MOLECULAR AND CELLULAR BIOLOGY, Oct. 2002, p. 7291–7301 Vol. 22, No. 20
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.20.7291–7301.2002

Cooperation between p53 Mutation and High Telomerase Transgenic Expression in Spontaneous Cancer Development

Eva González-Suárez,[1] Juana M. Flores,[2] and María A. Blasco[1]*

Department of Immunology and Oncology, National Center for Biotechnology, E-28049 Madrid,[1] and Department of Animal Pathology II, Facultad de Veterinaria, Universidad Complutense de Madrid, E-28040 Madrid,[2] Spain

Received 12 April 2002/Returned for modification 22 May 2002/Accepted 11 July 2002

Telomerase reintroduction in adult somatic tissues is envisioned as a way to extend their proliferative capacity. It is still a question, however, whether constitutive telomerase expression in adult tissues impacts the normal aging and spontaneous cancer incidence of an organism. Here, we studied the aging and spontaneous cancer incidence of mice with transgenic telomerase expression in a wide range of adult tissues, K5-Tert mice. For this, we maintained large colonies of K5-Tert mice for more than 2 years. K5-Tert mice showed a decreased life span compared to wild-type cohorts associated with a higher incidence of preneoplastic and neoplastic lesions in various tissue types. Neoplasias in K5-Tert mice were coincident with transgene expression in the affected tissues. These observations suggest that high telomerase activity may cooperate with genetic alterations that occur with age to promote tumorigenesis. Indeed, we demonstrate here that increased cancer incidence and the reduced viability of K5-Tert mice are aggravated in a $p53^{+/-}$ genetic background, indicating that telomerase cooperates with loss of p53 function in inducing tumorigenesis. Altogether, these results demonstrate that constitutive high levels of telomerase activity result in a decreased life span associated with an increased incidence of neoplasias as the organism ages.

Telomeres are large nucleoprotein complexes that cap the ends of eukaryotic chromosomes, ensuring genome stability and cell viability (1). Telomere dysfunction, due to loss of either telomeric DNA (TTAGGG repeats in vertebrates) or telomere-binding proteins, has been demonstrated to trigger end-to-end chromosomal fusions and age-related pathologies in mice (10). The telomerase enzyme is able to compensate for telomere loss as a consequence of DNA replication (the end replication problem), preventing telomere shortening to a critical length (6). Telomerase is composed of two essential components, an RNA molecule (Terc, telomerase RNA component) and a catalytic subunit (Tert, telomerase reverse transcriptase) (22).

Adult somatic tissues generally lack or have low levels of telomerase activity, and telomeres shorten with increasing age (13). Telomerase reintroduction in adult somatic tissues is envisioned as a putative gene therapy for those age-related diseases that are provoked by telomere exhaustion (4). In particular, telomerase has been proposed as a potential therapy for human premature aging syndromes characterized by a faster rate of telomere shortening, such as the Werner syndrome and dyskeratosis congenita, among others (19, 26, 29). This is supported by the fact that telomerase reintroduction through the germ line is able to elongate critically short telomeres and prevent chromosomal aberrations in the context of the telomerase-deficient mouse model (3, 15, 18, 26). Furthermore, the rescue of short telomeres by telomerase also prevents the appearance of aging phenotypes in these mice (26).

The enthusiasm for using telomerase in gene therapy was also supported by previous reports showing that telomerase is sufficient to immortalize some primary cells in culture without inducing changes associated with transformation (17, 20). Constitutive telomerase expression in cells cultured for prolonged times, however, can result in undesired epigenetic changes such as amplification of the c-*myc* oncogene (28). In addition, telomerase activity together with mutant Ras and T antigen expression seems to be sufficient to transform primary human cultured cells (12), suggesting that telomerase could have an active role in tumorigenesis. Indeed, high levels of telomerase activity are one of the hallmarks of human cancer (27), reflecting the fact that telomere maintenance by telomerase is necessary for indefinite cell growth.

For all of the above, transgenic mouse models with constitutive telomerase expression in adult tissues are necessary to evaluate the potential risks of a telomerase-based therapy in the context of the organism, as well as to understand the role of telomerase during tumorigenesis. In this regard, two recent reports described mice with transgenic telomerase expression in different target tissues, such as the stratified epithelia (9) and the heart (23). In both cases, forced telomerase expression resulted in a higher proliferation rate of the target tissues upon mitogenic stimulus, although no spontaneous tumors were observed in adult mice. These results supported the notion that telomerase is not an oncogene. It is important to note, however, that the transgenic mice expressing telomerase in the stratified epithelia, K5-Tert, showed a higher incidence of tumors upon chemical carcinogenesis of the skin, suggesting that telomerase upregulation could cooperate with other genetic alterations to promote tumorigenesis.

To address whether K5-Tert telomerase transgenics have a normal aging pattern, we generated large colonies of two independent K5-Tert lines and of the corresponding wild-type littermates and determined their life spans and their sponta-

* Corresponding author. Mailing address: Department of Immunology and Oncology, National Center for Biotechnology, E-28049 Madrid, Spain. Phone: 34 915854846. Fax: 34 913720493. E-mail: mblasco@cnb.uam.es.

neous tumor incidence. In addition, we generated K5-Tert transgenics in both $p53^{-/-}$ and $p53^{+/-}$ genetic backgrounds to directly address whether telomerase upregulation cooperates with p53 mutation in accelerating tumorigenesis. Altogether, the results presented here indicate that constitutive telomerase expression in adult tissues has a negative impact on the normal life span of the mice, which is coincident with an increased incidence of spontaneous tumors as they age. These deleterious effects of telomerase overexpression are aggravated in a $p53^{+/-}$ genetic background, indicating that telomerase upregulation cooperates with p53 loss in inducing tumors as the mice age. Importantly, since both wild-type and K5-Tert mice have long telomeres (9), the results presented here suggest a role of telomerase in promoting tumorigenesis that is independent of its role in net telomere elongation.

MATERIALS AND METHODS

Generation of transgenic K5-Tert mice in wild-type and mutant p53 genetic backgrounds. K5-Tert and $p53^{-/-}$ mice have been described elsewhere (9, 16). For the current study, several K5-Tert lines (T1, T3, T4, T5, and T8, described by González-Suárez et al. [9]) were aged at the National Center for Biotechnology animal facility (see below). In addition, large colonies of mice of the following genotypes were obtained and aged at the National Center for Biotechnology animal facility: littermate wt/$p53^{+/+}$ and wt/$p53^{-/-}$ (wild-type for the transgene and null for p53) and T8 K5-Tert/$p53^{-/-}$ (heterozygous for the transgene and null for p53), as well as littermate wt/$p53^{+/-}$ (wild-type for the transgene and heterozygous for p53), T1 K5-Tert/$p53^{+/-}$ (heterozygous for the transgene and for p53), T8 K5-Tert/$p53^{+/-}$ (heterozygous for the transgene and for p53), and T8 K5-Tert/$p53^{+/+}$ mice (heterozygous for the transgene and wild-type for p53). It is important to note that large colonies of the above-mentioned genotypes were generated in the same genetic background to avoid any effects on tumor susceptibility due to the genetic background.

Mouse handling. Mice were housed at the National Center for Biotechnology barrier area, where pathogen-free procedures are employed in all mouse rooms. Quarterly health monitoring reports have been negative for all pathogens in accordance with Federation of European Laboratory Animal Science Associations recommendations.

Statistical analysis. To calculate the statistical significance of the differences in spontaneous tumor development between the wild-type and K5-Tert mouse lines, the following tumor severity values were established according to the pathologies present at the time of death: 1, no preneoplastic or neoplastic lesion; 2, preneoplastic lesions (hyperplasia or hyperkeratosis) in only one tissue; 3, preneoplastic lesions in more than one tissue; 4, one tumor (lymphoma, sarcoma, adenoma, etc.); 5, one tumor and preneoplastic lesions, or one tumor and metastasis of this tumor (i.e., multicentric lymphomas); and 6, more than one tumor of different origins.

We assigned a tumor severity value to each of the mice analyzed, and statistical calculation was done with Microsoft Excel. For statistical significance, Student's *t* test values were calculated.

