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Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes

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Telomerase transgenics are an important tool to assess the role of telomerase in cancer, as well as to evaluate the potential use of telomerase for gene therapy of age-associated diseases. Here, we have targeted the expression of the catalytic component of mouse telomerase, mTERT, to basal keratinocytes using the bovine keratin 5 promoter. These telomerase-transgenic mice are viable and show histologically normal stratified epithelia with high levels of telomerase activity and normal telomere length. Interestingly, the epidermis of these mice is highly responsive to the mitogenic effects of phorbol esters, and it is more susceptible than that of wild-type littermates to the development skin tumors upon chemical carcinogenesis. The epidermis of telomerase-transgenic mice also shows an increased wound-healing rate compared with wild-type littermates. These results suggest that, contrary to the general assumption, telomerase actively promotes proliferation in cells that have sufficiently long telomeres and unravel potential risks of gene therapy for age-associated diseases based on telomerase upregulation.

Keywords: epithelial tumors/gene therapy/mTERT/skin carcinogenesis/telomerase-transgenic mouse

Introduction

Telomeres consist of tandem DNA repeats and specific proteins and protect the chromosome ends from degradation, recombination and DNA repair activities (reviewed in Blackburn, 1991; Greider, 1996). In this regard, loss of telomeric function, either by loss of telomeric repeats or by mutation of telomeric proteins, is associated with increased chromosomal instability and loss of cell viability (Counter *et al*., 1992; Blasco *et al*., 1997; van Steensel *et al*., 1998; Samper *et al*., 2000).

Telomerase is the cellular reverse transcriptase that synthesizes telomeric repeats *de novo* (Greider and Blackburn, 1985; reviewed in Nugent and Lundblad, 1998). It is composed of a catalytic subunit known as TERT (telomerase reverse transcriptase) (Harrington *et al*., 1997a; Kilian *et al*., 1997; Lingner *et al*., 1997; Meyerson *et al*., 1997; Nakamura *et al*., 1997; Greenberg *et al*., 1998; Martín-Rivera *et al*., 1998), an RNA molecule or TERC (telomerase RNA component), which is used as template for the addition of new telomeric repeats (Greider and Blackburn, 1989; Singer and Gottschling, 1994; Blasco *et al*., 1995; Feng *et al*., 1995), and associated proteins (Collins *et al*., 1995; Harrington *et al*., 1997b; Nakayama *et al*., 1997; Gandhi and Collins, 1998; Mitchell *et al*., 1999).

Telomerase activity is upregulated in the vast majority of human tumors as compared with normal somatic tissues. It has been shown that expression of the catalytic subunit of telomerase, TERT, in cultured human primary cells reconstitutes telomerase activity and allows immortal growth (Bodnar *et al*., 1998; Kiyono *et al*., 1998; Jiang *et al*., 1999; Morales *et al*., 1999). Furthermore, TERT-mediated telomerase activation is able to cooperate with oncogenes in transforming cultured primary human cells into neoplastic cells (Hahn *et al*., 1999). In addition, it has been shown recently that TERT-driven cell proliferation results in activation of the c-*myc* oncogene (Wang *et al*., 2000). These findings in cultured cells have opened up the possibility that telomerase upregulation, which occurs in >90% of all human tumors (reviewed in Shay and Bacchetti, 1997), may contribute actively to tumor growth (reviewed in Weitzman and Yaniv, 1999). On the other hand, telomerase activity reconstitution in adult somatic cells or tissues is envisaged as a potential approach for gene therapy of age-related diseases. To address directly if tissue-specific telomerase overexpression has an impact on tumor susceptibility or normal tissue biology, we targeted expression of the catalytic component of mouse telomerase, mTERT (Greenberg *et al*., 1998; Martín-Rivera *et al*., 1998), to basal keratinocytes of stratified epithelia. Here, we describe the construction and characterization of these mice.

Results and discussion

In vivo reconstitution of telomerase activity in transgenic K5-mTERT mice

We used the 5′-regulatory region of the bovine keratin K5 gene (Ramírez *et al*., 1994; Murillas *et al*., 1995) to target mTERT expression to basal keratinocytes (see Figure 1A for details of the bovine K5 promoter region used to express mTERT) (Materials and methods). Eight different founder K5-mTERT transgenic mice were identified (T1–T8) that showed increased telomerase activity in the skin of the tail compared with wild-type littermates (Figure 1B). Two of these founders, T1 = 8043 and T8 = 7664 (highlighted in red in Figure 1B) showed the highest *in vivo* reconstitution of telomerase activity in the tail skin. All the results described here were obtained using

the K5-mTERT mice derived from the T1 founder, K5-mTERT (T1) (asterisk in Figure 1B), and were confirmed, where indicated, with the T8 founder-derived K5-mTERT transgenics, K5-mTERT (T8), to rule out possible non-specific effects due to transgene insertion.

In agreement with the known expression pattern of keratin K5 (Ramírez *et al*., 1994; Murillas *et al*., 1995), the stratified epithelia of K5-mTERT (T1) mice tested, including uterus, trachea and skin, showed significant upregulation of telomerase activity as compared with the corresponding littermate wild-type tissues (Figure 1B and C). This telomerase upregulation coincided with increased mTERT mRNA levels in K5-mTERT transgenic skin (between 25- and 50-fold) compared with wild-type controls (data not shown). Other tissues of the K5-mTERT (T1) mice where the transgene is not expressed (i.e. liver, brain, lung, ovary and whole thymus) did not show upregulation of telomerase activity as compared with wild-type littermates (Figure 1C).

