by Kit and PI 3-kinase in other cell types is less clear. In primary oocytes of cuboidal follicles perhaps Kit signaling through PI 3-kinase, Rac and JNK may activate transcription factors involved in the expression of growth factors critical in mediating follicle growth. In spermatogonia, Kit signaling through PI 3-kinase, Akt and suppression of the pro-apoptotic functions of Bad or the forkhead transcription factor FKHRL1 may be critical for cell survival (Datta *et al*., 1997, 1999; Brunet *et al*., 1999). Furthermore, signaling through PI 3-kinase, Rac and Jnk may be critical for proliferation of spermatogonia. However, in Leydig cells it is difficult to speculate about mechanisms for Kit signaling through PI 3-kinase, as a role for Kit in this cell type is not yet understood.

Although no defects were observed in steady-state hematopoiesis, skin tissue mast cells and melanogenesis in mutant animals, it is possible that the mutation affects stress hematopoiesis and/or homing properties of hematopoietic progenitors and possibly functional mast cell responses. A more detailed analysis of such questions is in progress. Many naturally occurring murine W and human Piedbald mutations have been identified (Besmer, 1997). However, mutations affecting Tyr719 of the Kit receptor have never been observed. In the light of our results this is not surprising: all W and Piedbald mutations have an easily observed pigmentation phenotype, while the Kit^{Y719} mutation apparently does not affect melanogenesis and instead may be associated more with infertility.

The effects of the Kit^{Y719F} mutation on gametogenesis are quite impressive. Whereas analysis of gonads during embryonic development and in the newborn indicates no effect of the mutation on PGC proliferation and survival, strong effects are seen both on spermatogenesis and oogenesis. Since PGCs are not affected by the mutation and normal numbers of germ cells are present at the time of birth, mutant phenotypes in the postnatal gonads could be analyzed in detail. Two lines of evidence suggest a role for Kit signaling in follicle development. First, in Sl^{pan} mutant mice a severe phenotype is observed with follicle development arrested at the one-layered cuboidal stage (Huang *et al*., 1993). Secondly, *in vivo* use of antagonistic anti-Kit antibody suggests multiple roles for Kit in follicle development, at the one-layered cuboidal stage, the stage of follicular fluid formation of pre-antral follicles and at the penultimate stage of ovarian follicle development prior to ovulation (Yoshida *et al*., 1997). In the Kit^{Y719F}/Kit^{Y719F} mutant mice follicle development is delayed and impaired at the one- to three-layered cuboidal stages; however, the block is not complete and some

follicles mature to become antral and ovulatory follicles. Thus, the Kit^{Y719F} mutation affects early follicle growth and development but not the later stages. Furthermore, compared with the Sl^{pan} mutation the developmental block of the Kit^{Y719F} mutation may take effect a little later, possibly because of Kit signaling pathways that are not affected by the mutation. The impairment/delay in follicle development at the cuboidal stages suggests a defect in the communication between germ cell and somatic cell (Bachvarova *et al*., 1993; Huang *et al*., 1993). Thus, Kit signaling may control the production of factor(s) involved in granulosa cell differentiation and proliferation. GDF-9 and BMP-15 are two transforming growth factor-β family growth and differentiation factors produced by oocytes that are thought to mediate proliferation and differentiation of granulosa cells in follicles (Dong *et al*., 1996; Dube *et al*., 1998). In fact GDF-9 function is required for follicle development and in mice carrying a GDF-9 loss-of-function mutation follicle development is arrested at the cuboidal stage (Dong *et al*., 1996). It had been proposed previously that Kit may control the expression of GDF-9 because of the similar mutant phenotypes of Sl^{pan} and GDF-9 knock-out mice (Yoshida *et al*., 1997). However, no significant differences in GDF-9 and BMP-15 mRNA expression levels determined by *in situ* hybridization were observed between control and Kit^{Y719F}/Kit^{Y719F} juvenile ovaries (G.Rothschild, H.Kissel and K.Manova, unpublished observation). Local autocrine and paracrine factors play key roles in pre-antral follicle development until hormonal mechanisms determine folliculogenesis (Gougeon, 1996; Eppig *et al*., 1997; Vanderhyden and Macdonald, 1998), and pre-antral oocytes from 12-day-old ovaries have been shown to secrete factors that prevent premature steroidogenic differentiation of granulosa cells (Vanderhyden and Tonary, 1995; Vanderhyden and Macdonald, 1998). In oocytes the Kit^{Y719F} mutation could impair the production of such an inhibitory signal. As such, Kit^{Y719F}/Kit^{Y719F} ovaries contain anovular follicles with disorganized granulosa cells and interstitial cells.

