

Enhancers and Suppressors of Testicular Cancer Susceptibility in Single- and Double-Mutant Mice

Man-Yee Josephine Lam, Kirsten K. Youngren and Joseph H. Nadeau¹

*Department of Genetics, Comprehensive Cancer Center and Center for Computational Genomics,
Case Western Reserve University, Cleveland, Ohio 44106*

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ABSTRACT

Susceptibility to spontaneous testicular germ cell tumors (TGCTs), a common cancer affecting young men, shows unusual genetic complexity. Despite remarkable progress in the genetics analysis of susceptibility to many cancers, TGCT susceptibility genes have not yet been identified. Various mutations that are inherited as Mendelian traits in laboratory mice affect susceptibility to spontaneous TGCTs on the 129/Sv inbred genetic background. We compared the frequency of spontaneous TGCTs in single- and double-mutant mice to identify combinations that show evidence of enhancer or suppressor effects. The lower-than-expected TGCT frequencies in mice with partial deficiencies of TRP53 and MGF-SLJ and in 129.MOLF-Chr19 (M19) consomic mice that were heterozygous for the *A'* mutation suggest that either these genes complement each other to restore normal functionality in TGCT stem cells or together these genes activate mechanisms that suppress incipient TGCTs. By contrast, the higher-than-expected TGCT frequencies in *Mgf^{S^h}*-M19 compound heterozygous mice suggest that these mutations exacerbate each other's effects. Together, these results provide clues to the genetic and molecular basis for susceptibility to TGCTs in mice and perhaps in humans.

TESTICULAR germ cell tumors (TGCTs) are the most common cancer affecting young men. The incidence of TGCTs has doubled within the last 50 years, predominately in Eastern Europe, indicating that environmental factors affect susceptibility (BUETOW 1995). Genetics also contributes to TGCT susceptibility, with an increased risk of 8- to 10-fold among brothers and 4-fold among sons of affected individuals (FORMAN *et al.* 1992; LINDELOF and EKLUND 2001). Despite its prevalence, little is known about the genetic control of TGCT susceptibility. Mapping studies have revealed many weak autosomal linkages (LEAHY *et al.* 1995; BISHOP 1998) but only one significant linkage at Xq27 (RAPLEY *et al.* 2000). To date, TGCT susceptibility genes have not yet been identified in humans. Linkage studies have been difficult in part because of the limited number of multi-generational pedigrees with sufficient numbers of affected individuals. The 129/Sv inbred strain of laboratory mice is an important model for unraveling the complexity of the genetic control of susceptibility and resistance to TGCTs (STEVENS and HUMMEL 1957). Linkages and genes discovered in studies of these mice could be used to guide studies in humans.

TGCTs are rare in most inbred mouse strains and occur spontaneously at a rate of 1–5% in the 129/Sv inbred strains (STEVENS and HUMMEL 1957). Various

characteristics of TGCTs in these strains suggest that 129/Sv mice are an important model for pediatric germ cell tumors that occur in humans (STEVENS 1967; JIANG and NADEAU 2001; LAM and NADEAU 2003). These tumors originate during fetal development and are composed of various cell types and tissues at different stages of differentiation. In both humans and mice, males develop pediatric germ cell tumors before puberty and these tumors are classified histologically as nonseminomas.

In mice, primordial germ cells (PGCs) are the earliest recognizable precursors of gametes and arise outside the gonads (MOLYNEAUX *et al.* 2001). During embryogenesis, PGCs are first identified around embryonic day 7 (E7) at the primitive streak. The PGCs leave the yolk sac, migrate through the dorsal mesentery of the gut, and arrive at the genital ridges by E11.5. As the PGCs migrate toward the genital ridges, they proliferate, with the number increasing from ~40 at E7 to ~2000 at E11.5 when they arrive at the genital ridges. At E13.5, PGCs enter G₁ mitotic arrest and remain arrested until a few days after birth (DONOVAN *et al.* 1998). This period from E11.5 to E13.5 is thought to be when TGCT development begins. It has been hypothesized that PGCs fail to enter G₁ mitotic arrest and continue to divide into pluripotent stem cells called embryonal carcinoma cells that in turn become tumors composed of various cells and tissue types (JIANG and NADEAU 2001).

Several mutations that are inherited as Mendelian traits affect TGCT susceptibility in 129/Sv mice. These mutations must be congenic on the 129/Sv inbred ge-

¹Corresponding author: Genetics Department, Case Western Reserve University, 2109 Adelbert Rd., Cleveland, OH 44106.
E-mail: jhn4@po.cwru.edu

netic background to exert their influence, demonstrating that these mutant genes act together with 129/Sv-derived genes to control susceptibility.

The Steel (*Sl*) and White-spotting (*W*) mutants were first identified as coat color variants (SILVER 1995). *Sl* mutants result from molecular lesions in the mast cell growth factor (*Mgf*) gene (COPELAND *et al.* 1990) and *W* mutants from lesions in the *kit* oncogene, which encodes the c-kit tyrosine kinase cell surface receptor for the MGF ligand (CHABOT *et al.* 1988). Mice with mutations in these genes are defective in melanogenesis, gametogenesis, and hematopoiesis. The MGF-KIT signaling pathway is required for PGC survival and proliferation during germline development (DOLCI *et al.* 1991).

