Thrombospondin-1 Gene Expression Affects Survival and Tumor Spectrum of p53-Deficient Mice

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***In vitro* and *in vivo* data indicate that thrombospondin-1 (TSP1) inhibits tumor progression in several ways including direct effects on cellular growth and apoptosis in the stromal compartment. To evaluate the importance of TSP1 for the progression of naturally arising tumors *in vivo*, we have crossed TSP1-deficient mice with p53-deficient mice. In p53-null mice, the absence of TSP1 decreases survival from 160 ± 52 days to 149 ± 42 days. A log-rank test comparing survival curves for these two populations yields a two-sided P value of 0.0272. For mice that are heterozygous for the p53-null allele, survival is 500 ± 103 days in the presence of TSP1 expression, and 426 ± 125 days in its absence (P = 0.0058). Whereas TSP1 expression did not cause a measurable change in the incidence of the majority of tumor types, a statistically significant (P ≤ 0.05) decrease in the incidence of osteosarcomas is observed in the absence of TSP1. To determine more directly if host TSP1 inhibits tumor growth, B16F10 melanoma and F9 testicular teratocarcinoma cells have been implanted in C57BL/6J and 129Sv TSP1-null mice, respectively. The B16F10 tumors grow approximately twice as fast in the TSP1-null background and exhibit an increase in vascular density, a decrease in the rate of tumor cell apoptosis, and an increase in the rate of tumor cell proliferation. Increased tumor growth is also observed in the absence of TSP1 on the 129Sv genetic background. These data indicate that endogenous host TSP1 functions as a modifier or landscaper gene to suppress tumor growth. (Am J Pathol 2001, 159:1949–1956)**

The p53 tumor suppressor gene product is a transcription factor that induces growth arrest and apoptosis.¹ Mutations in the p53 gene are common in human cancers. In the mouse, the complete absence of p53 expression results in a dramatic decrease in survival, with all of the mice succumbing to various types of cancer within 9 months.^{2–4} Lymphomas are observed with the highest frequency in these mice; however, tumors from a wide range of cell lineages are observed. Mice that are heterozygous for p53 have an increased life span but eventually succumb to a more evenly distributed range of lymphomas, sarcomas, and carcinomas. Thus, mice that are deficient in p53 gene expression are a valuable model for assaying the effects of other gene mutations on the progression of naturally occurring tumors.

The loss of p53 function in fibroblasts from patients with Li-Fraumeni Syndrome has been shown to correlate with a reduction in thrombospondin-1 (TSP1) protein expression and a switch in the angiogenic phenotype from inhibitory to stimulatory.^{5,6} Moreover, p53 can also regulate TSP1 and angiogenesis in cultured fibroblasts and in some human breast tumors.⁷ However, in other tissues, such as brain, skin, and bladder, p53 does not seem to regulate TSP1 expression.^{8–10}

The thrombospondins are a family of extracellular calcium-binding proteins.^{11–13} Of the five family members, TSP1 is the most extensively characterized because it is readily purified from blood platelets. Through its interaction with proteoglycans, other matrix proteins, growth factors, and membrane receptors, TSP1 directs the assembly of multiprotein complexes that modulate cellular phenotype. TSP1 also directly activates transforming growth factor-β (TGF-β) and can affect the activity of various extracellular proteases including plasmin, elastase, and cathepsin.^{14–18} Biological processes frequently involve a balance of stimulatory and inhibitory factors.^{19–21} The multiprotein complexes that are formed on the cell membrane in response to TSP1 modulate cellular phenotype by shifting these balances. The resulting changes in extracellular protease or angiogenic ac-

Supported by grant HL28749 from the National Heart, Lung, and Blood Institute of the National Institutes of Health; and grants CA64239 and CA17007 from the National Cancer Institute. R. O. Hynes is a Howard Hughes Medical Institute Investigator.

Accepted for publication August 17, 2001.

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tivity are important at sites of tissue remodeling during wound healing and tumor progression.