Of interest also is the observation of ovarian cysts and tubular hyperplasia in Kit^{Y719F}/Kit^{Y719F} ovaries. This is reminiscent of W^{x}/W^{v} mice that have been shown to develop hyperplasia of the rete ovarii and complex tubular adenomas (Murphy, 1972). Kit^{Y719F}/Kit^{Y719F} ovaries are depleted of maturing follicles at an early age and hyperplastic changes of the interstitium and the germinal epithelium can be detected. The expression of *Kit* and *KL/Mgf* in the ovarian tumor may suggest a role for granulosa and/or thecal cell hyperplasia and furthermore the absence of functional oocytes may upregulate steroidogenesis and contribute to the hyperplastic changes observed (Murphy and Beamer, 1973; Risma *et al*., 1995). At this time a functional relationship between gonadotropic hormones and *Kit*/KL signaling is not understood, although LH has been shown to downregulate *Kit* expression in theca and interstitial tissue without affecting *Kit* expression in oocytes (Motro and Bernstein, 1993). *KL/Mgf* expression is also found in some theca and interstitial cells (Motro and Bernstein, 1993). However, whether or not Kit or KL/Mgf plays an active role in the hyperplastic changes of the interstitium and in ovarian tumorigenesis remains to be determined.

In the postnatal testis the Kit receptor is expressed starting at P4–P6 in gonocytes and persists until the preleptotene spermatocyte stage (Manova *et al*., 1990; Yoshinaga *et al*., 1991). Several lines of evidence have suggested an essential role for Kit receptor signaling in spermatogenesis. Tubules of chimeric Sl/Sl^{d} // +/+ mice contain both differentiated and depleted tubule sections, possibly due to the lack of differentiation of spermatogonia in patches of Sl/Sl^{d} tubules (Nakayama *et al*., 1988). Regenerative differentiation after surgical reversal of *Sl* and *W* mutant cryptorchid testis was impaired at the level of proliferation and differentiation from spermatogonia A to B and at meiotic division (Nishimune *et al*., 1980; Koshimizu *et al*., 1991). A defect in germ cell differentiation is also evident in Sl^{17H} mutant males (Brannan *et al*., 1992) and after administration of *Kit* receptor blocking antibody (Yoshinaga *et al*., 1991; Packer *et al*., 1995). Results from *in vitro* studies may suggest a role for *Kit* at the time of gonocyte migration from the center of tubules to the basal membrane at P4–P6 (Orth *et al*., 1997). Furthermore, progression of purified spermatocytes through meiosis mediated by the Sertoli cell line 15P is Kit dependent (Vincent *et al*., 1998). Our analysis of testis from Kit^{Y719F}/Kit^{Y719F} mice indicates normal numbers of germ cells at the time of birth and normal development until P6 and P7 when the majority of spermatogonia have migrated basally. This is important since studies in the rat have suggested a role in this early migratory phase of gonocytes from the adluminal compartment to the basal membrane (Orth *et al*., 1997). Subsequently, development in the mutant mice is impaired and at P10 the cellularity of tubules is reduced. There is a concomitant increase in the frequency of apoptotic germ cells and mutant germ cells fail to enter meiosis. Thus, tubules are rapidly depleted causing sterility. Accordingly, Kit-mediated PI 3-kinase signaling is critical for the development of male germ cells during the premeiotic stages. Whether the failure to enter meiosis may be attributed to a block in the proliferation and survival of spermatogonia, the differentiation of spermatogonia from type A to type B or failure of type B spermatogonia or preleptotene spermatocytes to initiate meiosis as reported by Vincent *et al.* (1998) is difficult to discern at this time. It is noteworthy that some type A spermatogonia are observed on P10 but the number of proliferating cells as determined by Ki-67 staining is reduced compared with controls. This may imply that the mutation affects both survival and proliferation of type A spermatogonia. Furthermore, the increase in apoptosis occurs when preparation for meiosis is under way, in agreement with the notion that the dying cells are primary spermatocytes.