Stevens transferred several *Sl* and *W* mutations onto the 129/Sv inbred strain to study tumorigenesis and found that only *Sl* and *Sl^l* alleles cause a significant increase in the frequency of TGCTs, whereas other alleles of *Sl* and *W* mutants did not affect susceptibility (L. C. STEVENS, personal communication). *Mgf^{Slj}* is a spontaneous mutation that results from an ~640-kb deletion that includes the *Mgf* gene (BEDELL *et al.* 1996). Homozygous *Mgf^{Slj}/Mgf^{Slj}* mice are PGC deficient and embryonic lethal (STEVENS 1967). Heterozygous 129/Sv-*Mgf^{Slj}/+* males have a tumor incidence more than double (12–14%) the rate in their wild-type controls (5%; STEVENS 1967).

Trp53 is a tumor suppressor gene that arrests cells to allow proper repair of DNA damage (KASTAN *et al.* 1991). To study the role of TRP53 in tumorigenesis, a null mutation was created by homologous recombination in mouse embryonic stem (ES) cells (DONEHOWER *et al.* 1992; JACKS *et al.* 1994). Mice homozygous for the null allele appear normal but are prone to a variety of tumors, with lymphomas being the most common (DONEHOWER *et al.* 1992; JACKS *et al.* 1994). The spectrum of tumors varies among TRP53-deficient mice on different genetic backgrounds (DONEHOWER *et al.* 1995). Loss of TRP53 appears to accelerate existing tumor predisposition in a background-dependent manner. In a mixed C57BL/6 and 129/Sv genetic background, only 9% of the TRP53-deficient mice develop testicular tumors, whereas on a pure 129/Sv genetic background, 35% of the TRP53-deficient mice develop testicular tumors (DONEHOWER *et al.* 1995).

A genome scan suggested that at least one gene on chromosome 19 of the MOLF/Ei inbred strain dramatically increases susceptibility to TGCTs (COLLIN *et al.* 1996). 129.MOLF-Chr19 is a chromosome substitution strain (CSS), also known as a consomic strain, in which chromosome (Chr) 19 of 129/Sv is replaced by MOLF-derived chromosome 19 on the 129/Sv background (MATIN *et al.* 1999). Homosomic mice, in which both copies of chromosome 19 are derived from MOLF/Ei, have a tumor incidence of ~80%; ~50% of these males have bilateral tumors as compared to the TGCT incidence of 5% with most cases being unilateral in 129/Sv

males (MATIN *et al.* 1999). Heterosomic animals, which have only one copy of the MOLF-derived chromosome 19, have a tumor incidence of 20%, and only 2–3% of the affected animals have bilateral tumors (MATIN *et al.* 1999; YOUNGREN *et al.* 2003).

A^y is a mutation with dominant effects at the Agouti (*a*) locus on mouse chromosome 2 (MICHAUD *et al.* 1994). The *A^y* mutation results from an ~170-kb deletion of the entire coding region of the *Raly* gene, which is proximal to the Agouti gene. This deletion places the coding region of Agouti under the transcriptional control of the *Raly* promoter, resulting in ubiquitous expression of the agouti gene product (MICHAUD *et al.* 1993, 1994; KHREBTUKOVA *et al.* 1999). The *A^y* phenotype is associated with obesity, diabetes, and yellow coat color (BULTMAN *et al.* 1992). *A^y* mice also show increased susceptibility to pulmonary and mammary gland tumors in females, induced skin tumors in both sexes, and hepatomas in males (STEVENS 1967). Remarkably, 129/Sv mice carrying the *A^y* mutation have approximately one-tenth the incidence of testicular germ cell tumors as the wild-type littermates (STEVENS 1967).

Testicular cancer is a complex trait that may require as many as six independently segregating genes with additive and recessive effects (STEVENS 1967). The low frequency of spontaneous TGCTs in the 129/Sv inbred strain and the multigenic basis of the cancer together make it difficult to dissect the genetic control of susceptibility of TGCTs. We used single gene mutations that are inherited as Mendelian traits to gain clues to the nature of genes and pathways that affect TGCT susceptibility. The various mutations on the 129 genetic background increase or decrease penetrance perhaps by activating novel pathways involved in tumorigenesis. The interaction of these Mendelian traits provides a unique opportunity to study TGCT development. In other model systems, interactions between different pairs of genes have provided unique insights into developmental pathways and cancer susceptibility (MOORE *et al.* 1990; OCA LUNA *et al.* 1995; LAMOREUX *et al.* 2001; MONTES DE PARANT and LOZANO 2003). We therefore tested interactions between pairs of TGCT susceptibility genes to evaluate their effect on tumor frequencies, laterality of the tumors, and parental effects to reveal more about the molecular and developmental pathways leading to tumorigenesis.

MATERIALS AND METHODS

Mice: 129S1/SvImJ (JR002448, previously known as 129/Sv and 129S3/SvImJ), 129S1/Sv-*p^{-t} Tyr⁺ Kit^{Slj}/+* (JR000090), B6.Cg-*A^y* (JR000021), and 129-*Trp53^{tm1Tj}* (JR002080) were obtained from the Jackson Laboratory (Bar Harbor, ME). The nomenclature for 129 substrains has been revised by the Jackson Laboratory (http://www.informatics.jax.org/mgihome/nomen/strain_129.shtml) and the recommended designations were used in this article. The 129.MOLF-Chr19 CSS (N15F2+) was described previously (MATIN *et al.* 1999) and

was obtained from our research colony. Mice were maintained in the Case Western Reserve University Animal Resource Center on a 12-hr:12-hr light:dark cycle and fed Lab Diet 5010.

