Knockout mice reveal a tumor suppressor function for Testin

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The Testin (TES) gene was previously identified as a putative human tumor suppressor gene at 7q31.2, a region that is frequently deleted in hematopoietic malignancies, as well as in epithelial tumors. To determine whether TES acts as a tumor suppressor *in vivo*, we generated a Tes knockout mouse and then used it in an established model of carcinogen-induced gastric cancer. In mice a zinc-deficient (ZD) diet enhances cellular proliferation in the forestomach and susceptibility to *N*-nitrosomethylbenzylamine (NMBA)-induced carcinogenesis. Five-week-old Tes wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice were divided into four groups: mice fed a zinc-sufficient diet (ZS); mice fed a ZD diet; ZS fed plus NMBA-treated mice (ZS+NMBA), and ZD fed plus NMBA-treated mice (ZD+NMBA). After 4 weeks, the ZS+NMBA and ZD+NMBA groups were treated with three intragastric doses of NMBA. Animals were killed 8 weeks after NMBA administration: 25% of +/+ mice developed benign lesions; 88% of +/− showed multiple papillomas, atypical glandular metaplasia, and squamous cell carcinomasl; and 81% of −/− mice displayed very large papillomas, squamous cell carcinomas, and adenocarcinomas. A statistically significant difference in tumor incidence was found between +/− versus +/+ and −/− versus +/+ ($P < 0.0001$). These data suggest that Tes functions as a tumor suppressor gene *in vivo*.

carcinogen-induced tumorigenesis | gastric cancer | TES | gene targeting

Human FRA7G is a common fragile site that harbors a region of frequent loss of heterozygosity. Deletions at 7q31.1–31.2 have been detected in several types of leukemia (1), as well as tumors of the breast (2), ovary (3), prostate (4), colon (5), stomach (6), kidney (7), and head and neck (8). These data strongly imply the existence of a tumor suppressor (TS) gene at 7q31.1–31.2. We isolated the human Testin (TES) gene as one putative TS gene in this region and showed that the loss of its expression in several primary tumors and cancer cell lines is due predominantly to the methylation of its promoter (9). The TES gene product (Tes) is a LIM domain only protein that contains three C-terminal LIM motifs. LIM domains are responsible for protein–protein interactions that coordinate intracellular and extracellular pathways. In particular, Tes is a cytoskeleton-associated protein that localizes along actin stress fibers, at cell-cell-contact areas, and at focal adhesion plaques. Tes interacts with a variety of cytoskeletal proteins, including zyxin, mena, VASP, talin, and actin.

Tes overexpression enhanced cell spreading and decreased cell motility (10). RNA interference knockdown of Tes led to a loss of actin stress fibers, suggesting a role of the protein in actin cytoskeleton regulation (11). Overexpression of Tes also reduced cell growth in Ovcar5 (ovarian), HeLa (cervical), and T47D (breast) carcinoma cell lines and had an inhibitory effect on T47D anchorage-independent growth in soft agar (12). Moreover, adenoviral transduction of TES into a breast cancer (T47D) and a uterine sarcoma (Mes-SA) cell line inhibited tumorigenesis in nude mice (13). To investigate the *in vivo* role of Testin, we have generated Tes-knockout mice and, in turn, used them in a well established model of carcinogen-induced gastric cancer (14).

Materials and Methods

Generation of Tes Knockout Mice. An SVJ/129 genomic phage library was screened to isolate a 4,675-bp fragment containing mouse exon 3. In particular, exon 3 is located in the middle of the 5′ EcoRI/KpnI fragment. The fragment was amplified by PCRs to insert multiple stop codons before the exon coding region and a StuI restriction site in the middle of the same exon, where a PGK-Neo cassette was cloned (EcoRI F:5′-GGG-CTG-CAG-GAA-TTC-TCA-TCC-CTT-3′; EcoRI R:5′-GAA-GGC-CTT-CAT-TCT-AAA-TTA-TCA-GCT-TGG-CGA-GGG-TGG-TGT-ACT-3′; KpnI F:5′-GAA-GGC-CTA-GTC-AGA-TGG-CAT-TCC-CAT-GT-3′; KpnI R:5′-TCA-CTA-TAG-GGC-GAA-TTC-GGT-ACC3-′). The targeting vector (50 μg) was electroporated into RW4 ES cells (Genome Systems, St. Louis). Two positive homologously recombinated clones were injected into 3.5-day-old blastocysts to generate female and male chimeras that were crossed to C57BL/6J mice. The germ-line +/− progeny gave rise to B6129 hybrid mice that were Tes +/+, +/− or −/− and were produced in the Kimmel Cancer Center animal facility at Thomas Jefferson University (Philadelphia).