Interstitial Leydig cells have a critical endocrine function in spermatogenesis and are the primary source of testicular testosterone. Leydig cells develop postnatally from mesenchymal progenitor cells and they proliferate and differentiate to form immature Leydig cells prepubertally (Ge *et al*., 1996). By the end of puberty, immature rat Leydig cells have divided once and have terminally differentiated into adult Leydig cells. Postnatal mouse Leydig cells express high levels of Kit receptor and Kit expression is maintained throughout postnatal life (Manova *et al*., 1990; Yoshinaga *et al*., 1991). However, little is known about a role for *Kit* in Leydig cell development and/or function. In mutant Kit^{Y719F}/Kit^{Y719F}

mice starting at P21 Leydig cells are increased in number and they continue to proliferate as indicated by Ki-67 staining. This is in contrast to normal Leydig cells, which have a very low mitotic index. In adult mutant testis the interstitial space is expanded and filled out by proliferating Leydig cells. The Leydig cell hyperplasia may arise either from a cell autonomous effect of the mutant Kit receptor in Leydig cells or alternatively as the consequence of a secondary effect. Both *AMH* and *AMH receptor* knock-out mice develop Leydig cell hyperplasia at 10 weeks or older (Mishina *et al*., 1996). While *AMH* is expressed by Sertoli cells, the *AMH receptor* is expressed by Leydig cells (Racine *et al*., 1998; Lee *et al*., 1999). Thus, the Leydig cell hyperplasia suggests that *AMH* negatively regulates Leydig cell proliferation. While in Kit^{Y719F}/Kit^{Y719F} mice *AMH* expression in Sertoli cells is not appreciably affected (data not shown), *KL/Mgf* expression is upregulated in Sertoli cells. KL/Mgf over-expression in Sertoli cells therefore may stimulate mutant Kit receptors in Leydig cells inducing hyperplasia. Another issue concerns the endocrine function of the Leydig cells in the mutant mice. If the Leydig cells in adult mutant mice are mature, levels of serum testosterone should be higher than control, since Leydig cell numbers are increased greatly in these mice. However, serum testosterone levels are normal and LH serum levels are increased >7-fold. This could mean that on a per cell basis the Leydig cells in the mutant mice produce less testosterone than normal adult Leydig cells due to a failure to differentiate fully and/or to impairment of steroidogenesis. If the Leydig cells are deficient in androgen production prior to puberty, development of the normal feedback relationship will not occur and LH levels may become chronically elevated. Thus, the combination of hyperplasia and elevated LH levels caused by feedback as a result of subnormal testosterone levels may be responsible for the apparently normal testosterone levels in the adult mutant mice. Therefore, these results suggest that the *Kit* receptor may have a role in Leydig cell differentiation and/or steroidogenesis.