Real-time quantitative RT-PCR for Tert mRNA detection in K5-Tert and wild-type tissues. Two to five mice of each genotype were sacrificed, and total RNA was prepared from the indicated tissues (Table 3) or tumors. Total RNA was extracted with Trizol (Gibco-BRL, Gaithersburg, Md.). Reverse transcription (RT) was performed with 5 μg of total RNA, random hexameres as primers, and Superscript II reverse transcriptase (Gibco-BRL) following the manufacturer's instructions. Real-time PCR was done with a Light-Cycler instrument (Roche, Mannheim, Germany). Each PCR contained, in 15 μl, 1× Fast Start DNA Master SYBR Green I mix (Roche), 3 mM $MgCl_2$, 0.4 μM (each) the sense and antisense primers, and 2 μl of the corresponding diluted cDNA. For each mRNA, Tert expression was corrected by the actin content in each sample.

The PCRs employed a set of primers specific for the Tert gene (A in Fig. 3): TERT-F, 5′ GGA TTG CCA CTG GCT TCC G 3′, and TERT-R, 5′TGC CTG ACC TCC TCT TGT GAC 3′. Parallel RT-PCRs were carried out for actin with specific primers ACTIN-F (5′GGC ACC ACA CCT TCT ACA ATG 3′) and ACTIN-R (5′GTG GTG GTG AAG CTG TAG 3′). In both cases, reaction mixes were incubated for 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 68°C, and 5 s at 80°C. Fluorescence was acquired during every 80°C step and analyzed with the Light Cycler software. In all cases, each PCR was repeated at least twice. Some of the samples were run in a gel to confirm that the specific band had the expected size (278 bp), as well as to rule out possible contaminants not distinguishable by their melting points (not shown).

Real-time PCR strategy for detection of transgene expression in K5-Tert tumors in wild-type and p53 mutant backgrounds. Tumors from wild-type and K5-Tert mice in either the wild-type or p53 mutant genetic background were frozen, total RNA was extracted, and reverse transcription was performed as described before. Each reaction contained, in 20 μl, 2 mM $MgCl_2$, 10% dimethyl sulfoxide, 0.4 μM (each) sense and antisense primers (B in Fig. 3), 1 U of AmpliTaq polymerase (Roche), and 2 μl of the corresponding diluted cDNA. Reactions were performed with a hot start, followed by 40 cycles of 20 s at 95°C, 30 s at 62°C, and 30 s at 68°C. The primers used for K5-Tert amplification were a forward primer in the 5′ untranslated region (K5-UTR, 5′ CTG CTC TTT CTC TCC AGC ACG 3′) and a reverse primer in the internal region of the Tert coding sequence (TERT, 5′GAG TCT CTG CAC AAC CCT GG 3′) (B in Fig. 3). The expected size of the K5-Tert-specific band is 522 bp (see Fig. 4). The band was cloned and sequenced to demonstrate that it amplified the specific product (not shown).

Histopathological analyses. Full-body histopathological analysis was performed on all the animals that died or were sacrificed. Tumoral and normal tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections of 4 mm were stained with hematoxylin-eosin. Images were captured with an Olympus-Vanox microscope at 20× magnification.

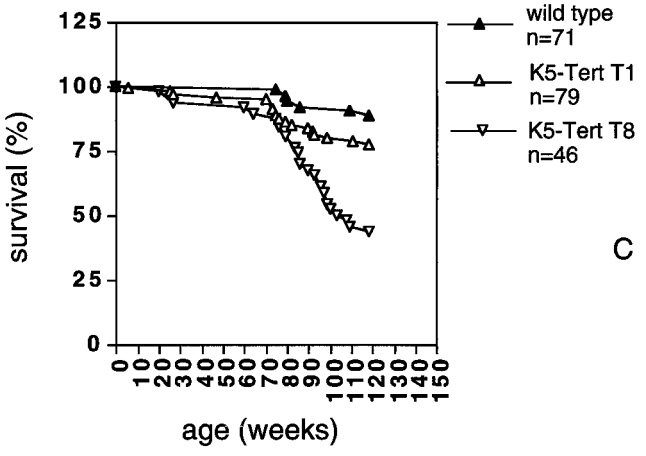
RESULTS

Reduced viability with age of K5-Tert telomerase transgenic mice compared to wild-type littermates. Transgenic mice overexpressing the murine telomerase reverse transcriptase gene (Tert) under the control of the bovine keratin 5 (K5) promoter, K5-Tert, were previously described by us (9). K5 promoter targets Tert expression to basal keratinocytes of stratified epithelia in the mouse (21; this paper). We have previously shown that adult K5-Tert mice show histologically normal stratified epithelia; however, upon chemical carcinogenesis, the transgenic epithelia showed a higher susceptibility to developing papillomas than those of the corresponding wild-type littermates (9). Furthermore, the skin of K5-Tert transgenics showed a faster rate of wound healing and a higher proliferation rate upon mitogenic stimuli than that of the corresponding wild-type controls (9).

To study the impact of telomerase overexpression on spontaneous cancer incidence in aging of K5-Tert tissues, we maintained five independent K5-Tert founder lines (T1, T3, T4, T5, and T8) for more than 2 years (9). Lines T1 and T8 showed the highest transgenic telomerase activity expression, lines T4 and T5 showed high to intermediate levels, and line T3 had low levels of transgenic telomerase expression (9). Only a reduced number of mice of lines T3, T4, and T5 were included in the study. In the case of lines T1 and T8, with the highest telomerase expression, and the wild-type controls, we generated large mouse colonies of 79, 46, and 71 mice, respectively (Fig. 1A).

Transgenic K5-Tert mice from both the T1 and T8 lines showed increased mortality with age compared with the corresponding wild-type cohorts in the same genetic background. As shown in Fig. 1A, 20% of T8 mice and 15% of T1 mice died in the first 80 weeks of life, whereas only 4% of wild-type mice died during this time. Differences in survival were even more evident after week 110 of life, when only 45% and 78% of T8 and T1 mice survived, respectively, compared to 90% of the wild-type counterparts (Fig. 1A). The differences in life span between the T1 and T8 K5-Tert transgenic lines could be due to (i) small differences in Tert levels (9) or (ii) positional effects due to transgene integration in the genome.

A



B

genotype	mice that died*	mice with tumors*, %	mice showing > 1 tumor, %
wild-type	15	6/15 (40%)	2/15 (13 %)
K5-Tert T1	32	22/32 (69%)	7/32 (22 %)
K5-Tert T8	30	23/30 (77%)	9/30 (30 %)

*Mice that died spontaneously. Preneoplastic lesions (hyperplasia, hyperkeratosis) were not included.

C

genotype	mice killed*	mice with tumors, %	mice showing > 1 tumor, %
wild-type	12	6/12 (50%)	2/12 (17 %)
K5-Tert T1	14	11/14 (79%)	3/14 (21 %)
K5-Tert T8	9	6/9 (67%)	2/9 (22 %)

*Mice sacrificed in parallel (91-124 weeks old). Preneoplastic lesions (hyperplasia, hyperkeratosis) were not included.

FIG. 1. (A) Decreased life span of K5-Tert transgenic mice compared to the wild-type controls. Survival curves for wild type (71 mice), T1 K5-Tert (79 mice), and T8 K5-Tert (46 mice). (B) Percentage of mice of each genotype that presented tumors at the time of spontaneous death. Preneoplastic lesions were not included as tumors. (C) Percentage of mice of each genotype that presented tumors at the time of sacrifice. Preneoplastic lesions were not included as tumors.

Increased incidence of spontaneous preneoplastic and neoplastic lesions in aged K5-Tert mice compared to wild-type controls. A full-body histopathological analysis was performed on each of the moribund wild-type, T1, and T8 K5-Tert mice. We classified the neoplastic and preneoplastic lesions according to the cell type of origin. Table 1 shows the number of mice that presented the indicated lesions at the time of death out of the total number of mice analyzed. See below for calculations of global tumor incidence per genotype, as well as for statistical analyses.

Preneoplastic lesions in stratified epithelia. Table 1 shows the number of mice of each genotype that showed preneoplastic lesions (hyperkeratosis or both hyperplasia and hyperkeratosis) in the indicated stratified epithelia (Table 1). To compare the incidence of preneoplastic lesions per genotype, we determined the number of mice of each genotype that developed preneoplastic lesions in any of the stratified epithelia at the time of death (Table 2). In addition to the moribund wild-type and T1 and T8 K5-Tert mice, we also included T3, T4, and T5 K5-Tert mice that were sacrificed in parallel (>75 weeks old) (Table 2).