Construction of a 129-*A^y* congenic strain: To transfer the *A^y* mutation onto the 129/Sv background, C57BL/6J-*A^y* mice were crossed to 129S1/SvImJ for 11 generations. Genes in the 129 background are essential for tumorigenesis, and therefore to perform the interaction tests, *A^y* must be transferred to and tested on the 129/Sv background.

Construction of 129-*A^y*/+, MOLF-Chr 19 congenic consomic mice: 129-*A^y* mice were crossed to our 129.MOLF-Chr 19 CSS. Heterosomic 129.MOLF-Chr 19 mice carrying *A^y* were then backcrossed to 129.MOLF-Chr 19. *A^y* progeny from these backcrosses were typed for simple sequence length polymorphisms along the length of chromosome 19. *A^y* progeny that were homosomic for chromosome 19 were selected to establish the test colony and wild-type littermates were used as controls.

Genotyping: DNA for PCR genotyping was obtained from samples of tail tissue. Each tail sample was digested in 89 μ l of water, 10 μ l 10 \times PCR buffer, and 1 μ l proteinase K (10 mg/ml).

***Trp53*:** In this study, we purchased the *Trp53*/+ mutant strain developed by JACKS *et al.* (1994) from the Jackson Laboratory. This strain has a *Trp53* mutant allele different from that reported by DONEHOWER *et al.* (1992). In both strains, *Trp53* heterozygous mutants rarely develop TGCTs, whereas they are more common in *Trp53* homozygous mutants. TGCT frequencies of the heterozygous or the homozygous *Trp53* mutants were not reported by JACKS *et al.* (1994), whereas HARVEY *et al.* (1993) reported that their homozygous mutants developed TGCT at 35% and that the heterozygous mutants were reported to rarely develop TGCTs.

A three-primer PCR assay was used to distinguish wild-type from heterozygous *Trp53* animals. The three primers are X7, 5'-TATACTCAGAGCCGCCT-3'; Neo19, 5'-CTATCAGACA TAGCGTTGG-3'; and X6.5, 5'-ACAGCGTGGTGGTACCT TAT-3'. Primers X7 and Neo19 amplify a 600-bp fragment identifying the *Neo* insert. Primers X7 and X6.5 amplify a 400-bp fragment from the untargeted *Trp53* allele. PCR amplification was carried out in a 96-well block MJ Research (Watertown, MA) PTC-200 thermal cycler. The reagents were 0.15 μ l (0.75 units) *Taq* polymerase (Invitrogen, San Diego), 2.5 μ l 10 \times PCR buffer (magnesium free), 0.3 μ l 10 mM dNTPs, 1 μ l 25 mM magnesium chloride, 0.2 μ l of each primer (0.1 μ M), 1 μ l DNA (25 ng), and 19.35 μ l dH₂O in a final volume of 25 μ l. PCR conditions were as follows: initial denaturation step for 94 $^{\circ}$ for 2 min followed by 35 cycles of 94 $^{\circ}$ for 1 min, 60 $^{\circ}$ for 1 min, 72 $^{\circ}$ for 1 min, final extension of 72 $^{\circ}$ for 5 min, and then 4 $^{\circ}$ for 15 min. PCR products were resolved on a 2% agarose gel and visualized with ethidium bromide.

Mgf^{S^h}: The breakpoints of the *Mgf^{S^h}* deletion are not known and as a result a PCR genotyping assay is not available for the *Mgf^{S^h}* mutation. *Mgf^{S^h}*/+ mutant heterozygotes have a light coat color in the belly and the tips of the tail and digits are pink.

***A^y*:** *A^y* progeny were identified on the basis of their yellow coat color.

TGCT assays: Males were autopsied at 3–4 weeks of age and testes were examined macroscopically for evidence of TGCTs. Twenty pairs of wild-type testes from each cross were examined histologically to confirm the macroscopic observations. Previous work involving validation of visual inspection with histological assessments showed that TGCTs rarely escape detection (MATIN *et al.* 1999; YOUNGREN *et al.* 2003).

Data analysis: We used χ^2 contingency tests (with 1 d.f.) to test for parental effects and to determine whether the observed TGCT frequencies were significantly different from the expected additive frequency. To evaluate the frequencies of bilat-

eral and unilateral TGCTs, the formula by YOUNGREN *et al.* (2003) was used to calculate the expected frequency of unaffected, unilateral, and bilateral cases in the *Mgf^{S^h}* and *M19* double heterozygotes, and contingency tests (with 2 d.f.) were used to compare the expected frequency and observed numbers of unaffected and unilaterally and bilaterally affected mice.

RESULTS

Control tests—TGCT frequencies in single-mutant mice: We used the frequency of TGCTs in single-mutant mice to calculate the expected frequency in double-mutant mice. If mutant genes have functionally independent and additive effects on tumorigenesis, the expected tumor frequency in double-mutant mice should be the sum of the frequencies in the relevant single-mutant mice. Rather than simply using TGCT frequencies reported in the literature, we measured the frequencies in our colony (Table 1). We also characterized the laterality of the tumors and tested for parental effects by comparing the tumor frequencies in reciprocal crosses that were not previously tested.