Genotyping. DNA from G418-resistant ES colonies was digested with the restriction enzyme NcoI (Roche). Digestions were electrophoresed on 0.7% agarose gels and blotted to nylon membranes. The membranes were hybridized with a 700-bp probe corresponding to the 3′SmaI/NcoI fragment (Fig. 1*B*). Homologously recombinated clones were identified for the presence of two bands: a 4,675-bp band for the wild-type allele and a 3,513-bp band for the recombinated allele.

Mice genotyping was performed either by Southern blotting or PCR using three primers: forward, 5′-CCA-CAT-GGG-TTA-TAG-TCA-CAC-TGG-3′; reverse, 5′-GCA-GCC-ACT-GGA-TTG-GTC-AAG-ATC-ATA3-′; and NeoR: 5′-TCT-TGT-TCA-ATG-GCC-GAT-CCC-ATA-TTG-GCT-3′. The forward/reverse and forward/NeoR pairs of primers amplified the 480-bp wild-type fragment and the recombinant 1,000-bp fragment, respectively.

Mouse Embryonic Fibroblasts (MEFs). Twelve-days-pregnant, heterozygous females were killed, and embryos were extracted. The head and internal organs were discarded. The remainder was homogenized in DMEM (Sigma) with 10% FBS (HyClone) and

Abbreviations: TS, tumor suppressor; MEF, mouse embryonic fibroblast; PCNA, proliferating cell nuclear antigen; NMBA, *N*-nitrosomethylbenzylamine; ZD, zinc deficient; ZS, zinc sufficient.

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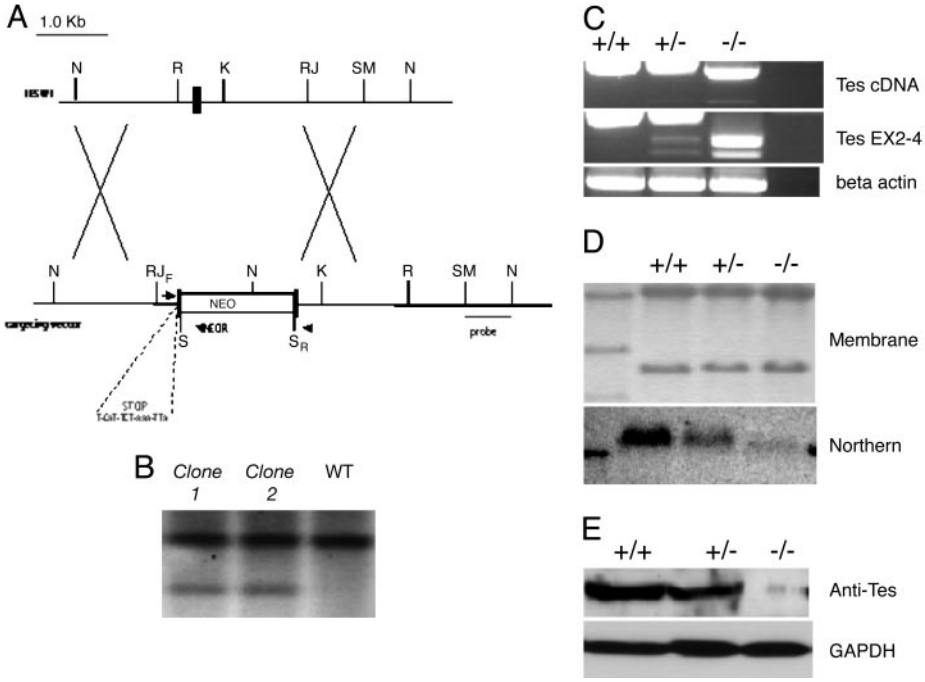

Fig. 1. Targeted disruption of the Tes gene. (*A*) Mouse Tes Exon 3 wild-type locus and the targeting vector. (*B*) Southern blot of the homologously recombinated ES clones (clones 1 and 2) and the wild-type counterpart. (*C*) RT-PCRs performed on MEF +/+, +/−, and −/− RNA: amplification of Tes mouse complete cDNA, of Tes mouse EX2-EX4 cDNA fragment, and β-actin control. (*D*) Northern blot on MEF +/+, +/−, −/− RNA: stained membrane and PhosphorImager picture after exposure using as a probe the EX4-EX7 Tes cDNA amplified fragment. (*E*) Anti-Tes Western blot and GADPH control.