As shown in Table 2, there was a higher incidence of preneoplastic lesions in the moribund T1 and T8 mice than in the wild-type controls, 50% and 34.7% compared to 13.3%, respectively. Representative images of preneoplastic lesions in the stratified epithelia of moribund K5-Tert mice are shown in Fig. 2a to c. Histopathological analysis of the T3, T4, and T5 K5-Tert mice revealed hyperplasias and hyperkeratosis only in T4 and T5 K5-Tert lines, which have intermediate and high levels of telomerase activity, respectively, but not in the T3 K5-Tert line, with low levels of telomerase activity (see Table 2). These results suggest that the incidence of spontaneous preneoplastic lesions in the stratified epithelia of K5-Tert mice is a direct consequence of telomerase activity reconstitution in the different transgenic lines.

Preneoplastic and neoplastic lesions in nonstratified epithelial tissues. Histopathological analysis of moribund T1 and T8 K5-Tert mice showed an increased frequency of preneoplastic and neoplastic lesions in a wide range of nonstratified epithelial tissues compared to moribund wild-type mice (Table 1). The tissues analyzed included the salivary glands, the glandular stomach, the intestine, the liver, the lung, and the male and female genital organs. Figure 2d shows a severe hyperplasia in the glandular stomach of a K5-Tert mouse; this type of lesion was never found in the moribund wild-type cohorts (Table 1). Similarly, the K5-Tert male genital organs, including the prostate, prepucial glands, seminal vesicles, and testes, showed preneoplastic and neoplastic lesions that were not found in any of the wild-type cohorts (Table 1). Representative images of some of the lesions are shown in Fig. 2e and 2f.

We also detected a higher incidence of tumors in the liver and lung of K5-Tert mice compared to similarly aged wild-type mice. Liver hepatocellular carcinomas and liver lymphomas were present in the K5-Tert mice but were never found in the wild-type cohorts (Table 1) (Fig. 2g and 2h). Other liver tumors such as histiocytic sarcomas were also detected in wild-type mice at a similar frequency. In addition, carcinomas of the mammary gland were detected in T1 and T8 K5-Tert mice but never in the wild-type cohorts. It is interesting that T8 K5-Tert mice showed a higher incidence of sarcomas in various tissues than the T1 K5-Tert line. Again, this difference in tumor susceptibility between T1 and T8 K5-Tert lines could be due to (i) small differences in Tert levels and/or telomerase activity levels or (ii) positional effects due to transgene integration in the genome.

TABLE 1. Lesions in moribund mice[a]

Tissue	Organ	Pathology	No. of mice/total no. examined (% of mice)		
			Wild type (n = 15)	T1 K5-Tert (n = 26)	T8 K5-Tert (n = 23)
Stratified epithelia	Skin	Hyperkeratosis only	0/15 (0)	1/26 (3.8)	1/23 (4.3)
		Hyperplasia & hyperkeratosis	1/15 (6.7)	5/26 (19.2)	1/23 (4.3)
		Sebaceous glands adenoma	0/15(0)	0/26 (0)	1/23 (4.3)
	Esophagus	Hyperkeratosis only	1/15 (6.7)	2/26 (7.7)	3/23 (13)
		Hyperplasia & hyperkeratosis	0/15 (0)	3/26 (11.5)	1/23 (4.3)
	Forestomach	Hyperkeratosis only	2/15 (13.3)	6/26 (23)	5/23 (21.7)
		Hyperplasia & hyperkeratosis	1/15 (6.7)	6/26 (23)	3/23 (13)
	Vagina	Abscess	0/5 (0)	0/15 (0)	1/8 (12.5)
Other epithelial tissues	Salivar glands	Histiocytic sarcoma	0/15 (0)	0/26 (0)	1/23 (4.3)
	Glandular stomach	Hyperplasia	0/15 (0)	1/26 (3.8)	4/23 (17.4)
	Intestine	Lymphoma	1/15 (6.7)	0/26 (0)	0/23 (0)
		Liposarcoma (anus)	0/10 (0)	0/26 (0)	1/23 (4.3)
	Liver	Cysts	1/15 (6.7)	1/26 (3.8)	1/23 (4.3)
		Hemangiosarcoma	1/15 (6.7)	1/26 (7.7)	2/23 (8.7)
		Histiocytic sarcoma	1/15 (6.7)	0/26 (0)	1/23 (4.3)
		Hepatocellular carcinoma	0/15 (0)	0/26 (0)	2/23 (8.7)
		Lymphoma	0/15 (0)	2/26 (7.7)	1/23 (4.3)
		Hemangioma	0/15 (0)	1/26 (3.8)	1/23 (4.3)
	Lung	Bronchioloalveolar adenoma	1/15 (6.7)	0/26 (0)	2/23 (18.7)
		Bronchioloalveolar carcinoma	0/15 (0)	1/26 (3.8)	1/23 (14.3)
		Lymphoma	0/15 (0)	2/26 (7.7)	0/23 (0)
	Prostate	Hyperplasia	0/10 (0)	2/11 (18.2)	0/15 (0)
	Prepucial glands	Adenoma	0/10 (0)	1/11 (9.1)	0/15 (0)
	Seminal vesicles	Hemangioma	0/10 (0)	0/11 (0)	1/15 (6.7)
	Testes	Leydig cells carcinoma	0/10 (0)	0/11 (0)	1/15 (6.7)
	Mammary glands	Hyperplasia	1/5 (20)	1/15 (6.7)	1/8 (12.5)
		Solid carcinoma	0/5 (0)	1/15 (6.7)	0/8 (0)
		Adenocarcinoma	0/5 (0)	0/15 (0)	2/8 (25)
	Ovary	Cyst	1/5 (20)	1/15 (6.7)	2/8 (25)
		Cystadenoma	0/5 (0)	0/15 (0)	1/8 (12.5)
		Granulose cell tumor	0/5 (0)	1/15(6.7)	0/8 (0)
	Uterus	Histiocytic sarcoma	1/5 (20)	0/15 (0)	0/8 (0)
		Endometrial hyperplasia	1/5 (20)	2/15 (13.3)	1/8 (12.5)
		Hemangiosarcoma	0/4 (0)	1/15 (6.7)	0/8 (0)
	Kidney	Lymphoma	0/15 (0)	1/26 (7.7)	0/23 (0)
Lymphoid tissues	Spleen	Hyperplasia	1/15 (6.7)	0/26 (0)	2/23 (18.7)
		Lymphoma	2/15 (13.3)	7/27 (25.9)	4/23 (17.4)
		Histiocytic sarcoma	1/15 (6.7)	0/26 (0)	0/23 (0)
		Fibrosarcoma	0/15 (0)	0/26 (0)	1/23 (4.3)
		Hemangiosarcoma	1/15 (6.7)	0/26(0)	0/23 (0)
	Lymph nodes	Hyperplasia	0/15 (0)	1/26 (3.8)	0/23 (0)
		Lymphoma	0/15 (0)	8/26 (30.7)	2/23 (18.7)
		Histiocytic sarcoma	0/15 (0)	0/26 (0)	3/23 (13)

[a] The numbers in parentheses indicate the percentage of moribund mice of each genotype that presented the indicated lesion. Some of these mice showed more than one lesion, as shown in Fig. 1B. For wild-type mice, 10 males and 5 females were analyzed; for T1 K5-Tert mice, 11 males and 15 females were studied; for T8 K5-Tert mice, 15 males and 8 females were analyzed. Note that this table depicts the number of mice of each genotype that presented each given pathology, whereas Fig. 1B shows the percentage of mice of each genotype that died with tumors (irrespective of tumor origin).

Tumors in lymphoid tissues. Moribund K5-Tert mice showed elevated frequencies of hyperplasias, lymphomas, and sarcomas in the lymph nodes, as well as hyperplasias and lymphomas in the spleen, compared to similarly aged moribund wild-type mice (Table 1).

Tumor incidence by genotype and statistical significance. The results presented above indicate that two independent K5-Tert transgenic lines, T1 and T8 K5-Tert, showed a modest but consistent increased incidence of spontaneous preneoplastic and neoplastic lesions as they aged compared to the wild-type cohorts. In particular, we determined that 69% and 77% of the moribund T1 and T8 transgenic mice, respectively, showed tumors, compared with only 40% of the wild-type mice (Fig. 1B; the preneoplastic lesions are not included in this calculation). Furthermore, 22% and 30% of the T1 and T8 transgenic mice with tumors, respectively, showed more than one tumor, compared to 13% of the wild-type mice (Fig. 1B).