Materials and methods

Targeting construct to modify the 129/Sv Kit locus

An 18 kb fragment of the mouse c-*kit* gene (Gokkel *et al*., 1992) including exons 8–17 was isolated by screening a genomic 129/SvJ mouse library (ΘFixII; Stratagene) with a ^{32}P-labeled PCR probe derived from the Kit kinase insert region. A replacement targeting vector was constructed using a 2.5 kb *Kpn*I–*Bam*HI fragment including exon 14 and a 4 kb *Bam*HI fragment including exons 15–17. Site-directed mutagenesis was performed on a 0.6 kb *Bam*HI–*Kpn*I fragment containing exon 15, introducing the substitution mutation Y719F (GAATAT→GAATTC) thereby creating a new *Eco*RI restriction site. A *loxP* flanked neomycin resistance gene expression cassette driven by the PGK promotor was isolated from the pKS*loxP*NT plasmid (kindly provided by A.Joyner, Skirball Institute, New York University Medical Center) inserted at a *Bam*HI site in intron 14 thus deleting the restriction site. The presence of the Y719F mutation and exon 14–17 sequences in the targeting construct was confirmed by sequence analysis. A herpes simplex virus thymidine kinase expression cassette driven by polyoma enhancer sequences (Py-Tk) was attached to the long arm of homology to enrich for homlogous recombinants by negative selection using gancyclovir. The targeting construct was linearized using a unique *Not*I restriction site outside the region of homology for transfection of ES cells.

Isolation of recombinant ES cell clones and generation of Kit^{Y719F}/Kit^{Y719F} mutant mice

Transfection of ES cells (CJ7) with the targeting construct and ES cell culture were carried out essentially as recently described (Tajima *et al*., 1998b) following standard protocols. Recombinant ES cell clones were identified by Southern blot analysis using ES cell DNA digest with the restriction endonuclease *Bam*HI and a 500 bp PCR-derived, ^{32}P-labeled hybridization probe spanning exons 10–12 (probe 1; Figure 1A). In C57BL/6J and Balb/C DNA, probe 1 detects a polymorphic *Bam*HI fragment of 5.5 kb instead of the 7.3 kb fragment found in 129/SvJ DNA. The presence of the mutation was confirmed by Southern blotting and digestion with *Eco*RI restriction using a 600 bp PCR-derived ^{32}P probe including intron 15 (probe 2; Figure 1A). A total of 2400 W9.5 (Lau *et al*., 1994) and CJ7 (Swiatek and Gridley, 1993) ES cell clones were screened giving rise to five clones containing a correctly targeted allele carrying the mutation. One clone recombined correctly incorporating the neomycin cassette at the *Bam*HI site but containing wt sequence at Y719.

Heterozygous mutant ES cells were injected into C57BL/6J blastocysts and chimeric males were backcrossed for germline transmission to C57BL/6J females. Two of the injected CJ7 clones successfully contributed to the germline and gave rise to $Kit^{Y719F-neo}$/+ heterozygous animals (C57BL/6J × 129Sv). For excision of the neomycin resistance gene $Kit^{Y719F-neo}$/+ heterozygous females were mated with EIIa*Cre* (Lakso *et al*., 1996) transgenic males (BalbC) and the F_1 generation was screened for loss of *neo* by using a *Bam*HI restriction digest and Southern blotting (probe 1). Excision of the *neo* gene was confirmed by re-hybridizing the filters with a neo-specific probe and the presence of the Y719F point mutation by Southern blotting using *Eco*RI-digested DNA (probe 2). F_1 Kit^{Y719F}/+ heterozygous mice were then intercrossed to generate homozygous mutant Kit^{Y719F}/Kit^{Y719F} mice (C57BL/6J × 129Sv × BalbC).

Histological analysis and in situ hybridization

Testis and ovaries were dissected and fixed in Bouin's fixative at 4°C overnight. The tissues were dehydrated in rising concentrations of ethanol, embedded in paraffin, sectioned at 8 μm and stained in Gill's H&E. To evaluate mast cell numbers in the skin, dorsal skin pieces were briefly dried on a paper towel, fixed in Bouin's at 4°C overnight, dehydrated, embedded in paraffin, sectioned at 8 μm and stained with Toluidine Blue (pH 4) without H&E counterstaining to facilitate quantitation. The length of the counted section was determined and the number of cells/cm calculated.