50 units/ml penicillin and 100 μg/ml streptomycin. The supernatant was plated in 35-mm dishes.

RT-PCR and Northern Blot. RT-PCR was performed to amplify the mouse transcript from exon 2 to exon 4 and the entire Tes mouse cDNA. The primers used are the following: EX2 forward, 5′-ATG-GGC-TTA-GGT-CAT-GAG-CAA-GGA-3′; EX4 reverse, 5′-TCA-TCT-CAG-AGG-GAA-ACT-TCA-CGT-CAC-CCA-3′; cTES F, 5′-TCC-GAC-GCC-CAG-TAT-GGA-3′; cTES reverse, 5′-TTA-GGA-CAT-CAT-CCT-CTT-ACA-CTC-CAC-GGA-3′.

A Northern blot probe was obtained amplifying the cDNA mouse transcript from exon 4 to exon 7 with the following primers: EX4 forward, 5′-GGC-AGT-ACA-TGC-AGA-TGC-TGC-CTA-3′; EX7 reverse, 5′-GTG-GAT-GCG-TGC-CAG-CTG-AAG-TTG-3′.

Immunoblot Analysis and Proliferating Cell Nuclear Antigen (PCNA) Immunohistochemistry. Anti-Tes antibody was produced by Zymed (San Francisco, CA). Rabbits were immunized with the following mouse peptide sequence: (C)GNRHAPAAVASK-DKSAES-AMIDE. Purified antibody was used at 1:200 dilution in immunoblotting. Specificity was tested on murine protein lysates by peptide inhibition.

After deparaffinization and rehydration in a graded alcohol series, sections were incubated with mouse anti-PCNA monoclonal antibodies (Santa Cruz Biotechnology) at a 1:250 dilution, followed by incubation with biotinylated goat anti-mouse antibodies and streptavidin horseradish peroxidase. The location of PCNA was visualized by incubation with 3-amino-9-ethylcarbazole substrate–chromogen system (DakoCytomation, Carpinteria, CA). Cells with a red reaction product in the nucleus were defined as positive for PCNA.

Chemicals and Diet. *N*-nitrosomethylbenzylamine (NMBA) was purchased from Ash Stevens (Detroit). Custom-formulated, egg white-based zinc-deficient (ZD) and zinc-sufficient (ZS) diets containing 1.5 and 75 ppm zinc, respectively, were prepared by Harlan Teklad (Madison, WI). ZD diet is identical to ZS diet

Table 1. Experimental design

	Number of mice per experimental group				
Genotype	ZD	ZD + NMBA	ZS	ZS + NMBA	Total
+/+	6	15	5	13	39
+/−	7	29	5	21	62
−/−	8	16	5	21	50
Total	21	60	15	55	151

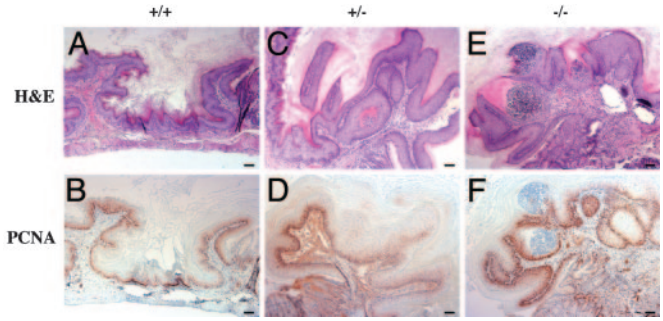

Fig. 2. Histopathology and cell proliferation in forestomach of Tes mice on a ZD diet. Wild-type mouse shows a slightly thickened forestomach epithelium (*A*) with PCNA-positive nuclei restricted to the basal and suprabasal cell layers (*B*). Heterozygous mouse forestomach shows upward and downward outgrowths (*C*) with abundant PCNA-positive nuclei in these areas (*D*). Homozygous mouse forestomach displays deep downgrowth and focal hyperplastic lesions (*C*) with abundant PCNA-positive nuclei in these areas (*F*). (Scale bar, 100 mm.)