To increase the number of mice studied, we sacrificed wild-type and T1 and T8 transgenic mice (between 91 and 124 weeks old) and determined the percentage of these mice that showed spontaneous tumors at the time of sacrifice (Fig. 1C). In this case, 79% and 67% of the sacrificed T1 and T8 transgenic mice, respectively, showed tumors, compared to 50% of the wild-type controls (Fig. 1C). Similarly, 21% and 22% of the

TABLE 2. Telomerase activity levels and incidence of preneoplastic lesions in stratified epithelia (skin, esophagus, and forestomach) of aged (>75 weeks old) wild-type and K5-Tert mice from the indicated founder lines[a]

Mice	No. of mice[b]	Level of telomerase activity[c]	No. of mice that showed preneoplastic lesions/no. examined (% with lesions)[d]
Wild type (moribund)	15	−	2/15 (13.3%)
T1 (moribund)	26	++++	13/26 (50%)
T8 (moribund)	23	++++	8/23 (34.7%)
T3 (sacrificed)	2	+	0/2 (0%)
T4 (sacrificed)	9	++	5/9 (55.5%)
T5 (sacrificed)	3	+++	3/3 (100%)

[a] Mice from lines T1 and T8 were moribund at the time of the analysis. Mice from lines T3, T4, and T5 were sacrificed at similar ages.
[b] Number of mice >75 weeks of age subjected to histopathological analysis (see also Table 1 for T1 and T8 mice).
[c] From González-Suárez et al. (9).
[d] Percentage of mice that showed preneoplastic lesions in any of the stratified epithelia of the organism. For instance, 2 of 15 wild-type mice analyzed showed lesions in stratified epithelia; the 13 other mice did not show preneoplastic lesions in any of the stratified epithelia analyzed (skin, esophagus, or forestomach).

T1 and T8 transgenic mice with tumors, respectively, contained more than one tumor, compared to only 17% of the wild-type controls (Fig. 1C).

To calculate the statistical significance of the differences between genotypes in the incidence and severity of the preneoplastic and neoplastic lesions, we assigned a tumor severity value to each mouse analyzed (see Materials and Methods). Student's *t* test analysis indicates that moribund T1 and T8 K5-Tert mice showed a significantly increased tumor susceptibility compared to the wild-type controls, $P = 0.044$ and $P = 0.033$, respectively (see Materials and Methods).

K5-driven Tert mRNA expression in the mouse is not restricted to stratified epithelia. As mentioned above, the known expression pattern of keratin 5 in the mouse includes all the stratified epithelia (21). In agreement with this, we observed an elevated frequency of lesions in the stratified epithelia of aged T1 and T8 K5-Tert mice compared to the age-matched littermate controls (Tables 1 and 2). However, the fact that we also observed an elevated frequency of spontaneous neoplasias in nonstratified epithelial tissues as well as in nonepithelial tissues from the K5-Tert mice suggested that transgene expression was not restricted to the stratified epithelia.

To demonstrate this, we developed a real-time PCR-based method that allows quantification of both endogenous and transgenic Tert expression at the mRNA level. The primers used were designed against sequences flanking an intron of the Tert gene to avoid detection of a putative contamination with endogenous Tert genomic DNA (see A in Fig. 3; Table 3). As expected, all stratified epithelia, including skin, oral mucosa, tongue, esophagus, forestomach, trachea, and vagina, showed a 10- to 100-fold increase in Tert mRNA expression compared to the corresponding wild-type tissues (see asterisk in Table 3). In addition, nonstratified epithelial tissues, in which we did not expect transgene expression, such as the prepucial glands, penis, seminal glands, testes, uterus, glandular stomach, thymus, lymph nodes, and lung, also showed an increase of between 10- and 100-fold in Tert mRNA levels compared to the wild-type tissues (Table 3). The colon, liver and spleen showed lower but still significantly elevated Tert mRNA expression compared to the corresponding wild-type tissues (Table 3). Tert mRNA expression was not elevated in the small intestine, kidney, ovary, heart, and brain of the K5-Tert mice compared to the corresponding wild-type tissues (Table 3). These results indicate that, although highly expressed in the stratified epithelia, K5-Tert is also expressed in a wide spectrum of adult tissues in the mouse. This broad expression pattern of the K5-Tert transgene is the most likely explanation for the wide tumor spectrum detected in aged T1 and T8 K5-Tert mice.

Specific K5-Tert mRNA expression in K5-Tert tumors. Telomerase activity has been described to be upregulated in murine tumors despite the fact that mice have very long telomeres (1, 2). Similarly, quantitative real-time RT-PCR with Tert-specific primers (primers A in Fig. 3) showed that Tert is upregulated in tumors derived from both wild-type and K5-Tert mice (not shown). With this set of primers, however, we could not distinguish between endogenous and transgenic Tert in the tumors derived from K5-Tert mice.

To specifically address whether cells expressing the K5-Tert mRNA contributed to the formation of spontaneous tumors in aged moribund K5-Tert mice, we designed a second RT-PCR strategy that distinguishes endogenous Tert from transgenic K5-Tert expression. For this, we took advantage of the fact that transgenic K5-Tert mRNA contains a 5′ UTR region that is not present in the endogenous Tert transcript (see B in Fig. 3). In particular, we designed a forward primer directed against the 5′ UTR region of keratin 5 and a reverse primer directed against the Tert coding sequence (see B in Fig. 3). This pair of primers cannot amplify the endogenous Tert mRNA (Fig. 4). These primers also distinguish between specific K5-Tert mRNA amplification and a possible contamination of K5-Tert genomic DNA. The expected size for the specific K5-Tert mRNA amplified product is 522 bp, and in the case of genomic DNA contamination, it would be 1,096 bp.

We cloned and sequenced the 522-bp band and demonstrated that it specifically amplifies the K5-Tert mRNA (not shown). With this strategy, we were able to detect K5-Tert mRNA in 9 of 15 K5-Tert tumors analyzed (60%) (Fig. 4 shows examples). This does not rule out that, in addition to K5-Tert, the endogenous Tert is also upregulated in these tumors or in the K5-Tert tumors that are negative for transgene expression. The K5-Tert tumors with detectable specific K5-Tert mRNA expression included skin papillomas, liver hemangiosarcomas, liver histiocytic sarcomas, hepatocellular carcinomas, spleen hyperplasias, and spleen lymphomas. Figure 4 shows specific K5-Tert expression in two K5-Tert liver hemangiosarcomas, one K5-Tert spleen lymphoma, and one K5-Tert skin papilloma (9). As a positive control, a K5-Tert skin sample was used (Fig. 4). As expected, none of the wild-type tumors, zero of four tumors tested, showed transgene expression. As an example, Fig. 4 shows undetectable K5-Tert-specific expression in a hemangiosarcoma from a moribund wild-type mouse.

Aggravated loss of viability and spontaneous tumor incidence of K5-Tert mice in p53 mutant backgrounds. To test whether forced telomerase expression in adult tissues cooperates with p53 loss to promote cancer development during aging, we generated large colonies of T1 and T8 K5-Tert mice as