TSP1 supports attachment and migration of various carcinoma and melanoma cell lines.^{22–24} In addition, TSP1 inhibits proliferation of endothelial cells *in vitro* and inhibits angiogenesis *in vivo*.²⁵ These effects are mediated by various receptors on the cell surface, including proteoglycans, integrins, integrin-associated protein (IAP), CD36, and several receptors that remain to be fully characterized.¹¹ A 50,000-d protein that is expressed in tumor tissue is included in the latter group.²⁶ This receptor, CD36, and some proteoglycans appear to bind to the type 1 repeats of TSP1. Fusion proteins or peptides that contain type 1 repeat sequences inhibit angiogenesis, inhibit proliferation of melanoma cells, and inhibit tumor growth.^{23,27–29} In addition, a peptide from the procollagen homology region has been shown to inhibit angiogenesis.²⁷ Inhibition of angiogenesis by TSP1 is mediated by CD36 on endothelial cells.^{30,31} Recent data indicate that CD36 associates with integrins and tetraspanins on the platelet membrane.³²

TSP1 is expressed in normal breast, colon, and bladder epithelium.^{10,33–35} In general, TSP1 expression is significantly reduced in cancer cells that arise in these tissues. However, lobular carcinomas in the breast express significantly higher levels of TSP1 and TSP2 than normal tissue.^{10,33–35} TSP1 expression shows an inverse correlation with vascular density in bladder and colon cancer but does not correlate with vascular density in some other tissues such as ductal breast carcinoma.^{10,33–35} Whereas TSP1 may be down-regulated in tumor cells, relatively high levels of TSP1 and TSP2 mRNA and protein are associated with stromal fibroblasts.^{33,36} In addition, activated monocytes and macrophages contribute TSP1 to the tumor environment as do endothelial cells.³⁷ These data raise the possibility that TSP1 produced by stromal cells may function to inhibit tumor growth. If this is the case, then the *Thbs1* gene would represent a landscaper for tumor progression.³⁸ Landscaper genes are expressed by cells within the immediate environment of the transformed tumor cells and act to modify their ability to form tumors. Whereas the genetic modifications that occur in tumor cells have been studied extensively, the effect of stromal cell gene expression has been primarily overlooked.³⁹

Systemic treatment of tumor-bearing mice with TSP1 or peptides that are derived from the type 1 repeats inhibits experimental tumor growth.^{28,29,40} Moreover, increased expression of TSP1 in v-Src-transformed NIH 3T3 cells, transformed endothelial cells, human breast adenocarcinoma MDA-MB-435, or human skin carcinoma cells reduces the size and vascular density of the tumors that are produced when these cells are implanted in mice.^{41–45}

All of the tumor studies that have been performed to date have investigated the effects of TSP1 on tumors arising from the transplantation of fully transformed cells. To establish a role for TSP1 in the progression of spontaneously occurring tumors arising at orthotopic sites *in vivo*, we have crossed mice that are deficient in TSP1 with mice that are deficient in p53. Mice that lack TSP1 and are either homozygous or heterozygous for the p53-null

allele have a decrease in survival when compared with mice that express wild-type levels of TSP1. Moreover, a decreased incidence of osteosarcoma is observed in p53-deficient mice in the absence of TSP1. We also show that B16F10 and F9 experimental tumors grow more rapidly in *Thbs1*-null mice. Increased B16F10 tumor cell proliferation and vascular density are observed in the absence of TSP1 whereas the apoptotic index is decreased. These data establish the TSP1 gene as a modifier or landscaper gene for tumor progression.

Materials and Methods

Production of Mouse Strains

TSP1-deficient mice were crossed with p53-deficient mice that were kindly provided by Dr. Tyler Jacks (Massachusetts Institute of Technology).^{3,46} All of the mice in the experiment were derived from two p53-null males and four TSP1-null females. The two p53-null males were littermates from a heterozygous cross. The four TSP1-null females were offspring of a single TSP1-null cross. Both strains of mice were on a mixed C57BL/6J and 129Sv background. Double-heterozygous offspring were used to produce mice that were deficient in both gene products. Double-homozygous males were crossed to females that were homozygous for the *Thbs1*-null allele and heterozygous for the p53-null allele. The progeny of these crosses were homozygous for the *Thbs1*-null allele and homozygous or heterozygous for the p53-null allele. Both populations were followed for survival and tumor spectrum. Control groups that were homozygous for the wild-type *Thbs1* allele and homozygous or heterozygous for the p53-null allele were established in the same way. All genotyping was done using the polymerase chain reaction (PCR) with DNA prepared from portions of the tail as described by Laird and co-workers.⁴⁷ Genotyping for *Thbs1* and p53 was done by PCR as described previously.^{3,46}