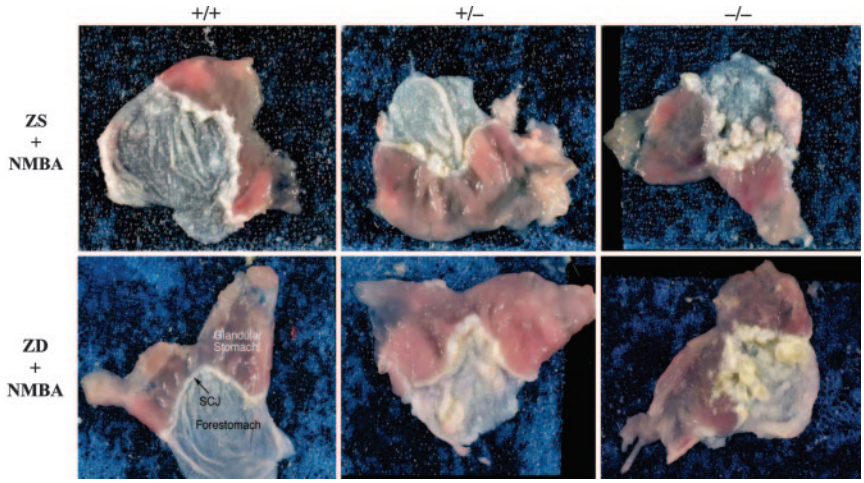

Fig. 3. Gross anatomy of ZD+NMBA- and ZS+NMBA-treated Tes +/+, +/−, and −/− mouse forestomachs. ZS, zinc-sufficient; ZD, zinc-deficient; NMBA, *N*-nitrosomethylbenzylamine.

except for the concentration of elemental zinc, and ZS diet is nutritionally complete (14).

Carcinogenicity Study. Five-week-old Tes wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice were randomly distributed into four groups: mice fed a ZS diet, mice fed a ZD diet, ZS fed plus NMBA-treated mice (ZS+NMBA), and ZD fed plus NMBA-treated mice (ZD+NMBA). After 4 weeks, ZD+NMBA and ZS+NMBA groups were treated with three intragastric doses of NMBA at 2 mg per kg of body weight. All animals were killed at 8 weeks after NMBA treatment. After anesthetization with isoflurane (Ohmeda, Madison, WI), the mice were killed and subjected to complete autopsies. Whole stomachs were excised and opened longitudinally. Tumors >0.5 mm in diameter were mapped and counted. The whole forestomach was fixed in buffered formalin and embedded in paraffin; 4-μm-thick cross-sections were cut. Sections were either stained with hematoxylin and eosin for histopathology or left unstained for immunohistochemical studies.

Statistics. Tumor incidence according to pathological diagnosis and tumor multiplicity according to macroscopic results were compared among the three genotypes by using χ^2 tests of association and nonparametric Wilcoxon Rank Sum tests. The same comparison of tumor incidence and multiplicity were performed by using χ^2 tests of association and nonparametric Kruskal–Wallis for each diet separately. In addition, logistic regression models were applied to the data to compare the odds of developing a tumor between the three genotypes, controlling for diet and sex. Exact methods were used.

Table 2. Effect of Tes insufficiency on NMBA-induced forestomach tumor incidence

Genotype	Total number of NMBA-treated mice	Number of mice bearing tumors (%)	Number of tumors/forestomach
+/+	28	7 (25)	2.4 ± 2.5
+/−	50	44 (88)	6.1 ± 3.7
−/−	37	30 (81)	5.6 ± 3.5

Tumor incidence (χ^2 *P* value): +/− versus +/+, $P < 0.0001$; −/− versus +/+, $P < 0.0001$; +/− versus −/−, $P < 0.3827$. Number of tumors/forestomach (Kruskal–Wallis *P* value): +/− versus +/+, $P < 0.0001$; −/− versus +/+, $P < 0.0001$; +/− versus −/−, $P < 0.5012$.

Results and Discussion

Generation of Tes Knockout Mice. Genomic DNA of the mouse Tes locus containing exon 3 was isolated from an SVJ/129 phage library. The targeting vector was designed as follows (Fig. 1*A*): multiple stop codons readable in all of the possible reading frames were located before exon 3, and a pGK-Neo cassette was inserted into the exon. The targeting vector was electroporated into ES cells. After G418 selection, ES clones were screened by Southern blotting: two homologously recombinated clones (Fig. 1*B*) were identified and injected into blastocysts. All of the chimeras went through germ line, and their progeny were crossed. Genotyping was performed by Southern blotting and PCR.