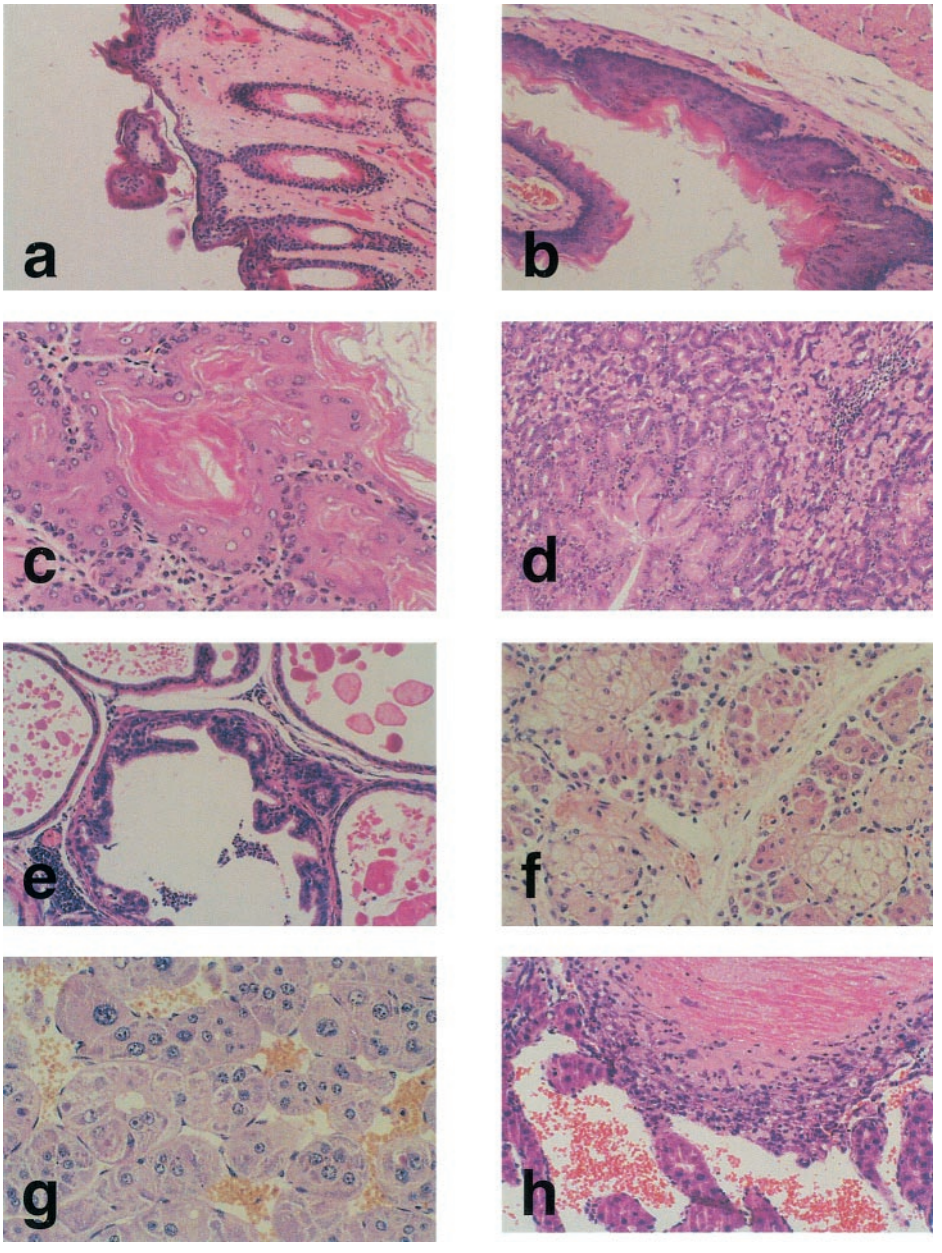


FIG. 2. Examples of preneoplastic and neoplastic lesions in K5-Tert transgenics. Magnifications, ×20. (a) A skin section showing areas of hyperplasia and hyperkeratosis. (b) An esophagus section showing areas of hyperplasia and hyperkeratosis. (c) Forestomach showing areas of hyperplasia and hyperkeratosis. (d) Glandular stomach showing areas of hyperplasia. (e) Prostate showing hyperplasia. (f) Adenoma in the prepucial glands. (g) Hepatocellular carcinoma in the liver. (h) Hemangiosarcoma of the liver.

well as of the corresponding wild-type controls in both $p53^{-/-}$ and $p53^{+/-}$ genetic backgrounds. Deletion of the p53 tumor suppressor in mice, $p53^{-/-}$, results in a dramatic decrease in the life span of these mice coincident with an increased incidence of tumors, particularly, lymphomas (7, 14, 16; this paper). Heterozygous $p53^{+/-}$ mice also show a reduced life span and an increased tumor incidence compared to the wild-type controls, although the appearance of tumors is delayed in time with respect to the $p53^{-/-}$ mice (14, 16; this paper).

For the current analysis, large colonies of mice of the following genotypes were obtained and studied: wt/$p53^{+/+}$ and wt/$p53^{-/-}$ (wild-type for the transgene and null for p53) and T8 K5-Tert/$p53^{-/-}$ (heterozygous for the transgene and null for p53), as well as wt/$p53^{+/-}$ (wild-type for the transgene and heterozygous for p53), T1 K5-Tert/$p53^{+/-}$ (heterozygous for the transgene and for p53), T8 K5-Tert/$p53^{+/-}$ (heterozygous for the transgene and for p53), and T8 K5-Tert/$p53^{+/+}$ (heterozygous for the transgene and wild-type for p53). We found no significant differences in life span between wt/$p53^{+/+}$ and T8 K5-Tert/$p53^{+/+}$ controls at week 80 after birth (Fig. 5A). This is in agreement with the fact that the negative impact of high telomerase on life span is only seen at older ages, as

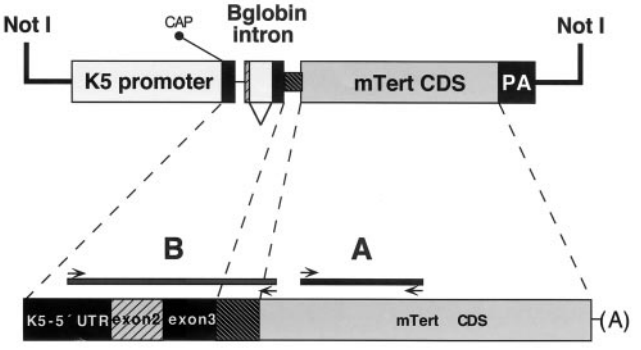


FIG. 3. Scheme of the K5-Tert construct used for transgenesis. The primers used for PCR detection of both endogenous and transgenic Tert (A primers) or only transgenic Tert (B primers) are shown.

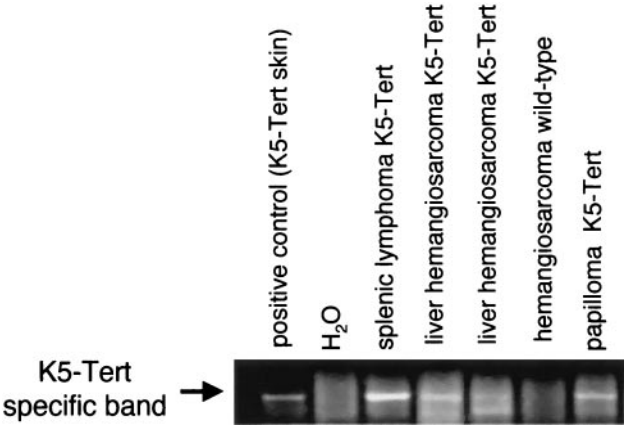


FIG. 4. Specific detection of transgenic K5-Tert mRNA expression in tumors derived from K5-Tert mice. The primers used are shown in Fig. 3 (B primers). The K5-Tert mRNA-specific band is indicated with an arrow (522 bp in length). RNA was obtained from the indicated tumor as described (Materials and Methods). A K5-Tert skin sample was used as a positive control, and a tumor derived from a wild-type mouse was used as a negative control. The 522-bp band was cloned and sequenced to demonstrate that it amplified the specific product (not shown).

shown above. T8 K5-Tert/p53$^{-/-}$ and wt/p53$^{-/-}$ cohorts showed a reduced life span compared to that of mice with wild-type p53 (Fig. 5A). In particular, all of the T8 K5-Tert/p53$^{-/-}$ and wt/p53$^{-/-}$ mice died 40 weeks after birth (Fig. 5A). Therefore, there were no significant differences in life span between T8 K5-Tert/p53$^{-/-}$ and wt/p53$^{-/-}$ mice, indicating that high telomerase expression in adult tissues does not significantly affect viability in a p53 null background, most likely because these mice die very rapidly of tumors (Table 4).

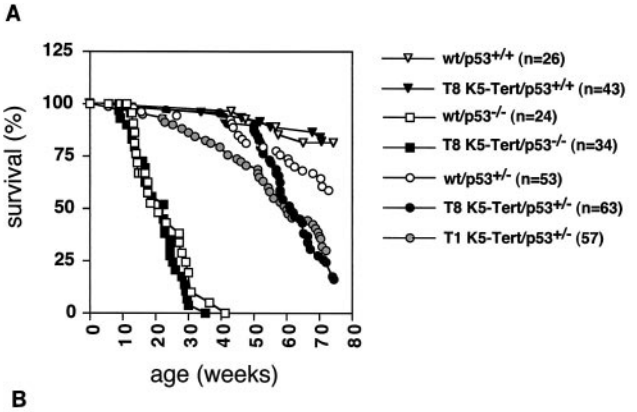
Interestingly, the moribund K5-Tert/p53$^{-/-}$ cohorts had a reproducibly higher tumor incidence than the control wt/p53$^{-/-}$ mice. In particular, 100% of the K5-Tert/p53$^{-/-}$ mice died of tumors, compared to 83% of the wild-type cohorts (Fig. 5B). In addition, K5-Tert/p53$^{-/-}$ mice showed a broader spectrum of preneoplastic and neoplastic lesions than the wt/p53$^{-/-}$ mice (Table 4). Indeed, most of the lesions, except for lymphomas and sarcomas, were detected only in the K5-Tert/p53$^{-/-}$ mice but not in the wt/p53$^{-/-}$ cohorts (Table 4). In addition, the incidence of lymphomas in the thymus and in the lymph nodes was also increased in the K5-Tert/p53$^{-/-}$ mice compared to the wt/p53$^{-/-}$ cohorts (Table 4). These results suggest that transgenic telomerase expression in adult tissues favors tumorigenesis, in this case cooperating with p53 deficiency.