Mgf^{S^h}: *Mgf^{S^h}*/+ heterozygotes typically have a TGCT frequency of 12–14%, on the basis of surveys of exceptionally large numbers of mice (STEVENS 1967). We found that *Mgf^{S^h}*/+ heterozygotes had similar frequencies of TGCTs in reciprocal crosses ($\chi^2 = 0.13$, $P > 0.05$), suggesting that parental factors did not affect susceptibility. The TGCT frequency in the pooled data of 170 *Mgf^{S^h}*/+ heterozygotes was 12% (Table 1A).

***Trp53*:** The rate of affected *Trp53*/+ heterozygotes has not been reported (DONEHOWER 1995). We found that *Trp53*/+ heterozygotes had similar TGCT frequencies in reciprocal crosses ($\chi^2 = 0.17$, $P > 0.5$). In the interaction crosses between *Trp53*/+ and *Mgf^{S^h}*/+ heterozygous mutants, the single mutant *Trp53*/+ littermates pooled from reciprocal crosses had a tumor frequency of 15% (Table 5).

129.MOLF-Chr19 (M19): The tumor frequency of 129.MOLF-Chr19 was reported to be 82% with equal frequencies of unilateral and bilateral tumors (MATIN *et al.* 1999). 129.MOLF-Chr19 mice were backcrossed to 129/Sv mice to obtain heterosomic animals (only one copy of MOLF-Chr19). We found that heterosomic mice had similar frequencies of TGCTs in reciprocal crosses ($\chi^2 = 0.88$; $P > 0.1$). The tumor frequency in the pooled sample of 300 heterosomic animals was 33% and tumors were mainly unilateral (29%; Table 1B).

***A^y*:** The tumor frequency of 129/Sv mice carrying the *A^y* mutation was first reported by STEVENS (1967) to be ~1%, whereas their wild-type littermates had a frequency of ~8% ($\chi^2 = 24.4$, $P < 0.001$). To verify Stevens' discovery, we made a 129/Sv-*A^y* congenic strain and then transferred the *A^y* mutation onto the 129.MOLF-Chr19 CSS background. This CSS strain has a tumor frequency of ~80% and therefore serves as a statistically powerful way to quickly determine whether *A^y* suppresses tumori-

TABLE 1
Control tests

Genotype	Sample size	% unilateral tumors	% bilateral tumors	% total affected animals
A. Control test between 129/Sv and <i>Sl^l/+</i> : overall tumor frequency of <i>Sl^l/+</i> and wild-type controls				
<i>Sl^l/+</i>	170	11 (19)	1 (2)	12 (21)
<i>+/+</i>	191	8 (15)	0	8 (15)
B. Control test between M19 and 129/Sv: overall tumor frequency of heterosomic animals				
Heterosomic	300	29 (86)	4 (13)	33 (99)

genesis; detecting a 10-fold reduction from the 5% TGCT frequency in 129/Sv would require a survey of significantly more mice than is required to assess TGCT susceptibility in 129.MOLF-Chr19 *A^l/+* consomic congenic mice. A smaller survey was conducted in our lab with 129-*A^l/+* mice having a tumor frequency of ~4% and their wild-type littermates of ~13% ($\chi^2 = 2.3$; $P < 0.5$), which represents an ~3-fold, but not significant, reduction. The results obtained were not statistically significant probably because of the modest number of mice that were surveyed ($N_{+/+} = 40$; $N_{A^l/+} = 50$).

Double-mutant interaction tests: Double-mutant mice were used to test for interactions between TGCT susceptibility genes. We evaluated tumor frequency, tumor laterality, and parental effects in the double mutants.

***Mgf^{Sl^l}* and M19 double heterozygote:** The double-heterozygous mutants of the interaction test between *Mgf^{Sl^l}/+* and M19 revealed a higher-than-expected TGCT susceptibility (Table 2). There was no evidence of parental effects between the reciprocal crosses ($\chi^2 = 0.83$; $P > 0.1$); the data for the two crosses were therefore pooled. The control M19/*+/+* heterozygote littermates had a TGCT frequency of 32%, which was not significantly different from the 33% frequency that was found in the control test (Table 2). If M19 and *Mgf^{Sl^l}* acted additively, the expected tumor frequency in the double-heterozygous mutants would be 45% [= 33% (M19/*+/+*) + 12% (*Mgf^{Sl^l}/+*)]. The observed TGCT frequency of 57% in the 215 double-heterozygous mutants was significantly higher than expected ($\chi^2 = 9.7$; $P < 0.005$). The increase in TGCT frequency resulted largely from an increase in the frequency of bilateral tumors,

which increased in frequency from 6% in M19/*+/+* mice to 19% in M19/*+/+ Mgf^{Sl^l}/+* mice. By contrast, the frequencies of unilateral TGCTs in these two groups of mice were similar, suggesting that M19 and *Mgf^{Sl^l}* interacted to increase the frequency of bilateral tumors only.