Total RNA derived from Tes +/+, +/−, and −/− MEFs was analyzed by RT-PCR (Fig. 1*C*). Primers were designed to amplify the Tes transcript from exon 2 to exon 4 (567 bp). The 567-bp product was present only in the +/+ and +/−. In the −/−, as well as in the +/−, 440- and 388-bp products were amplified, suggesting that Tes null and heterozygous mice were producing aberrant transcripts. The aberrant bands were purified from agarose gel and sequenced. Sequence analysis revealed that both the transcripts contained exons 2 and 4 and a partial part of the 3′ half end of exon 3. The 440-bp band was spliced to the acceptor located at base pair 127 of exon 3, immediately after the neomicine insertion site. The 388-bp band was spliced 52 bp downstream.

To determine whether the remaining Tes cDNA was transcribed, the whole Tes cDNA was amplified (Fig. 1*C*). In the +/+, only the wild-type transcript was present. In the +/− a wild-type band and two aberrant bands were seen. The two aberrant transcripts were the only bands produced in the −/− and showed the same 52-bp shift found in EX2–4 PCR. These results were confirmed by Northern blotting (Fig. 1*D*). Thus, in

Table 3. Logistic regression modelling of tumor outcome

Variable	Comparison	Estimated OR	95% CI for OR	*P* value
Genotype	+/− vs. +/+	38.9	7.6, 307.5	<0.0001
	−/− vs. +/+	27.6	5.0, 241.0	<0.0001
Sex	Female vs. male	6.6	1.5, 40.2	0.0066

Tumor outcome is defined as ''yes'' or ''no.'' OR, odds ratio.

GENETICS

Table 4. Effect of dietary ZD on forestomach tumorigenesis in Tes knockout mice

Genotype	Tumor incidence (%)		Number of tumors/forestomach	
	ZD	ZS	ZD	ZS
+/+	5/15 (33.3)	2/13 (15.4)	2.7 ± 3.2	2.0 ± 1.5
+/−	26/29 (89.7)	18/21 (85.7)	6.8 ± 3.7	5.0 ± 3.5
−/−	16/16 (100)	14/21 (66.7)	6.0 ± 3.2	5.2 ± 3.8

Tumor incidence (Fisher's exact *P* value): ZD groups: −/− versus +/+, $P < 0.0001$; +/− versus +/+, $P < 0.0001$; +/− versus −/−, $P < 0.54$. ZS groups: −/− versus +/+, $P < 0.0002$; +/− versus +/+, $P < 0.0001$; +/− versus −/−, $P < 1.4$. Tumor multiplicity (Kruskal–Wallis *P* value): ZD groups: −/− versus +/+, $P < 0.005$; +/− versus +/+, $P < 0.005$; +/− versus −/−, $P < 0.4$. ZS groups: −/− versus +/+, $P < 0.005$; +/− versus +/+, $P < 0.007$; +/− versus −/−, $P < 0.9$.

Table 5. Diet comparison

Factor	Level	Total	No tumor	Tumor	*P* value*
Diet	ZD	60	13 (21.7%)	47 (78.3%)	0.0525
	ZS	55	21 (38.2%)	34 (61.8%)	

*χ^2 test.

the Tes mice, the null allele produced two aberrant transcripts in which the first half of exon 3 was deleted. The 3′ half of exon 3 had two acceptors that were used from either one of the transcripts to continue the transcription up to the end of the gene.

To determine whether Tes null and heterozygous mice produced any Tes protein, we performed anti-Tes Western blotting on MEF lysates by using anti-Tes antibody (Fig. 1*E*). The antibody was produced by peptide rabbits immunization and recognized the terminal part of the murine Tes exon 4. According to the RT-PCR and Northern blotting results, exon 4 is present in both of the transcripts (Fig. 1*D*). If any protein was produced, it should be detected by the antibody. As shown in Fig. 1*E*, no protein was found in −/− lysates, and the proportion of Tes protein in the three genotypes suggests that the trascriptional product was not translated from one allele of the +/− and from both alleles of the −/− mice.