This fact was more evident when wt/p53$^{+/-}$, T1 K5-Tert/p53$^{+/-}$, and T8 K5-Tert/p53$^{+/-}$mice were analyzed. In this case, T1 K5-Tert/p53$^{+/-}$ and T8 K5-Tert/p53$^{+/-}$ mice showed a reduced life span compared to the wt/p53$^{+/-}$ controls (Fig. 5A). In particular, 70 weeks after birth, most of the T1 K5-Tert/p53$^{+/-}$ and T8 K5-Tert/p53$^{+/-}$ mice had died, whereas 60% of the wt/p53$^{+/-}$ had survived (Fig. 5A). Extensive histopathological analysis of large numbers of moribund mice revealed a significantly higher tumor incidence and a wider tumor spectrum in the T1 K5-Tert/p53$^{+/-}$ and T8 K5-Tert/p53$^{+/-}$ mice than in the wt/p53$^{+/-}$ littermates (Fig. 5B; Table 5). In particular, 90% and 91% of the T8 K5-Tert/p53$^{+/-}$ and T1 K5-Tert/p53$^{+/-}$ mice, respectively, showed tumors at the

TABLE 3. Increase in Tert mRNA levels[a]

Increase (fold)	Organ(s) or tissue(s)
1	Small intestine, kidney, ovary, heart, brain
<10	Colon, liver, spleen
10	Oral mucosa,* tongue,* trachea, thymus, glandular, stomach, lymph nodes, lung
10–100	Skin,* esophagus,* forestomach,* vagina,* prepucial glands, penis, seminal glands, testis, uterus

[a] *, stratified epithelia.



B

genotype	mice with tumors*, %	mice showing > 1 tumor, %
wild-type/p53$^{-/-}$	20/24 (83%)	14/24 (58 %)
T8 K5-Tert/p53$^{-/-}$	29/29 (100%)	16/29 (55 %)
wild-type/p53$^{+/-}$	16/22 (73%)	6/22 (27 %)
T8 K5-Tert/p53$^{+/-}$	36/40 (90%)	11/40 (27 %)
T1 K5-Tert/p53$^{+/-}$	32/35 (91%)	20/35 (57 %)

*Mice that died with tumors out of the total mice that died (Fig. 5). Preneoplastic lesions (hyperplasia, hyperkeratosis) were not included.

FIG. 5. (A) Survival curves of wild-type and K5-Tert mice in the p53$^{-/-}$ and p53$^{+/-}$ genetic backgrounds. The number of mice of each genotype used in the analysis is indicated. (B) Percentage of mice of each genotype that presented tumors at the time of spontaneous death. Preneoplastic lesions were not included as tumors.

TABLE 4. Preneoplastic and neoplastic lesions in 18 moribund wild-type and 18 T8 K5-Tert mice in a $p53^{-/-}$ genetic background after a full-body histopathological analysis[a]

Tissue	Organ	Pathology	No. of mice with lesion/no examined (% with lesion)	
			wt/$p53^{-/-}$ (n = 18)	T8 K5-Tert/$p53^{-/-}$ (n = 18)
Stratified epithelia	Skin	Hyperplasia/hyperkeratosis	1/18 (5.5)	2/18 (11.1)
	Esophagus	Hyperkeratosis	1/18 (5.5)	0/18 (0)
		Hyperplasia/hyperkeratosis	1/18 (5.5)	1/18 (5.5)
	Forestomach	Hyperkeratosis	0/18 (0)	1/18 (5.5)
		Hyperplasia/hyperkeratosis	3/18 (16.6)	1/18 (5.5)
Other epithelial tissues	Glandular stomach	Hyperplasia & dysplasia	0/18 (0)	1/18 (5.5)
	Intestine	Hemangiosarcoma	0/18 (0)	1/18 (5.5)
		Lymphoma	0/18 (0)	1/18 (5.5)
	Liver	Hemangioma	0/18 (0)	1/18 (5.5)
	Prepucial glands	Adenoma	0/11 (0)	1/10 (10)
		Squamous cell papilloma	0/11 (0)	1/10 (10)
	Uterus	Carcinoma	1/7 (14.3)	0/8 (0)
	Peritoneum	Hemangiosarcoma	0/15 (0)	2/18 (11.1)
		Undifferentiated tumor	0/15 (0)	1/18 (5.5)
Lymphoid tissues	Thymus	Lymphoma	3/18 (16.6)	16/26* (61.5)
		Multicentric lymphoma	7/18 (38.9)	7/18 (38.9)
	Spleen	Hyperplasia	0/18 (0)	2/18 (11.1)
		Lymphoma	7/18 (38.9)	10/18 (55.5)
	Lymph nodes	Lymphoma	9/18 (50)	8/18 (44.4)
Mesenchymatous tissues	Subcutaneous	Fibrosarcoma	3/18 (16.6)	2/18 (11.1)
	Tissue	Hemangiosarcoma	0/18 (0)	3/18 (16.6)

[a] In the case of wt/$p53^{-/-}$ mice, 11 males and 7 females were studied. In the case of T8 K5-Tert/$p53^{-/-}$ mice, 10 males and 8 females were analyzed. Eight extra mice were included in the case of the thymus because they showed lymphomas even though the rest of the organs were not analyzed. Note that this table depicts the number of mice of each genotype that presented each given pathology, whereas Fig. 5B shows the percentage of mice of each genotype that died of tumors (irrespective of the tumor origin).

time of death, whereas only 73% of the wt/$p53^{+/-}$ controls showed tumors (Fig. 5B). In addition, T1 K5-Tert/$p53^{+/-}$ and T8 K5-Tert/$p53^{+/-}$ mice showed a higher incidence of preneoplastic lesions in stratified epithelia (skin, esophagus, oral cavity), as well as tumors in other tissues that were not affected in the moribund wt/$p53^{+/-}$ mice studied (prepucial glands, vagina, salivary glands, lymphoid tissues; Table 5). The differences in the incidence and severity of the preneoplastic and neoplastic lesions between wild-type and T1 K5-Tert/$p53^{+/-}$ or T8 K5-Tert/$p53^{+/-}$ mice were statistically significant ($P < 0.05$) (Materials and Methods).

As described for the single K5-Tert transgenics, a significant proportion of tumors from K5-Tert transgenics in both the $p53^{-/-}$ and $p53^{+/-}$ genetic backgrounds showed K5-Tert mRNA expression by RT-PCR (Fig. 3, B primers). In particular, 8 of 25 K5-Tert/$p53^{+/-}$ tumors analyzed showed detectable transgenic Tert mRNA expression. The K5-Tert/$p53^{+/-}$ tumors studied included thymic lymphomas, keratoacanthomas, papillomas, and tumors of the salivary glands and the prepucial glands (not shown). As expected, none of the tumors that appeared in the wt/$p53^{+/-}$ mice showed K5-Tert mRNA expression (0 of 10 tumors analyzed).

Altogether, these results indicate that transgenic telomerase expression in adult mouse tissue cooperates with p53 loss in inducing tumorigenesis.

DISCUSSION

The negative impact of telomerase deficiency and telomere shortening on tumor development in the context of an organism has been extensively addressed in the past by using the telomerase-deficient mouse model (3, 5, 8, 11, 24, 25). Those studies have been crucial in evaluating the efficiency of a putative telomere-based anticancer therapy. Similarly, mouse models of telomerase overexpression in adult tissues are important to determine any possible risks of a putative telomerase-based gene therapy for age-related diseases.

To study the impact of high telomerase expression on both aging and spontaneous cancer rates, we used a transgenic mouse that overexpresses telomerase in adult tissues, K5-Tert mice (9). K5-Tert mice show transgenic telomerase expression in most of the adult tissues, as shown here with a quantitative real-time PCR approach. We generated large colonies of two different K5-Tert lines as well as of the corresponding wild-type controls and let them age at our animal facility. The results shown here indicate that high telomerase expression in a wide range of adult tissues has a negative impact on the viability of the mice compared to wild-type controls.