***Mgf^{Sl^l}* and M19 congenic strains:** Two congenic strains (A2 and C2; YOUNGREN *et al.* 2003) were used in heterozygous state to localize the region on MOLF-Chr19 that interacted with *Mgf^{Sl^l}* to increase susceptibility to bilateral tumors. A2 congenic mice have the proximal and the middle segment of MOLF-Chr19, whereas C2 congenic mice have the distal and the overlapping middle segment (with A2) of MOLF-Chr19. Parental effects between the reciprocal A2/*+* congenic crosses were not significant ($\chi^2 = 0.04$; $P > 0.5$). The tumor frequency in the pooled data for the double-heterozygous mutants A2/*+/+ Mgf^{Sl^l}/+* was 30% and these tumors were mainly unilateral (Table 3A).

In contrast, a modest parental effect was detected in the interaction cross between C2/*+* and *Mgf^{Sl^l}/+* mice ($\chi^2 = 4.0$; $P < 0.025$). A total of 97 double-heterozygous mutants of parental crosses between C2 females \times *Mgf^{Sl^l}/+* males were examined and 10% of the double-heterozygous mutants developed TGCTs, of which only 2% were bilateral tumors (Table 3B). In the reciprocal cross of *Mgf^{Sl^l}/+* females \times C2 males, 20% of 89 double-heterozygous mutants developed TGCTs, of which only 3% were bilateral tumors (Table 3C). Double-heterozygous mutants from both congenic crosses had low tumor frequencies and, importantly, rarely developed bilateral tumors. It appears that *Mgf^{Sl^l}* did not interact separately

TABLE 2
Interaction test between M19 and *Mgf^{Sl^l}* in heterozygous mutants: overall tumor frequency of M19/*+/+ Sl^l/+* and wild-type controls

Genotype	Sample size	% unilateral tumors	% bilateral tumors	% total affected animals
M19/ <i>+/+ Sl^l/+</i>	215	38 (82)	19 (40)	57 (122)
M19/ <i>+/+ +/+</i>	225	26 (58)	6 (14)	32 (72)

TABLE 3
Localization of the region on MOLF-Chr19 that interacts with *Mgf^{SlJ}*

Genotype	Sample size	% unilateral tumors	% bilateral tumors	% total affected animals
A. Interaction cross between congenic strain A2 and <i>Sl^J</i> mice: overall tumor frequency of A2/+ <i>Sl^J/+</i> and wild-type controls				
A2/+, <i>Sl^J/+</i>	130	23 (30)	7 (9)	30 (39)
A2/+, +/+	163	15 (24)	1 (1)	15 (25)
B. Interaction cross between ♀ C2 × ♂ <i>Sl^J/+</i>				
C2/+, <i>Sl^J/+</i>	97	8 (8)	2 (2)	10 (10)
C2/+, +/+	84	13 (11)	0	13 (11)
C. Interaction cross between ♀ <i>Sl^J/+</i> × ♂ C2				
C2/+, <i>Sl^J/+</i>	89	18 (16)	2 (2)	20 (18)
C2/+, +/+	71	8 (6)	0	8 (6)

with either the proximal or the distal segments of the MOLF-Chr19 to increase the incidence of bilateral tumors.

M19 homosomics and *Mgf^{SlJ}* congenic heterozygotes: Homosomic-congenic mutants were generated by transferring *Mgf^{SlJ}* to the 129.MOLF-Chr19 strain. If M19 and *Mgf^{SlJ}* acted additively, the expected tumor frequency in the M19/M19 *Mgf^{SlJ}/+* mice would be 94% [= 82% (M19/M19) + 12% (*Mgf^{SlJ}/+*)]. The TGCT frequency in the homosomic congenic-heterozygous mutants of the reciprocal crosses was similar ($\chi^2 = 3.65$; $P > 0.05$). The homosomic-congenic mutant had the expected high tumor frequency with an affected rate in the pooled data of 92% (Table 4), which was not significantly different from the expected additive frequency of 94% ($\chi^2 = 0.4$; $P > 0.5$). The M19/M19 +/+ littermates had a TGCT frequency of 80%, which was not significantly different from the expected tumor frequency (MATIN *et al.* 1999). Previously, we reported (Table 2) that the double-heterozygous mutants had a significant increase of bilateral tumors; this was not observed in the homosomic-congenic mutants.

***Trp53*/+ and *Mgf^{SlJ}/+* double-mutant heterozygotes:** The interaction test between *Trp53*/+ and *Mgf^{SlJ}/+* mutants revealed surprising and remarkable results. The tumor frequencies in the reciprocal parental crosses were not significantly different ($\chi^2 = 0.5$; $P > 0.1$). The

pooled data for 93 wild-type littermates showed a tumor frequency of 2%, whereas in 91 *Trp53*/+ single-mutant littermates the pooled tumor frequency was 15%. Of 87 *Mgf^{SlJ}/+* single-mutant littermates, 11% developed TGCTs. Thus, the wild-type and single-mutant littermates had the appropriate rates of affected males, as compared to the control data. These TGCT frequencies were used to calculate the expected additive TGCT frequency for the *Trp53*/+ *Mgf^{SlJ}/+* double-heterozygous mutants. If the two mutant genes acted additively, the expected additive tumor frequency would be 26% [= 15% (*Trp53*/+) + 11% (*Mgf^{SlJ}/+*)]. In the pooled data of 75 double-heterozygous mutants, only 7% developed a TGCT (Table 5). The double-heterozygous mutants had a tumor frequency fourfold lower than expected ($\chi^2 = 13.6$; $P < 0.001$), which suggests that partial deficiency of the tumor suppressor gene *Trp53* and heterozygosity for the *Mgf^{SlJ}* deletion suppressed development of TGCTs.