Study Design. To test whether Tes functions as a TS gene *in vivo*, we assessed its role in an established gastric carcinogenesis model (14). We also investigated whether dietary zinc deficiency that enhances cell proliferation in stomach epithelia and increases the susceptibility to NMBA-induced carcinogenesis in *p53*-deficient mice (14) has a similar effect in the Tes knockout mice. A total of 151 five-week-old Tes mice were used in this study (Table 1). Eighty-one (21 +/+, 36 +/−, 24 −/−) Tes mice were fed with a ZD diet, and 70 (18 +/+, 26 +/−, 26 −/−) animals were subjected to a nutritionally complete ZS diet. All mice were fed with their respective diet for the entire duration of the study.

After 4 weeks from the beginning of the dietary regimen, 15 +/+, 29 +/−, and 16 −/− ZD-fed Tes mice and 13 +/+, 21 +/−, and 21 −/− ZS-fed Tes mice were administered three intragastric doses of NMBA. To determine whether the induced phenotype was an effect of the carcinogenic treatment, the remaining 36 mice continued the ZD diet (6 +/+, 7 +/−, 8 −/−) or the ZS diet (5 +/+, 5 +/−, 5 −/−) until the end of the study. Eight weeks after NMBA treatment, all 151 mice were killed, and their stomachs were dissected and macroscopically and histologically examined.

NMBA-Induced Tumors. In general, within a genotype, cellular proliferation in mouse forestomach was enhanced by dietary ZD. Within a dietary group, the forestomach of +/− and −/− mice were more proliferative than the wild type, an observation consistent with previous findings in p53-deficient mice (14). The results of staining for PCNA, an endogenous marker for cell proliferation, are shown for ZD mice in Fig. 2. In ZD wild-type mice, cellular proliferation was mostly confined to the basal and suprabasal cell layers. By contrast, heterozygous and homozygous mice frequently displayed deep downward and upward growths and focal hyperplastic lesions (mostly in −/− mice) with abundant PCNA-positive nuclei present in these areas (Fig. 2).

Regardless of the zinc status of the diet, the majority of Tes +/+ mice had a large forestomach with a thin epithelium and a slightly thickened squamous columnar junction (SCJ) (Fig. 3). By contrast, heterozygous and homozygous Tes mice were characterized by the presence of a small forestomach with a very thick epithelium and SCJ dominated by lesions. As shown in Table 2, 25% of +/+, 88% of +/−, and 81% of −/− mice developed tumors. A statistically significant difference in tumor incidence was found between +/− versus +/+ and −/− versus +/+ ($P < 0.0001$), whereas the difference between +/− and −/− mice was not significant ($P = 0.3708$). This finding suggests that TES may be a one-hit TS gene, as are FHIT and other TS genes (15).

Table 2 gives the average number of tumors per genotype. The mean number of tumors was significantly higher for the +/− and −/− when compared to the +/+ animals ($P < 0.0001$). Again, no statistical difference was observed between +/− and −/− animals. By comparing the average surface area of the lesion, only +/− and −/− mice developed tumors >4 mm ($P = 0.007$).

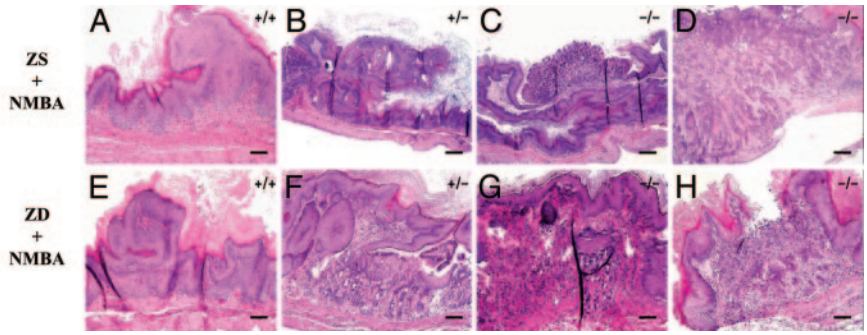

Fig. 4. Forestomach lesions in Tes knockout mice. Hematoxylin and eosin-stained sections are shown from ZS+NMBA-treated (*A–D*) and ZD+NMBA-treated (*E–H*) mice. ZS+NMBA-treated mice: papillomas in +/+ (*A*) and +/− (*B*) mice are shown, as are atypical glandular metaplasia (*C*) and squamous carcinoma (*E*) in −/− mice. ZD+NMBA-treated mice: a papilloma in a +/+ mouse is shown, as are atypical glandular metaplasia and invasive adenocarcinomas in +/− (*F*) and −/− (G) mice and squamous cell carcinoma in a −/− mouse (*H*). (Scale bar, 100 mm.)