The cause of the increased death rates in these mice is a modest but significantly higher incidence of cancer with increasing age than that shown by the wild-type cohorts. In addition, we demonstrate here that 60% of the tumors in the K5-Tert transgenics show transgene expression, supporting that the phenotypes observed in these mice are a direct consequence of the forced telomerase expression. This is also supported by the fact that the incidence of preneoplastic lesions in the stratified epithelia of five different K5-Tert transgenic lines (T1, T3, T4, T5, and T8) correlates with the levels of telomerase expression in those K5-Tert lines. It is important to note that, at earlier ages (up to 75 weeks), no significant differences between wild-type and K5-Tert telomerase transgenics were observed in terms of viability or cancer incidence,

TABLE 5. Preneoplastic and neoplastic lesions in moribund mice of the indicated genotype[a]

Tissue	Organ	Pathology	No. of mice with lesion/no. examined (% with lesion)		
			wt/p53$^{+/-}$ (n = 21)	T8 K5-Tert/p53$^{+/-}$ (n = 40)	T1 K5-Tert/p53$^{+/-}$ (n = 35)
Stratified epithelia	Skin	Hyperkeratosis	1/21 (4.7)	2/40 (5)	1/35 (2.8)
		Hyperplasia/hyperkeratosis	1/21 (4.7)	7/40 (17.5)	4/35 (11.4)
		Papillomas	0/19 (0)	0/40 (0)	1/35 (2.8)
		Keratoacanthoma	1/21 (4.7)	0/40 (0)	0/35 (0)
		Basal cell carcinoma	1/21 (4.7)	0/40 (0)	1/35 (2.8)
	Oral cavity	Hyperkeratosis	0/21 (0)	1/40 (2.5)	1/35 (2.8)
	Esophagus	Hyperkeratosis	1/21 (4.7)	2/40 (5)	2/35 (5.7)
		Hyperplasia/hyperkeratosis	2/21 (9.5)	6/40 (15)	4/35 (11.4)
	Forestomach	Hyperkeratosis	1/21 (4.7)	7/40 (17.5)	4/35 (11.4)
		Hyperplasia/hyperkeratosis	5/21 (23.8)	8/40 (20)	5/35 (14.3)
	Vagina	Fibrosarcoma	0/13 (0)	2/21 (9.5)	0/21 (0)
Other epithelial tissues	Salivary glands	Squamous cell carcinoma	0/21 (0)	1/40 (2.5)	1/35 (2.8)
	Intestine	Hypeplasia Peyer	0/21 (0)	1/40 (2.5)	0/35 (0)
		Hyperplasia	0/21 (0)	1/40 (2.5)	0/35 (0)
		Lymphoma	0/21 (0)	1/40 (2.5)	0/35 (0)
	Liver	Histiocytic sarcoma	0/21 (0)	1/40 (2.5)	1/35 (2.8)
		Hemangioma	0/21 (0)	0/40 (0)	2/35 (5.7)
		Hemangiosarcoma	1/21 (4.7)	2/40 (5)	1/35 (2.8)
		Lymphoma	0/21 (0)	2/40 (5)	4/35 (11.4)
	Lung	Histiocytic sarcoma	0/21 (0)	2/40 (5)	0/35 (0)
		Adenoma/adenocarcinoma	0/21 (0)	1/40 (2.5)	2/35 (5.7)
		Hemangiosarcoma	0/21 (0)	0/40 (0)	1/35 (2.8)
		Lymphoma	0/21 (0)	3/40 (7.5)	2/35 (5.7)
	Kidney	Lymphoma	1/21 (4.7)	3/40 (7.5)	4/35 (11.4)
	Prepucial glands	Adenoma	0/8 (0)	0/19 (0)	1/14 (7.1)
		Squamous cell carcinoma	0/8 (0)	1/19 (5.2)	0/14 (0)
	Mammary glands	Adenocarcinoma	1/13 (7.7)	0/21 (0)	1/21 (4.7)
	Uterus	Endometrial hyperplasia	2/13 (15.4)	2/21 (9.5)	1/21 (4.7)
		Histiocytic sarcoma	0/13 (0)	1/21 (4.7)	1/21 (4.7)
	Ovary	Hemangioma	0/13	0/21 (0)	1/21 (4.7)
	Peritoneum	Hemangiosarcoma	0/21 (0)	0/40 (0)	1/35 (2.8)
Lymphoid tissues	Thymus	Lymphoma	5/21 (34)	8/40 (20)	11/35 (31)
	Spleen	Hyperplasia	3/21 (14.3)	7/40 (17.5)	6/35 (17.1)
		Lymphoma	4/21 (19)	5/40 (12.5)	11/35 (31.4)
		Histiocytic sarcoma	0/21 (0)	2/40 (5)	1/35 (2.8)
		Mastocytoma	1/21 (4.7)	0/40 (0)	0/35 (0)
		Hemangioma	0/21 (0)	0/40 (0)	1/35 (2.8)
		Hemangiosarcoma	0/21 (0)	1/40 (2.5)	1/35 (2.8)
	Lymph nodes	Hyperplasia	0/21 (0)	3/40 (7.5)	1/35 (2.8)
		Lymphoma	1/21 (4.7)	6/40 (15)	8/35 (22.9)
		Histiocytic sarcoma	0/21 (0)	1/40 (2.5)	1/35 (2.8)
		Hemangioma	0/21 (0)	1/40 (2.5)	0/35 (0)
		Hemangiosarcoma	0/21 (0)	0/40 (0)	1/35 (2.8)
Mesenchymatous tissues	Muscle	rhabdomyosarcoma	0/21 (0)	1/40 (2.5)	1/35 (2.8)
	Bone	Osteosarcoma	5/21 (23.8)	12/40 (30)	6/35 (17.2)
	Subcutaneous tissue	Fibrosarcoma	4/21 (19)	9/40 (22.5)	5/35 (14.3)
		Hemangiosarcoma	0/19 (0)	1/40 (2.5)	2/35 (5.7)
		Mastocytoma (ear)	0/19 (0)	1/40 (2.5)	0/35 (0)

[a] In the case of wt/p53$^{+/-}$ mice, 8 males and 13 females were analyzed. In the case of T8 K5-Tert/p53$^{+/-}$ mice, 19 males and 21 females were analyzed. In the case of T1 K5-Tert/p53$^{+/-}$ mice, 14 males and 21 females were subjected to histopathological analysis. Note that this table depicts the number of mice of each genotype that presented each given pathology, whereas Fig. 5B shows the percentage of mice of each genotype that died of tumors (irrespective of the tumor origin).

indicating that high telomerase expression is not oncogenic per se, as also suggested by tissue-cultured cells immortalized by forced telomerase expression (17, 20). Indeed, the increased tumor incidence in K5-Tert mice was seen at old ages (>75 weeks), suggesting that other genetic alterations that occur with aging may cooperate with high telomerase expression in favoring tumorigenesis.

To study whether the higher incidence of neoplastic lesions that appear at old ages in the telomerase transgenics is a consequence of cooperation between high telomerase activity levels and other tumorigenic alterations, we generated telomerase transgenics in both p53$^{-/-}$ and p53$^{+/-}$ genetic backgrounds. The results obtained indicate that the deleterious effects on cancer and aging of high transgenic telomerase ex-

pression are dramatically aggravated in p53 mutant backgrounds, suggesting that high telomerase activity can cooperate with p53 mutations in promoting spontaneous cancer. It is interesting that K5-Tert and wild-type mice in a $p53^{-/-}$ genetic background died at similarly young ages (<40 weeks old). The absence of a K5-Tert-dependent effect on the viability of p53 null mice is likely to be due to the fact that p53 deficiency results in very rapid tumor development and death of the mice before the deleterious effects of telomerase overexpression can take place. Nevertheless, the telomerase K5-Tert transgenics in a p53 null background showed a higher incidence and a wider spectrum of tumors (some of which were never detected in the wild-type mice) than the wild-type counterparts. This effect was further enhanced when wild-type and telomerase K5-Tert transgenics were generated in a p53 heterozygous genetic background. In this case, the K5-Tert telomerase transgenics showed both a shorter life span and a higher incidence of tumors than the control wild-type mice.