RNA in situ hybridization, TUNEL method and Ki-67 staining

The tissues indicated were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight followed by ethanol dehydration the next day. Paraffin sections (8 μm) were prepared. Antisense probes were generated and hybridized essentially as described by Tomihara-Newberger *et al*. (1998). The following probes were used: Kit (Manova *et al*., 1990); KL/Mgf (Manova *et al*., 1993); GDF-9 (McGrath *et al*., 1995); and AMH (Mishina *et al*., 1996). The GDF-9 and AMH plasmids were kindly provided by Drs Se-Jin Lee and Robin Lovell-Badge.

For immunohistochemical analysis a Histomouse kit from Zymed (South San Francisco) was used following the manufacturer's instructions. Tissues were fixed in 4% paraformaldehyde or Bouin's fixative at 4°C overnight, dehydrated, embedded in paraffin and sectioned at 8 μm. Slides were deparaffinized, quenched in 1% H_2O_2 and washed in PBS. Antigen retrieval was performed for 10 min. After blocking, the primary antibody (1:200) was incubated in 2% bovine serum albumin (BSA)/PBS at 4°C overnight. The Ki-67 monoclonal antibody MM1 was from Novocastra Laboratories, Newcastle, UK.

For detection of apoptotic cells, tissues fixed in 4% paraformaldehyde and/or Bouin's were processed for the TUNEL reaction where biotinylated dUTP is added to DNA double-strand breaks using terminal transferase essentially as recently described (Manova *et al*., 1998). The procedure was based on the method of Gavrieli *et al*. (1992), with minor modifications (Manova *et al*., 1998).

Determination of peripheral blood parameters and mast cell numbers

Blood samples were drawn from the retro-orbital plexus or tail vein with a capillary pipet (Unopette; Becton-Dickinson, Rutherford, NJ). Platelet and white blood cell numbers were determined using a hemocytometer under a phase contrast microscope. The hematocrit was measured using a heparinized micro-hematocrit capillary tube (Fisher Scientific).

Determination of mast cell numbers in the skin of control and Kit^{Y719F}/Kit^{Y719F} mice was as described previously (Tajima *et al*., 1998a). Peritoneal mast cells were obtained from Kit^{Y719F}/Kit^{Y719F} and control mice by gentle lavage of the peritoneal cavity with 5 ml of PBS and mast cells were identified by staining with 0.1% Toluidine Blue.

Mast cell cultures

BMMCss from Kit^{Y719F}/Kit^{Y719F} and control mice were produced by culturing bone marrow in RPMI 1640 supplemented with 1 mM sodium pyruvate, 1 mM non-essential amino acids, 5.5×10^{-5} M β-mercaptoethanol, 0.075% sodium bicarbonate, 10% fetal bovine serum (RPMI complete) and 10% X63-derived interleukin (IL)-3 containing conditioned medium. Kit cell surface expression was monitored using anti-Kit monoclonal ACK2 antibody (1:60) coupled to fluorescein isothiocyanate (FITC) and FACS analysis.

Proliferation assay and determination of apoptosis

The proliferation assay was performed as described previously (Yee *et al*., 1994). Briefly, cells were starved of growth factors in complete RPMI for 12 h, 10^5 cells were seeded in 0.2 ml per well in triplicate in 96-well plates, followed by stimulation with KL/Mgf (200 ng/ml) or IL-3 (20 ng/ml) for 24 h. Cytokines were used at concentrations that produced a maximal proliferative response (data not shown). After 20 h, 0.5 μCi of [^{3}H]thymidine was added for 4 h. Cells were harvested and ^{3}H radioactivity was measured in a liquid scintillation counter.