Tumor Analysis

A complete necropsy was performed on mice that were found shortly after death or that were sacrificed because they had a tumor burden that was >10% of their body weight, they were moribund, or they displayed poor body condition. A ventral incision was used to open the abdominal and thoracic cavities and the intestines were extended. The top of the skull was removed to facilitate fixation of the brain. The open carcasses were placed in 10% formalin [3.7% formaldehyde in phosphate-buffered saline (PBS)] for fixation and storage until processing for histology. A portion of each organ was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Survival data were analyzed using the log-rank test for two-way comparisons (*Thbs1*+/-, p53-/- versus *Thbs1*-/-, p53-/-, and *Thbs1*+/-, p53+/- versus *Thbs1*-/-, p53+/- and two-sided *P* values are reported.⁴⁸ The incidence of the various tumor types was compared among the four genotypes using the 4 by 2 Pearson's chi-square analysis.⁴⁹ For determination of the *P*

Table 1. Distribution of Mice Studied for Survival and Tumor Spectrum

Genotype	Number of animals followed for survival	Mean age at death (days) $\pm$ SD	Number of animals analyzed by necropsy	Animals with metastasis*
TSP ^{+/+} , p53 ^{-/-}	66	160 $\pm$ 52	30 (46%)	14.3%
TSP ^{-/-} , p53 ^{-/-}	92	149 $\pm$ 42	36 (39%)	6.5%
TSP ^{+/+} , p53 ^{+/+}	43	500 $\pm$ 103	34 (79%)	12.5%
TSP ^{-/-} , p53 ^{+/+}	93	426 $\pm$ 125	64 (69%)	12.3%

*Given as a percentage of mice in which tumors were found.

value, two-way comparisons were used as above and *P* values were not adjusted for multiple comparisons.

Loss of Heterozygosity (LOH)

The analysis for LOH of the *p53* allele was performed on paraffin-embedded 10- μ m sections essentially as described by Bianchi and co-workers.⁵⁰ A probe for mouse *p53* was prepared using PCR with the forward primer 5'-ACA GCG TGG TGG TAC CTT AT-3' and the reverse primer 5'-TAT ACT CAG AGC CGG CCT-3'.³ The PCR product was subcloned into pBluescript KS (Invitrogen, Carlsbad, CA) and used as a probe for Southern blotting of the *p53* PCR products.⁵¹

Experimental Tumor Growth

The B16F10 murine melanoma cell line was obtained from the ATCC (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 μ g/ml penicillin, 50 U/ml streptomycin, and 2 mmol/L glutamine. The F9 murine testicular teratocarcinoma cell line was obtained from Dr. Lorraine Gudas (Cornell University Medical College) and cultured in 0.3% gelatin-coated dishes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 mg/ml penicillin, and 50 U/ml streptomycin. Five- to 8-week-old C57BL/6J male control mice (Taconic Farms, Germantown, NY) were acclimated, caged in groups of four or less, and their backs were shaved. The *Thbs1*-null allele was bred to the C57BL/6J background by eight backcrosses to C57BL/6J mice beginning with the chimeric males. Cultured B16F10 or F9 cells (2.5×10^5) were inoculated subcutaneously on the backs of C57BL/6J or 129Sv mice, respectively. Tumors were measured with a dial-caliper and the volumes were determined using the formula width² $\times$ length $\times$ 0.52. After 16 days, the mice were sacrificed and the tumors were cut and fixed in neutral-buffered formalin. For immunohistochemistry, paraffin-embedded tissue sections were deparaffinized and rehydrated as described previously.⁵² Sections were treated with 0.25% trypsin in PBS for 30 minutes at 22°C. Monoclonal anti-proliferating cell nuclear antigen (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-CD31 (PharMingen, La Jolla, CA) antibodies were used with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) to detect proliferating tumor cells or capillaries, respectively. For terminal dUTP nick-end labeling staining, the sections were treated with 20 μ g/ml of proteinase K for 15 minutes at 22°C. The slides were washed twice with PBS

and incubated in terminal deoxynucleotide transference (TdT) buffer (Life Technologies, Inc., Grand Island, NY) for 10 minutes at 22°C. The buffer was removed and 300 U/ml of TdT (Life Technologies, Inc.) and 2 nmol/L biotin-16-2'-deoxyuridine-5'-triphosphate (Boehringer Mannheim, Indianapolis, IN) was added for 30 minutes at 37°C. The slides were washed three times and developed with the Vectastain ABC kit (Vector Laboratories) according to protocols provided by the manufacturer.