It is important to note that both wild-type and K5-Tert mice have very long telomeres (9). Therefore, it is unlikely that the increased spontaneous cancer incidence of K5-Tert mice due to telomerase overexpression is mediated by the role of telomerase in telomere elongation, suggesting novel roles of telomerase independent of net telomere lengthening. This is also supported by the fact that murine tumors have been shown to upregulate telomerase activity despite the fact that mice have very long telomeres (1, 2, 4). In addition, the increased rate of wound healing as well as the higher incidence of 7,12-dimethylbenz[a]anthracene (DMBA)- plus tetradecanoyl phorbol acetate-induced papillomas described for K5-Tert mice also support this notion (9). Similarly, transgenic telomerase expression in the heart promotes cardiac muscle cell proliferation, hypertrophy, and survival of transgenic mice (23). Based on both mouse models for transgenic telomerase expression and evidence from cell culture models, a novel role of telomerase independent of net telomere elongation has recently been proposed by several authors (4, 9, 18). However, the precise mechanisms by which telomerase overexpression may promote survival are unknown.

In summary, the results presented here suggest cooperation between high telomerase activity and loss of the p53 tumor suppressor in inducing tumors during the normal aging process in the mouse. Therefore, the fact that adult somatic tissues have low or undetectable levels of telomerase activity can be envisioned as a tumor-protective mechanism. This notion is in accordance with the existence of a very well controlled balance between aging and tumor suppression in mammals and predicts that altering telomerase activity may affect both. Indeed, the telomerase knockout mouse model has provided evidence for this, as telomere shortening in this mouse accelerates aging at the same time that it suppresses tumor growth, at least in a p53 wild-type genetic background (5, 8, 11, 25). Finally, the finding that telomerase activity promotes tumorigenesis with aging and p53 mutation reveals possible risks of therapies based on telomerase reintroduction.

ACKNOWLEDGMENTS

We are indebted to Angel Zaballos (National Center for Biotechnology, Madrid, Spain) for help with quantitative real-time RT-PCR and to Rosa Serrano and Elisa Santos for mouse care and genotyping, respectively.

E.G.-S. is a Fellow of the Fondo de Investigaciones Sanitarias (FIS). M.A.B.'s laboratory is funded by the Cancer Swiss Bridge Award 2000, by the Ministry of Science and Technology (PM97-0133), Spain, by the EU (EURATOM/991/0201, FIGH-CT-1999-00002, and FIS5-1999-00055), and by the Department of Immunology and Oncology (DIO). The DIO is funded by the Spanish Council for Scientific Research and by Pharmacia Corporation.

REFERENCES

1. **Bednarek, A. K., Y. Chu, T. J. Slaga, and C. Aldaz.** 1997. Telomerase and cell proliferation in mouse skin papillomas. Mol. Carcinog. **20:**329–331.
1a. **Blackburn, E. H.** 1991. Structure and function of telomeres. Nature **350:** 569–573.
2. **Blasco, M. A., M. Rizen, C. W. Greider, and D. Hanahan.** 1996. Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. Nat. Genet. **12:**200–204.
3. **Blasco, M. A., H.-W. Lee, P. Hande, E. Samper, P. Lansdorp, R. DePinho, and C. W. Greider.** 1997. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell **91:**25–34.
4. **Bodnar, A. G., M. Ouellette, M. Frolkis, et al.** 1998. Extension of life-span by introduction of telomerase into normal human cells. Science **279:**349–352.
4a. **Broccoli, D., L. A. Godley, L. A. Donehower, H. E. Varmus, and T. de Lange.** 1996. Telomerase activation in mouse mammary tumors: lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation. Mol. Cell. Biol. **16:**3765–3772.
4b. **Chan, S.W.-L., and E. H. Blackburn.** 2002. New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. Oncogene **21:**553–563.
5. **Chin, L., S. E. Artandi, Q. Shen, et al.** 1999. p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. Cell **97:**527–538.
6. **Collins, K.** 2000. Mammalian telomeres and telomerase. Curr. Opin. Cell Biol. **12:**378–383.
7. **Donehower, L. A., M. Harvey, B. L. Slagle, et al.** 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature **356:**215–221.
8. **González-Suárez, E., E. Samper, J. M. Flores, and M. A. Blasco.** 2000. Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. Nat. Genet. **26:**114–117.
9. **González-Suarez, E., E. Samper, A. Ramírez, J. Flores, J. Martín-Caballero, J. L. Jorcano, and M. A. Blasco.** 2001. Increased epidermal tumors and increased skin, wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes. EMBO J. **20:**2619–2630.
10. **Goytisolo, F. A., and M. A. Blasco.** 2002. Many ways to telomere dysfunction. In vivo studies with mouse models. Oncogene **21:**584–591.
11. **Greenberg, R. A., R. C. Allsopp, L. Chin, G. Morin, and R. de Pinho.** 1999. Short dysfunctional telomeres impair tumorigenesis in the $INK4a^{D2/3}$ cancer-prone mouse. Cell **97:**515–525.
12. **Hahn, W. C., C. M. Counter, A. S. Lundberg, R. L. Beijersbergen, M. W. Brooks, and R. A. Weinberg.** 1999. Creation of human tumour cells with defined genetic elements. Nature **400:**464–468.
13. **Harley, C. B., A. B. Futcher, and C. W. Greider.** 1990. Telomeres shorten during ageing of human fibroblasts. Nature **31:**458–460.
14. **Harvey, M., M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel, A. Bradley, and L. A. Donehower.** 1993. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nat. Genet. **5:**225–229.
15. **Hemann, M. T., M. A. Strong, L. Y. Hao, and C. Greider.** 2001. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell **107:**67–77.
16. **Jacks, T., L. Remington, B. O. Williams, et al.** 1994. Tumor spectrum analysis in p53-mutant mice. Curr. Biol. **4:**1–7.
17. **Jiang, X.-R., G. Jimenez, E. Chang, et al.** 1999. Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. Nat. Genet. **21:**111–114.
18. **Lee, H.-W., M. A. Blasco, G. J. Gottlieb, J. W. Horner, C. W. Greider, and R. A. DePinho.** 1998. Essential role of mouse telomerase in highly proliferative organs. Nature **392:**569–574.
18a. **Mattson, M. P., W. Fu, and P. Zhang.** 2001. Emerging roles for telomerase in regulating cell differentiation and survival: a neuroscientist's perspective. Mech. Aging Dev. **122:**659–671.
19. **Mitchell, J. R., E. Wood, and K. Collins.** 1999. A telomerase component is defective in the human disease dyskeratosis congenital. Nature **402:**551–555.
20. **Morales, C. P., S. E. Holt, M. Ouellette, et al.** 1999. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. Nat. Genet. **21:**115–118.
21. **Murillas, R., F. Larcher, C. J. Conti, M. Santos, A. Ullrich, and J. L. Jorcano.** 1995. Expression of a dominant negative mutant of epidermal

growth factor receptor in the epidermis of transgenic mice elicits striking alterations on hair follicle development and skin structure. EMBO J. **14:** 5216–5223.
22. **Nugent, C. I., and V. Lundblad.** 1998. The telomerase reverse transcriptase: components and regulation. Genes Dev. **12:**1073–1085.
23. **Oh, H., G. E. Taffet, K. A. Youker, et al.** 2001. Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival. Proc. Natl. Acad. Sci. USA **98:**10308–10313.
24. **Rudolph, K. L., S. Chang, H.-W. Lee, M. Blasco, G. Gottlieb, W. C. Greider, and R. A. DePinho.** 1999. Longevity, stress response, and cancer in aging telomerase deficient mice. Cell **96:**701–712.
25. **Rudolph, K. L., M. Millard, M. W. Bosenberg, and R. A. De Pinho.** 2001. Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. Nat. Genet. **28:**155–159.
26. **Samper, E., J. M. Flores, and M. A. Blasco.** 2001. Restoration of telomerase activity rescues chromosomal instability and premature aging in Terc$^{-/-}$ mice with short telomeres. EMBO Rep. **2:**800–807.
27. **Shay, J. W., and S. Bacchetti.** 1997. A survey of telomerase activity in human cancer. Eur. J. Cancer **33:**787–791.
28. **Wang, J., G. J. Hannon, and D. H. Beach.** 2000. Risky immortalization by telomerase. Nature **405:**755–756.
29. **Wyllie, F. S., C. J. Jones, J. W. Skinner, et al.** 2000. Telomerase prevents the accelerated cell ageing of Werner syndrome fibroblasts. Nat. Genet. **24:**16–17.