In addition, heterozygous and homozygous mice developed an array of very large papillomas, dysplasia, *in situ* carcinomas, and squamous cell and adenocarcinomas. Wild-type mice only exhibited papillomas without progression to malignancy.

Logistic regression models were applied to the data to compare the odds of developing a tumor among the three genotypes while controlling for diet and sex (Table 3). Because almost all of the mice with the +/− and −/− genotypes have developed a tumor, exact methods were used. We estimate that the odds of developing a tumor are significantly greater for both the +/− and −/− genotypes (odds ratio = 38.9 and 27.6, respectively). In addition, the odds of a female developing a tumor are estimated to be 6.6 times higher than for males.

As shown in Table 4, there were no significant differences in tumor incidence between the +/− and −/− genotypes in either dietary group treated with NMBA. However, the incidence and multiplicity of tumors in both dietary groups were significantly higher in mice with +/− and −/− genotypes, as compared to those of the +/+ genotype. When the percentages of ZD+NMBA Tes mice with no tumors (21.7%) and with tumors (78.3%) were compared with ZS+NMBA Tes mice that did (38.2%) or did not develop tumors (61.8%), respectively (Table 5), a borderline statistically significant difference was found ($P = 0.05$). These data suggest that, in Tes NMBA-treated mice, diet differences did not influence tumor incidence and multiplicity.

By contrast, diet differences did affect tumor progression. Specifically, in ZD+NMBA-treated mice, five papillomas (33.3%, Fig. 4*E*) were found in wild-type mice. In heterozygous mice, 18 papillomas (62%, results not shown), three *in situ* carcinomas (10.3%, results not shown), four squamous cell carcinomas (Fig. 4*H*), and one squamous cell carcinoma together with an adenocarcinoma (17.2%, Fig. 4*F*) were found. Homozygous mice developed 10 papillomas (62.5%), one *in situ* squamous cell carcinoma (6.3%), four squamous carcinomas, and one adenocarcinoma (31.3%). ZS+NMBA-treated mice had the following tumor types: two papillomas (15.4%, Fig. 4*A*) in +/+, 15 papillomas (71.4%, results not shown) in +/−, and 10 papillomas (4.7%), two *in situ* squamous carcinoma (9.5%), and two squamous carcinomas in −/− mice (9.5%, Fig. 4*D*). To summarize, all of the lesions developed in wild-type NMBA-treated mice on either ZD or ZS diet were benign papillomas, whereas 30.8% of heterozygous and 37.5% of homozygous mice developed lesions with progression to malignancy. In ZS+NMBA-treated mice, 100% of the lesions present in +/− mice were benign, and 28.6% of the lesions in −/− mice were malignant. Thus, in heterozygous mice the ZD diet caused a third of the tumors to progress to malignancy. This finding is further evidence of TS function of TES/Tes and particularly of TES/Tes in gastric cancer.

Studies of loss of heterozygosity in primary gastric tumors have postulated the existence of a TS gene at 7q 31.1–35 that showed TES characteristics. Kaniyasu *et al.* (16) have demonstrated that deletion at D7S95 was closely associated with peritoneal dissemination of gastric carcinoma. Nishizuka *et al.* (6) found that the deletion of D7S522 (7q31.2–31.3) was correlated to the early stages of well differentiated tumors of the stomach. To explain this apparent contradictory results, Nishizuka *et al.* (17) have hypothesized that a cell adhesion molecule, such as E-cadherin, would be encoded by the putative tumor suppressor gene on 7q.

E-cadherin gene inactivation is associated with disseminated tumor growth (18) and occurs at an early stage (19). Tes is involved in the regulation of cell adhesion, cell motility, and the actin cytoskeleton; it colocalizes with α-catenin. TES/Tes perfectly matches the profile of the sought-after TS gene; it maps to 7q31.2, and is a cell adhesion molecule and a TS gene in induced gastric cancer.