Results

Survival

The survival of four genotypes was followed. The control populations had wild-type levels of TSP1 and were either heterozygous or homozygous for the *p53*-null allele. These groups have been extensively analyzed in several previous studies.^{2,3,53} Mice that inherited one or two copies of the *p53*-null allele had an average survival of 500 $\pm$ 103 days and 160 $\pm$ 52 days, respectively (Table 1). These values agree well with those previously reported by others.^{2,3,53} The experimental populations were homozygous for the *Thbs1*-null allele and were either heterozygous or homozygous for the *p53*-null allele. The mice that were homozygous for the *p53*-null allele lived an average of 149 $\pm$ 42 days in the absence of the *Thbs1* gene and 160 $\pm$ 52 days in its presence. A log-rank test comparing survival curves for these two populations yields a two-sided *P* value of 0.0272 (Figure 1). In populations that were heterozygous for the *p53*-null allele, a statistically significant difference in survival in the presence or absence of *Thbs1* gene expression was established (*P* = 0.0058) (Figure 1). The mice that were heterozygous for the *p53*-null allele lived an average of 500 $\pm$ 103 days (*n* = 43) in the presence of the *Thbs1* gene and 426 $\pm$ 123 days (*n* = 93) in its absence. A comparable decrease in survival was observed in the mice with lymphomas, sarcomas, or carcinomas, suggesting that the absence of TSP1 resulted in decreased survival of mice affected by various types of cancer.

Tumor Spectrum

Among the mice that were homozygous for the *p53*-null allele, lymphomas comprised approximately half of the tumors observed (Table 2). This was consistent with previous studies using this model in which 50 to 71% of the tumors were lymphomas.^{2,3,53} *Thbs1* gene expression

Figure 1. Survival of mice with various genotypes. The genotypes are identified as TTPp (*Thbs1*^{+/+}, *p53*^{-/-}), ttp (*Thbs1*^{-/-}, *p53*^{-/-}), TTPp (*Thbs1*^{+/+}, *p53*^{+/-}), and ttp (*Thbs1*^{-/-}, *p53*^{+/-}). The number of mice with each genotype that were followed for survival and the mean survival is given in Table 1.

did not affect the incidence of the majority of tumor types. A statistically significant ($P < 0.05$) decrease in the incidence of osteosarcomas was observed in the absence of the *Thbs1* gene in groups that were either heterozygous or homozygous for the *p53*-null allele. In addition, a statistically significant ($P < 0.05$) decrease in the incidence of hemangiosarcomas was observed in the group that was heterozygous for *p53* gene expression. Fewer sick mice were identified in time for necropsy in the *p53*-null population, as compared to the *p53* heterozygous group, presumably because the disease progressed more rapidly (Table 1). For both *p53* genotypes, fewer mice were found in time for necropsy in the absence of *Thbs1* gene expression (Table 1). These data suggest that the *Thbs1*-null mice had more severe, widespread disease that progressed more rapidly. Consistent with other studies that have used *p53*-null mice and have been able to identify the tumors in 71 to 96% of the mice, we were able to find tumors in 86 to 94% of our various populations.²⁻⁴

LOH

In some of the tumors that occur in *p53* heterozygous mice, the single copy of the wild-type allele is deleted resulting in LOH.^{3,53,54} Venkatachalam and co-workers⁵⁴ found that the wild-type *p53* gene remains functional in those tumors that have not undergone LOH. Similarly, a functional wild-type allele is observed in some human tumors in which one *p53* allele has been mutated.⁵⁵⁻⁵⁸ Thus, a reduction in *p53* gene dosage may be sufficient for tumor progression or wild-type *p53* activity may have been inactivated by another mechanism. PCR followed by Southern blotting revealed that 58% of 19 tumors that occurred in mice that were heterozygous for *p53* and lacked *Thbs1* gene expression were homozygous for the *p53*-null allele (Figure 2). This analysis was performed on a representative group of tumor types including four lymphomas (LOH in two of four), four fibrosarcomas (LOH in one of four), four osteosarcomas (LOH in three of four), five adenocarcinomas (LOH in three of five), and two