***A^v* and M19:** 129.MOLF-Chr19 carrying the *A^v* mutation had a reduced TGCT frequency compared to that of 129.MOLF-Chr19 wild-type littermates without *A^v* (Table 6). No evidence for significant parental effects was observed among progeny from reciprocal crosses. A total of 104 male progeny of 129.MOLF-Chr19 carrying the *A^v* mutation were examined and 40% developed TGCTs, in comparison to 65% of the 105 129.MOLF-

TABLE 4
Interaction test between M19 and *Sl^J* in homozygous-congenic mutants: overall tumor frequency of M19/M19 *Sl^J/+* and wild-type controls

Genotype	Sample size	% unilateral tumors	% bilateral tumors	% total affected animals
M19/M19, <i>Sl^J/+</i>	39	33 (13)	59 (23)	92 (36)
M19/M19, +/+	50	36 (18)	46 (23)	82 (41)

TABLE 5

Interaction test between *Trp53* and *Mgf^{Slj}*: overall tumor frequency of *Trp53/+ Slj/+* and littermate controls

Genotype	Sample size	% unilateral tumors	% bilateral tumors	% total affected animals
<i>Trp53/+</i> , <i>Slj/+</i>	75	7 (5)	0	7 (5)
<i>Trp53/+</i> , <i>+/+</i>	91	15 (14)	0	15 (14)
<i>+/+</i> , <i>Slj/+</i>	87	11 (10)	0	11 (10)
<i>+/+</i> , <i>+/+</i>	93	2 (2)	0	2 (2)

Chr19 without the *A^y* mutation that developed TGCTs ($\chi^2 = 12.4$, $P < 0.001$). These results show that *A^y* not only suppresses TGCT frequency in the 129/Sv inbred strain (STEVENS 1967) but also causes a 25% reduction of TGCT incidence in the 129.MOLF-Chr19 CSS.

DISCUSSION

The low incidence of spontaneous TGCTs in the 129/Sv inbred strain makes it difficult to study the genetic control of TGCT susceptibility in these mice. In this article, Mendelian mutations that are known to individually increase or decrease the frequency of TGCTs were used to test interactions as a way to learn more about the pathways that control TGCT susceptibility.

The pairwise gene interaction tests showed that several mutant genes enhanced or suppressed the development of TGCTs. The M19/+ *Mgf^{Slj}*/+ compound heterozygotes had a 27% increase in TGCT frequency over the expected additive rate. This increase resulted primarily from an unexpectedly high frequency of bilateral tumors. In contrast, an interaction between *Mgf^{Slj}* and *Trp53* suppressed the development of TGCTs by about fourfold in double heterozygotes, lowering the affected rate from the expected 26% to 7%, which is similar to the background rate of the 129/Sv strain. The *A^y* mutation also suppressed the TGCT frequency in the 129.MOLF-Chr19 CSS.

Traditionally, mutations that increase susceptibility to cancers have been studied to learn more about the mechanisms that promote tumorigenesis. In this study, we discovered two pairs of interacting genes that suppressed development of TGCTs and another pair of genes that enhanced susceptibility. These suppressors and enhancers may provide an important way to modu-

late the genetic and molecular pathways that lead to TGCTs in mice and perhaps in humans.

Enhancers of TGCT susceptibility: The *Mgf^{Slj}*/+ M19/+ double-heterozygous mutants showed that *Mgf^{Slj}* and at least two genes on chromosome 19 (one proximal and one distal) interacted to significantly increase the incidence of TGCTs. The increased frequency over the expected additive tumor frequency results from an increased incidence of bilateral tumors. Congenic strains that dissect the MOLF-Chr19 into two regions, proximal and distal, were crossed to the *Mgf^{Slj}*/+ mutants. We were, however, unable to localize the region on MOLF-Chr19 that interacted with *Mgf^{Slj}* to increase susceptibility to bilateral tumors. Heterozygous-congenic mutants showed that neither the proximal nor the distal end of the MOLF-Chr19 alone increased the incidence of bilateral tumors. These data complement the analysis of the panel of congenic strains to characterize TGCT susceptibility genes in the 129.MOLF-Chr19 CSS (YOUNGREN *et al.* 2003). A survey of TGCT frequencies in this panel of congenic strains suggests that several genes on MOLF-Chr19 affect TGCT susceptibility in both additive and epistatic manners and act as enhancers or suppressors. The various congenic strains with only one segment of MOLF-Chr19 did not have the high TGCT frequency similar to the 129.MOLF-Chr19 CSS, whereas double-congenic strains with both the proximal and distal end of MOLF-Chr19, but missing the small middle segment, resulted in a TGCT frequency similar to that of 129.MOLF-Chr19 CSS (YOUNGREN *et al.* 2003). The double-congenic strain showed epistasis between TGCT susceptibility genes on MOLF-Chr19. The increased TGCT frequency in *Mgf^{Slj}*/+ M19/+ mice may result from an interaction between *Mgf^{Slj}* and an interacting set of genes rather than from the individual component genes on chromosome 19. The increased susceptibility to bilat-

TABLE 6

Interaction test between M19 and *A^y*: overall tumor frequency of M19/M19 *A^y*/+ and wild-type controls

Genotype	Sample size	% unilateral tumors	% bilateral tumors	% total affected animals
M19/M19, <i>A^y</i> /+	104	32 (33)	9 (9)	40 (42)
M19/M19, <i>+/+</i>	105	40 (42)	26 (26)	65 (68)

eral tumors may therefore require at least three factors: *Mgf^{Slj}*, together with the proximal and distal portions of MOLF-Chr19. Interestingly, the homosomic-congenic mutants *Mgf^{Slj}/+* M19/M19 did not have an increased susceptibility to bilateral tumors, suggesting that the interaction observed in the double heterozygous mutants is complex and perhaps involves a dosage effect.