For apoptosis assays 10^6 cells were seeded in 2 ml per well in six-well plates. After treatment with apoptotic stimuli as described in the figure legends, cells were collected into FACS tubes, washed once with Annexin V binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.5% BSA) and incubated for 30 min in 100 μl of binding buffer containing 10 μl of Annexin V–FITC (Biosource) in the dark. Cells were washed twice with the binding buffer without BSA, resuspended in PBS and analyzed by FACS. The fraction of green fluorescent cells represented the fraction of cells undergoing apoptosis.

JNK in vitro kinase assays

Cells (10^7) were collected in lysis buffer (50 mM HEPES pH 7.4, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, 50 μM $ZnCl_2$, 25 mM NaF, 20 mM β-glycerophosphate, 50 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate), cleared lysates were normalized for protein content and JNK was immunoprecipitated with antibody-precoupled beads for 2 h at 4°C. The immunoprecipitates were washed three times in NP-40 buffer (1% NP-40, 2 mM sodium orthovanadate in PBS), once in LiCl buffer [10 mM Tris–HCl, 500 mM LiCl, 1 mM dithiothreitol (DTT)] and once in kinase buffer (12.5 mM MOPS pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM sodium orthovanadate, 1 mM DTT). JNK assays were performed as follows: 30 μl of kinase buffer containing 4 mg/ml GST–jun-1-135 (Verheij *et al*., 1996), 25 μM ATP and 1 μCi/ml [γ-^{32}P]ATP were added to the beads and reactions were incubated for 20 min at 30°C. Reactions were stopped by addition of sample buffer and proteins fractionated by SDS–PAGE.

Akt in vitro kinase assay

Cells (10^7) were collected in a Triton X-100 lysis buffer containing 1 mM microcystin, cleared lysates were normalized for protein content and Akt proteins were immunoprecipitated with anti-Akt antibody-precoupled beads for 2 h at 4°C. Immunoprecipitates were washed three times with NP-40 buffer, once with LiCl and once in a kinase buffer (12.5 mM MOPS pH 7.5, 12.5 mM β-glycerophosphate, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM sodium orthovanadate, 1 mM DTT), essentially as described previously (Franke *et al*., 1995), and Akt kinase assays were performed as follows: 30 μl of kinase buffer containing 4 mg/ml histone H2B (Boehringer Mannheim), 25 μM ATP and 1 μCi/ml [γ-^{32}P]ATP were added to the beads and reactions were incubated for 20 min at 30°C. Reactions were stopped by addition of sample buffer and proteins fractionated by SDS–PAGE.

Immunoprecipitation and Western blotting

Cells were lysed in Triton X-100 lysis buffer, cleared lysates were normalized for protein content and proteins were immunoprecipitated with beads coupled with polyclonal antibodies specific for Kit, JNK and Akt for 2 h at 4°C. Immunoprecipitates were washed three times in lysis buffer, resuspended in sample buffer, boiled for 5 min, subjected to SDS–PAGE and transferred to nitrocellulose. The membranes were blocked overnight in TBS–Tween containing 5% non-fat milk or 5% BSA for anti-phosphotyrosine blotting. Membranes were incubated for 1 h with the following antibodies: anti-Kit polyclonal antibody (1:500), anti-phosphotyrosine antibody (1:1000), anti-phospho-JNK antibody (1:50) and anti-p85 antibody (1:500). Proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

Measurement of serum LH and testosterone

Blood was allowed to stand at room temperature for 15 min. Serum was obtained by centrifugation of clotted blood and stored at –20°C until assay. The RIA for LH was performed according to a previously published procedure (Chandrashekar *et al*., 1988) using anti-rat LH antiserum (NIDDK-anti-rLH-S-11), rat LH standards (NIDDK-rLH-I-9) from the National Hormone and Pituitary Program, and radio-iodinated LH from Hazleton Washington (Catalog No. AG-0007; Vienna, VA). The tritium-based RIA for testosterone was performed as described previously (Cochran *et al*., 1981). The inter-assay variations of the LH and testosterone RIAs were 6.7 and 7.8%, respectively.

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