Figure 2. Analysis of LOH. DNA from tumors from nine mice that were heterozygous for the *p53*-null allele was amplified with primers for the *p53* wild-type (+) and null (-) alleles (lanes 1 to 9). The PCR products were Southern blotted with the wild-type PCR product. Lanes 1, 3, 4, 6, 7, and 8 were identified as having lost heterozygosity based on the absence or underrepresentation (lane 4) of the *p53* wild-type allele. Lane 10 shows the expression of the *p53* wild-type and null alleles in tail DNA from a *p53* heterozygous mouse.

squamous cell carcinomas (LOH in two of two). By comparison, significantly fewer (29%, $P \leq 0.005$) tumors in mice that were heterozygous for *p53* and wild type for *Thbs1* displayed LOH. This analysis was performed on four lymphomas (LOH in two of four), six osteosarcomas (LOH in one of six), three adenocarcinomas (LOH in none of three), and one undifferentiated sarcoma (LOH in none of one).

Growth of Experimental Tumors

To evaluate the effect of host TSP1 on tumor growth directly, the B16F10 melanoma and F9 testicular teratocarcinoma subcutaneous models were used in *Thbs1*-null and wild-type mice. Each tumor cell line was grown in the genetic background from which they were derived. The B16F10 melanomas were grown in C57BL/6J mice and the F9 testicular teratocarcinomas were grown in 129Sv. The rate of B16F10 tumor growth is twofold higher in the absence of *Thbs1* gene expression (Figure 3). The data shown in Figure 3 was obtained with eight mice in each group. A second experiment with four mice in each group gave comparable results (data not shown). Tumors in the *Thbs1*-null mice display a 51% increase in blood vessel density (Figure 4A). In addition, the lack of *Thbs1* gene expression results in a 37% decrease in the tumor cell apoptotic index and a 30% increase in tumor cell proliferative index (Figure 4, B and C). Whereas tumors were detectable in all of the mice injected with B16F10 cells, F9 tumors were observed in 4 of 11 wild-type mice after 14 days. By comparison, F9 tumors were observed in six of seven *Thbs1*-null mice after 14 days. The tumors in the *Thbs1*-null mice were 5.5 times larger than those grown in wild-type mice ($0.05 \leq P \leq 0.1$, data not shown).

Discussion

In *p53*-deficient mice, tumors can occur spontaneously and progress naturally in various tissues. The data presented here indicate that *Thbs1* gene expression significantly increases the survival of mice that are either homozygous or heterozygous for *p53* gene expression. The absence of the *Thbs1* gene decreases the life span of *p53* heterozygotes by 74 days, or ~15% of the life span of the *p53* heterozygotes that are wild type for *Thbs1*.

Figure 3. Growth of B16F10 tumors in TSP1-deficient and wild-type mice. Tumor cells (2.5×10^5) were inoculated subcutaneously on the backs of C57BL/6J wild-type (filled circles) or *Thbs1*-null mice (open squares). Tumor volume was determined with a caliper and calculated by the formula $0.52 \times \text{width}^2 \times \text{length}$.

Disruption of the *Thbs1* gene results in an increase in tumor LOH and an increase in tumor growth rate. We have shown that experimental B16F10 and F9 tumors do grow more rapidly in TSP1-deficient mice. In these experiments, we have not manipulated the level of TSP1 expression in the tumor cells. Thus, the difference in the rate of tumor growth is entirely because of TSP1 expression in the host stromal cells. Extensive TSP1 expression in tumor stroma has been documented.^{33,36} Our data are the first to show that TSP1 expression by stromal cells serves to make the tumor microenvironment less permissive for growth. However, because the mice used in this study were produced by crossing two distinct strains, we cannot preclude the possibility that genes that are tightly linked to the *Thbs1* or *p53* genes contribute to the observed effects. Tumor formation and growth is also in-

creased in TSP2-null mice.⁵⁹ Stromal TSP2 expression is markedly increased in chemically induced skin papillomas and carcinomas in wild-type mice. In TSP2-null mice, the incidence and growth rate of papillomas is increased. The tumors that form in the TSP1-null and TSP2-null mice are similar in that they display increased angiogenesis and decreased tumor cell apoptosis.