Suppressors of TGCT susceptibility: The interaction crosses revealed two new models to suppress TGCT susceptibility. Traditionally, genes that promote tumorigenesis are identified, with the ultimate goal of suppressing or preventing tumor formation by studying the mechanisms of tumorigenesis. Tumor suppression in mice such as the *Trp53*/⁺ *Mgf^{Slj}/+* and *A^y/+* M19/⁺ double-mutant mice, with their significantly reduced TGCT frequencies, may reveal clues to the genetic and molecular basis for modulating TGCT susceptibility.

***Trp53* and *Mgf^{Slj}*:** Mutations in *Trp53* and *Mgf^{Slj}* on the 129/Sv inbred genetic background each increased susceptibility to TGCTs. In the interaction cross between *Trp53*/⁺ and *Mgf^{Slj}/+*, the double mutants had a tumor frequency remarkably lower (about a fourfold decrease) than the expected rate resulting from the additive effects of the single mutants. These results suggest that interactions between apoptosis and MGF-KIT signal transduction modulate susceptibility to testicular cancer.

Trp53 mediates apoptosis and cell cycle arrest at the G₁/S stage (LEE *et al.* 1994), whereas the signaling pathway of the MGF-KIT prevents apoptosis and promotes cell division and growth of melanocytes, mast cells, and germ cells (DOLCI *et al.* 1991). In a cell culture system, MGF inhibits *Trp53*-mediated apoptosis and differentiation, but not G₁/S cell cycle arrest (ABRAHAMSON *et al.* 1995). Most studies that examine the relationship between KIT/MGF signaling and TRP53 involve cell culture systems (ABRAHAMSON *et al.* 1995; MATSUI *et al.* 2000), whereas JORDAN *et al.* (1999) used an *in vivo* model system to study the affect of *Trp53* on melanocytes, mast cells, and germ cells by taking advantage of the *Kit^{W^v}* mutant, which is homozygous viable.

Mouse mutants of the KIT receptor and its ligand, MGF, are defective in melanogenesis, gametogenesis, and hematopoiesis. Although the phenotypes of most of the *Kit* and *Sl* mutants are similar, STEVENS (1967) found contrasting results for several *Kit* and *Sl* alleles on the 129/Sv inbred background: *Sl* alleles (*Sl* and *Sl^l*) developed TGCTs more than twice as often as did the wild-type littermates, whereas none of the *Kit* mutants showed an increased susceptibility of TGCTs. In a separate study, the *Kit^{W^v}* mutant was crossed to *Trp53*-deficient mutants to study the role of *Trp53* in regulating cell death in the absence of MGF-KIT signaling (JORDAN *et al.* 1999). The double mutants that were deficient in TRP53 function did not increase the survival of melanocyte and mast cells, but showed instead an increased number of germ cells and restored fertility (JORDAN *et al.* 1999). Normally, MGF-KIT signals are induced

between E7.5 and E13 (MANOVA and BACHVAROVA 1991) to inhibit TRP53 function in PGC proliferation (ABRAHAMSON *et al.* 1995). The *Kit^{W^v}* mutants lack MGF-KIT signals and therefore cannot inhibit TRP53, leading to apoptosis of PGC and sterility. JORDAN *et al.* (1999) demonstrated that double mutants deficient in *Trp53* and *Kit* rescued sterility defects because apoptosis of male germ cells is *Trp53* dependent but apoptosis of melanocyte and mast cells is regulated by a *Trp53*-independent pathway. This experiment explains how fertility can be restored in mutants that lack the functions of KIT and MGF, but it does not readily explain the suppression of TGCT development in the double heterozygous mutants *Mgf^{Slj}/+* *Trp53*/⁺.

PGCs enter G₁ mitotic arrest at E13.5 until after birth (DONOVAN *et al.* 1998). If PGCs fail to enter G₁ mitotic arrest, they may become pluripotent stem cells, resulting in tumors of various cell and tissue types. The double-heterozygous mutants have a partial deficiency of TRP53 to inhibit apoptosis and bypass G₁ cell cycle check points, which could result in uncontrolled cell growth. In addition, the double-heterozygous mutants have the *Sl^l* deletion, which can involve loss of a tumor suppressor gene or MGF itself that results in an increase of TGCT frequency. Surprisingly, the partial deficiency of *Trp53* interacts with the *Mgf^{Slj}* mutation to suppress the development of TGCTs.