Atypical Glandular Metaplasia. Atypical glandular metaplasia was observed in one ZD+NMBA-treated +/− mouse (Fig. 4*F*) as well as three −/− ZS+NMBA-treated mice (Fig. 4*C*). ZD+NMBA-treated −/− mouse also showed an invasive adenocarcinoma in the forestomach (Fig. 4*G*). These observations are interesting in light of the rising incidence of human carcinomas near the gastroesophageal SCJ, where there occurs a progression from metaplastic epithelia to dysplasia and cancer. By analogy, we speculate that the atypical glandular metaplasia observed in Tes knockout mice is a precancerous lesion that can progress to malignancy, as shown in a Tes −/− mouse on a ZD diet (Fig. 4*G*).

In conclusion, by using genetically engineered mice that harbor a targeted disruption of the mouse Tes locus, we demonstrated that Tes heterozygous and homozygous ZD+NMBA- or ZS+NMBA-treated mice had a higher gastric tumor incidence and multiplicity than their wild-type littermates. Our results provide evidence that TES/Tes functions as a TS gene *in vivo*.

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1. Pedersen, B. & Ellegaard, J. (1994) *Cancer Genet Cytogenet.* **78,** 181–188.
2. Bieche, I., Khodja, A., Driouch, K. & Lidereau, R. (1997) *Clin. Cancer Res.* **3,** 1009–1016.
3. Edelson, M. I., Scherer, S. W., Tsui, L. C., Welch, W. R., Bell, D. A., Berkowitz, R. S. & Mok, S. C. (1997) *Oncogene* **14,** 2979–2984.
4. Latil, A., Cussenot, O., Fournier, G., Baron, J. C. & Lidereau, R. (1995) *Clin. Cancer Res.* **1,** 1385–1389.
5. Zenklusen, J. C., Thompson, J. C., Klein-Szanto, A. J. & Conti, C. J. (1995) *Cancer Res.* **55,** 1347–1350.
6. Nishizuka, S., Tamura, G., Terashima, M. & Satodate, R. (1997) *Br. J. Cancer* **76,** 1567–1571.
7. Shridhar, V., Sun, Q. C., Miller, O. J., Kalemkerian, G. P., Petros, J. & Smith, D. I. (1997) *Oncogene* **15,** 2727–2733.
8. Matsuura, K., Shiga, K., Yokoyama, J., Saijo, S., Miyagi, T. & Takasaka, T. (1998) *Anticancer Res.* **18,** 453–458.
9. Tatarelli, C., Linnenbach, A., Mimori, K. & Croce, C. M. (2000) *Genomics* **68,** 1–12.
10. Griffith, E., Coutts, A. S. & Black, D. M. (2004) *Cell Motil. Cytoskeleton* **57,** 133–142.
11. Griffith, E., Coutts, A. S. & Black, D. M. (2005) *Cell Motil. Cytoskeleton* **60,** 140–152.
12. Tobias, E. S., Hurlstone, A. F., MacKenzie, E., McFarlane, R. & Black, D. M. (2001) *Oncogene* **20,** 2844–2853.
13. Sarti, M., Sevignani, C., Calin, G. A., Aqeilan, R., Shimizu, M., Pentimalli, F., Picchio, M. C., Godwin, A., Rosenberg, A., Drusco, A., *et al.* (2005) *Clin. Cancer Res.* **11,** 806–813.
14. Fong, L. Y., Ishii, H., Nguyen, V. T., Vecchione, A., Farber, J. L., Croce, C. M. & Huebner, K. (2003) *Cancer Res.* **63,** 186–195.
15. Zanesi, N., Fidanza, V., Fong, L. Y., Mancini, R., Druck, T., Valtieri, M., Rudiger, T., McCue, P. A., Croce, C. M. & Huebner, K. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 10250–10255.
16. Kuniyasu, H., Yasui, W., Yokozaki, H., Akagi, M., Akama, Y., Kitahara, K., Fujii, K. & Tahara, E. (1994) *Int. J. Cancer* **59,** 597–600.
17. Nishizuka, S., Tamura, G., Terashima, M. & Satodate, R. (1998) *J. Pathol.* **185,** 38–43.
18. Tamura, G., Sakata, K., Nishizuka, S., Maesawa, C., Suzuki, Y., Iwaya, T., Terashima, M., Saito, K. & Satodate, R. (1996) *Jpn. J. Cancer Res.* **87,** 1153–1159.
19. Muta, H., Noguchi, M., Kanai, Y., Ochiai, A., Nawata, H. & Hirohashi, S. (1996) *Jpn. J. Cancer Res.* **87,** 843–848.