The difference in survival for the *p53*-null mice in the presence or absence of the *Thbs1* gene is considerably smaller than for mice that are *p53* heterozygotes. A correlation between the expression of p53 and TSP1 has been documented in fibroblasts from individuals with Li-Fraumeni syndrome.⁵ Mutations in *p53* also correlate with decreased TSP1 expression in melanoma, colon, and bladder cancer.^{34,35,60} Thus, the *p53*-null mice may have decreased TSP1 protein in some tissue even when both wild-type *Thbs1* alleles are present. Thus, the effect of TSP1 may not be fully appreciated on the *p53*-null background.

TSP1 may modulate tumor progression through direct effects on tumor cell growth and apoptosis, through effects on tumor cell growth via TGF- β , through inhibition of angiogenesis, or through mechanisms that remain to be determined. Neovascularization is an essential part of tumor growth.⁶¹ Control of tumor growth by inhibition of angiogenesis can be associated with an increase in the rate of tumor cell apoptosis rather than in a decrease in proliferative rate.⁶² Whereas it is difficult to compare tumors in the *p53* model because they are heterogeneous in tissue of origin and stage at the time that the mice die, the B16F10 model results in a more homogeneous population of tumors. In this study, an increase in vascular density and a decrease in tumor cell apoptosis was observed in tumors growing in *Thbs1*-null mice. In addition, we observed an increase in tumor cell proliferation. Thus, tumors may progress more rapidly in TSP1-deficient mice in part because there is increased angiogenesis. The

Figure 4. Histological analysis of B16F10 tumors in wild-type (filled bar) and TSP1-null (hatched bar) mice. Vessel counts (A) were determined on CD31-stained sections, apoptotic index (B) was determined on terminal dUTP nick-end labeling-stained sections and proliferative index (C) was determined on proliferating cell nuclear antigen-stained sections. The asterisk indicates statistically significant differences ($P < 0.005$).

Table 2. Tumor Distribution

	Genotype			
	<i>Thbs1</i> ^{+/+} <i>p53</i> ^{-/-}	<i>Thbs</i> ^{-/-} <i>p53</i> ^{-/-}	<i>Thbs1</i> ^{+/+} <i>p53</i> ^{+/-}	<i>Thbs1</i> ^{-/-} <i>p53</i> ^{+/-}
Number of mice	30	36	34	64
Number of tumors	42	39	48	90
Lymphoma*	70%	53%	32%	44%
Sarcoma	50%	39%	56%	44%
Fibrosarcoma	13%	8.3%	0%	13%
Hemangiosarcoma	20%	17%	8.8%	0%
Histocytic sarcoma	0%	5.6%	15%	17%
Liposarcoma	0%	2.8%	0%	0%
Osteosarcoma	13%	0%	29%	13%
Undifferentiated sarcoma	3.3%	5.6%	2.9%	1.6%
Carcinoma	3.3%	8.3%	35%	33%
Adenocarcinoma	0%	5.6%	18%	19%
Lung	0%	0%	12%	9.4%
Mammary	0%	0%	2.9%	4.7%
Pancreas	0%	2.8%	2.9%	1.6%
Small intestine	0%	2.8%	0%	0%
Hepatocarcinoma	0%	0%	0%	1.6%
Islet cell	0%	0%	0%	1.6%
Squamous cell carcinoma	3.3%	2.8%	18%	14%
Ear	0%	2.8%	18%	14%
Stomach	3.3%	0%	0%	0%
Malignant ependymoma	0%	0%	2.9%	1.6%
Benign tumors	17%	8.3%	15%	19%
Adenoma	0%	0%	5.9%	4.7%
Hair matrix tumor	0%	0%	2.9%	0%
Hemangioma	3.3%	0%	0%	0%
Fibrolipoma	0%	2.8%	0%	0%
Polyps	13%	5.6%	5.9%	13%
Stomach	3.3%	0%	2.9%	13%
Small intestines	0%	0%	2.9%	0%
Colon	6.7%	0%	0%	0%
Gut-ileoceleal	0%	2.8%	0%	0%
Rectum	0%	2.8%	0%	0%
Stomach papilloma	0%	0%	0%	1.6%

*The incidence of each tumor type observed is expressed as a percentage of the total number of mice necropsied.