Additional experiments are needed to learn more about the mechanisms that suppress the development of TGCTs, such as immunohistochemistry to determine the localization of *Trp53* within the testis of single- and double-mutant mice, measuring the levels of *Trp53*, and determining whether the interactions altered downstream or upstream the *Trp53*-mediated apoptosis or DNA repair pathway. From *in vitro* experiments, it does not seem likely that the MGF-KIT signaling pathway interacts directly with *Trp53* functions, because the expression of other *Trp53*-dependent and downstream genes such as *bax*, *p21*, and *mdm2* are not affected by the absence of the Steel factor (ABRAHAMSON *et al.* 1995).

***A^y*:** Previous studies showed that *A^y* mice have a greatly increased susceptibility to many different types of tumors (WOLFF *et al.* 1986; WOLFF 1987). However, 129/Sv male mice that are heterozygous for the *A^y* mutation showed a 10-fold reduction in TGCTs (STEVENS 1967). The mechanism for this TGCT suppression was not pursued and Steven's congenic strain was lost many years ago.

To verify findings of STEVENS (1967), we created a new 129-*A^y* congenic strain. 129/Sv mice normally develop TGCTs at a rate of 1–5%; Stevens therefore needed to examine a large number of males to find the 10-fold reduction. To detect a stronger effect with a smaller number of mice, we crossed *A^y* congenic mice to a strain that has a high TGCT incidence, 129.MOLF-Chr19 (MATIN *et al.* 1999). The M19/M19 strain of mice has a TGCT incidence of 80% (MATIN *et al.* 1999) and therefore serves as an excellent strain to test for TGCT

suppression with A^y . We found that the A^y mutation does indeed suppress TGCT formation and could serve a key role in understanding TGCTs and tumorigenesis in general.

Although the suppressive effect of A^y on TGCTs is exciting, little is known about the ways in which this mutation affects tumorigenesis. Because the A^y phenotype results from a large deletion upstream of agouti, two explanations for suppression are possible. The first is that the ubiquitous overexpression of agouti, because of the A^y mutation, results in TGCT suppression as a consequence of this unique expression pattern involving the melanocortin receptor signaling pathway. Another explanation for suppression is the deletion of *Raly* or other genes between *Raly* and agouti found in the 170-kb region upstream of agouti. The first model suggests that TGCT suppression may be linked to the ubiquitous expression of agouti. Agouti is normally expressed during development, in neonatal skin, and in the testis (YEN *et al.* 1994) and is responsible for the wild-type coat color seen in mice (SILVERS 1979). Agouti expression results in the switching of eumelanin to pheomelanin production by inhibition of α -melanocyte-stimulating hormone due to antagonism of melanocortin receptor 1 (BULTMAN *et al.* 1992; LU *et al.* 1994; CONE *et al.* 1996). However, in A^y mutants, ectopic agouti expression interferes with this switching pattern by preventing eumelanin production, leaving only pheomelanin production. This results in the uniform yellow coat associated with the mutation (CONE *et al.* 1996).

When expressed ubiquitously, it is possible that the agouti protein interacts with other receptors not previously characterized. Characterization of the agouti gene revealed that multiple transcripts of varying sizes were expressed in the testis but not in any other adult tissues sampled. None of the testis-specific transcripts were expressed in neonatal skin or during development and they therefore play an undetermined role independent of pigmentation and development (BULTMAN *et al.* 1992). In A^y mutants a unique transcript is ectopically expressed in all tissues examined (MICHAUD *et al.* 1993). It is possible that in the A^y mutant the ubiquitous overexpression of this larger agouti transcript found in all adult tissues interacts with the testis-specific transcripts, leading to a suppression of TGCTs.

The second model suggests that TGCT suppression results from loss-of-function for genes deleted in the 170-kb region upstream of agouti. This deletion causes the coding region of agouti to be under the transcriptional control of the *Raly* promoter, resulting in ubiquitous expression of the agouti gene product (MICHAUD *et al.* 1993). It is possible that deletion of the *Raly* coding exons or other genes within this region suppresses TGCTs. *Raly* is normally ubiquitously expressed and belongs to a family of RNA-binding proteins involved in pre-mRNA processing and embryonic developmental regulation (MICHAUD *et al.* 1993; KHREBTUKOVA *et al.*

1999). *Raly* functions in preimplantation development and its deletion accounts for the embryonic lethality associated with the A^y mutation (DUHL *et al.* 1994). Another gene located in the deletion between *Raly*'s promoter and agouti that could account for TGCT suppression is *eIF2s2*. *eIF2s2* is a eukaryotic translation initiation factor involved in protein synthesis. Phosphorylation and dephosphorylation of *eIF2s2* modulates protein translation (LODISH *et al.* 2000). Further studies involving engineered mutations are being pursued to identify the gene near A^y that suppresses TGCT susceptibility.

Implications for TGCTs in humans: Because of the prevalence of testicular cancer, an international effort is underway to identify susceptibility genes in humans (BISHOP 1998). However, lack of multigenerational pedigrees with several affected individuals has made the search difficult. Family studies typically have limited power to detect linkage for complex traits; nevertheless, numerous weak linkages have been identified (BISHOP 1998; LINDELOF and EKLUND 2001). This raises the possibility that additional linkages are yet to be discovered. Utilization of our mouse models provides an opportunity to control the genetics involved, thereby increasing the power of linkage studies. Approaches in the mouse and in humans will complement the search for genes involved in the initiation and progression of TGCTs.

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