changes in tumor cell apoptotic and proliferative indices may be because of decreased nutrients that would be associated with decreased vessel density or may be because of direct effects on the tumor cells. An increase in proliferative rate and a decrease in apoptotic index may contribute to genetic instability and an increase in LOH. The shortened life span of the *Thbs1*-null animals may be attributed to an increased ease of developing an angiogenic phenotype in the absence of this protein that is a potent natural inhibitor of angiogenesis. Whereas the pneumonia that is observed in *Thbs1*-null mice is patchy and does not result in a decreased life span, the possibility that decreased lung function may hasten the death of mice with a high tumor burden cannot be rigorously ruled out (J Lawler and RO Hynes, unpublished data).⁴⁶

Some of the effect of *Thbs1* gene expression on tumor progression may result from its ability to activate TGF- β .^{14,15} TGF- β , Rag 2 double-null mice display an increased frequency of adenoma and carcinoma of the cecum and colon.⁶³ Mice that are heterozygous for a TGF- β -null allele express levels of TGF- β protein that are 10 to 30% of normal levels.⁶⁴ These mice display enhanced tumor formation in response to chemical carcinogens. TSP1 is a major activator of TGF- β in many tissues and may influence the development of tumors indirectly via this inhibitory cytokine.¹⁵ In *Thbs1*-null mice that are

heterozygous for *p53*, we do observe a modest increase in benign tumors of the stomach, a tissue where TSP1 activation of TGF- β has been documented.

Contrary to this general trend of TSP1 as protective, there was one specific tumor type whose frequency was consistently and significantly decreased when TSP1 was absent, namely, osteosarcoma. In human osteosarcomas, high levels of TGF- β are associated with more severe disease and TSP1 may be stimulatory because of its ability to activate TGF- β .^{65,66} Although generally known as a negative regulator of cell growth, TGF- β can directly stimulate the growth of some tumor cells including those from colon,⁶⁷ melanoma,⁶⁸ and prostate,⁶⁹ and may be acting in this way on osteosarcomas. Alternatively, in osteosarcomas, TGF- β may play a key role in tumor angiogenesis, as it has recently been shown to do in renal cell carcinoma.⁷⁰

Metastases are observed in a small number of mice (two to seven in each of the four genotypes) in this study. In the absence of *p53* gene expression, metastases were observed in a greater percentage of the mice with normal levels of TSP1 (4 of 28, 14.3%) as compared to the mice that lacked TSP1 protein (2 of 31, 6.5%). Whereas the sample size is not large enough to permit a definitive explanation, it seems that this difference results from the decreased incidence of a tumor type, osteosarcomas,

that metastasized relatively frequently (7 of 22, 32%). Whereas a similar decrease in the incidence of osteosarcomas is also observed in *Thbs1*-null mice that are heterozygous for *p53* gene expression, the number of mice with metastases is not reduced (Table 1). This seems to be because of the fact that the decrease in metastatic osteosarcomas is offset by an increase in the number of carcinomas that metastasized in the absence of *Thbs1* gene expression (four of seven, 57%) as compared to mice with normal levels of TSP1 (one of four, 25%). We are currently exploring the role of *Thbs1* gene expression in genetic models of tumor progression that metastasize with higher frequency.

The data presented here indicate that TSP1 is a natural inhibitor of the growth of multiple types of spontaneously occurring tumors. As such, *Thbs1* is a modifier gene for tumor progression. In most cases *Thbs1* probably acts as a landscaper gene whose loss releases constraints on stromal endothelial cells, thereby making tumor development easier.³⁸ The data presented here are consistent with the proposal of Hanahan and Weinberg³⁹ that gene mutations in the stromal cells play a key role in tumor progression. Therapeutics or gene therapy approaches that are designed to up-regulate TSP1 expression may function to suppress tumor growth.

Acknowledgments

We thank Dr. Tyler Jacks for kindly providing the *p53*-null mice, Dr. Susan Crawford for help in obtaining some of the pathology data, Drs. Joan S. Chmiel and Ruth Lipman for assistance with the statistical analysis, Denise Crowley and the Dana-Farber/Harvard Cancer Center Rodent Histopathology Core for providing assistance with the histology, Ms. Tong Zi for providing valuable technical support, Dr. Lorraine Gudas for providing the F9 testicular teratocarcinoma cells, and Regina Prout and Alexis Bywater for preparing the manuscript.

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