Telomere length in wild-type and K5-mTERT transgenic basal keratinocytes

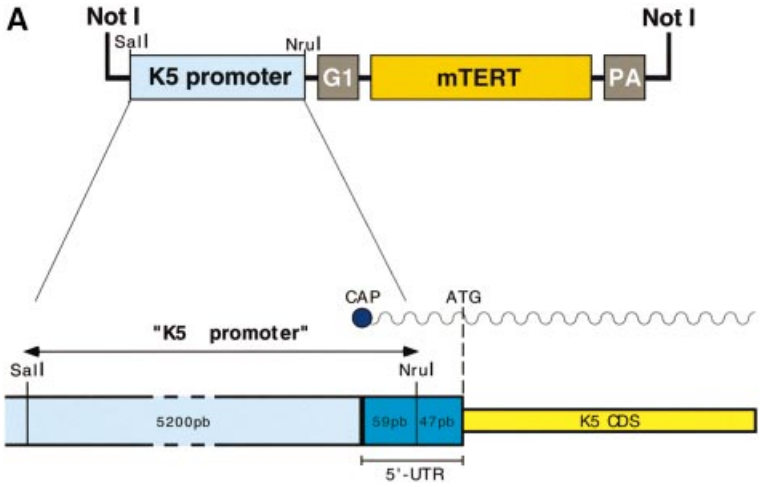
Previous studies using cultured human cells showed that TERT-mediated telomerase activity reconstitution resulted in extension or maintenance of telomere length (Bodnar *et al*., 1998; Kiyono *et al*., 1998; Hahn *et al*., 1999). We measured telomere length in K5-mTERT (T1) skin keratinocytes by quantitative fluorescence *in situ* hybridization (Q-FISH) on skin sections as previously described (González-Suárez *et al*., 2000). Figure 2A shows Q-FISH images of interphase nuclei in the skin of age-matched (8-week-old) littermate wild-type and K5-mTERT (T1) mice; the basal layer of skin keratynocytes is indicated with a yellow arrow. Quantification of the fluorescence intensity of telomere dots showed that telomeres in K5-mTERT (T1) epidermis are similar in length to those of age-matched littermate wild-type mice (Figure 2B; black bars). Primary keratinocyte cultures derived from newborn wild-type and K5-mTERT (T1) mice also showed similar telomere length using Flow-FISH, an independent quantitative FISH technique to measure telomere length that is based on flow cytometry (see Materials and methods) (Rufer *et al*., 1998) (Figure 2C). All together, these results suggest that TERT-driven telomerase upregulation in basal skin keratinocytes does not result in a significant extension of telomeres in the skin as compared with age-matched wild-type mice.

K5-mTERT stratified epithelia are histologically normal

K5-mTERT (T1 and T8) mice, ranging from newborn to 19 months old, were phenotypically indistinguishable from the corresponding age-matched wild-type littermates and showed no pathologies, no spontaneous development of epithelial tumors and no loss of viability with age (not shown). In agreement with this, histopathological analysis of stratified epithelia (skin, oral cavity, esophagus, forestomach and vagina) from 15- to 19-month-old K5-mTERT (T1 and T8, as indicated) and the corresponding age-matched littermate wild-type controls revealed no macroscopic or histological differences between genotypes, indicating that transgenic telomerase expression *per se* does not alter the stratified epithelium structure (Figure 3). These results suggest that potential telomerase reconstitution for gene therapy of epithelial aging-associated diseases would not have any deleterious effects on the structure of stratified epithelia.

Increased tumor susceptibility and mortality of K5-mTERT transgenics produced by chemical carcinogens

To study further the effects of telomerase overexpression on the normal biology and function of stratified epithelia, we first addressed whether transgenic telomerase expression impacts on epithelial tumor formation upon exposure to chemical carcinogens. The stages of initiation, promotion and tumor progression in the classical skin chemical carcinogenesis model are well characterized (Heckner *et al*., 1982). In wild-type mice, initiation using 7,12-dimethylbenz[*a*]anthracene (DMBA) and subsequent promotion with 12-*o*-tetradecanoylphorbol 13-acetate (TPA) leads to papillomas that are hyperplastic, well-differentiated, skin lesions. H-*ras* activation, loss of the p53 tumor suppressor and telomerase upregulation are reported in the majority of DMBA-initiated papillomas (Balmain *et al*., 1984, 1992; Quintanilla *et al*., 1986; Bednarek *et al*., 1997). Furthermore, when telomeres are critically short, as in late generation mice deficient for the RNA component of mouse telomerase, *Terc* (Blasco *et al*.,



1997; Lee *et al*., 1998), the absence of telomerase activity has a dramatic inhibitory impact on skin tumorigenesis (González-Suárez *et al*., 2000), indicating that tumor formation in skin requires telomere maintenance above a threshold length.

Age-matched (8- to 12-week-old) wild-type and K5-mTERT (T1) littermate mice received a single DMBA treatment followed by TPA–acetone treatment twice a week for 15 weeks (Materials and methods). Six weeks after the start of DMBA + TPA treatment, papillomas appeared in both wild-type and K5-mTERT (T1) mice, and these papillomas continued to grow in size throughout treatment (Figure 4A). The number of papillomas in K5-mTERT (T1) mice was significantly increased compared with wild-type littermates (Figure 4A and B). In particular, at week 15 (the end of TPA treatment), a total of 222 and 109 papillomas for K5-mTERT (T1) and wild-type mice, respectively, were counted (Figure 4A); this corresponds to 20.0 ± 9.8 and 9.9 ± 6.3 papillomas per mouse for K5-mTERT (T1) and wild-type mice, respectively (Figure 4B; Student's *t*-test $P = 0.0091$). After termination of TPA treatment at week 15 (asterisk in Figure 4A), many of the papillomas regressed in both genotypes, and a similar percentage of them progressed to larger lesions,

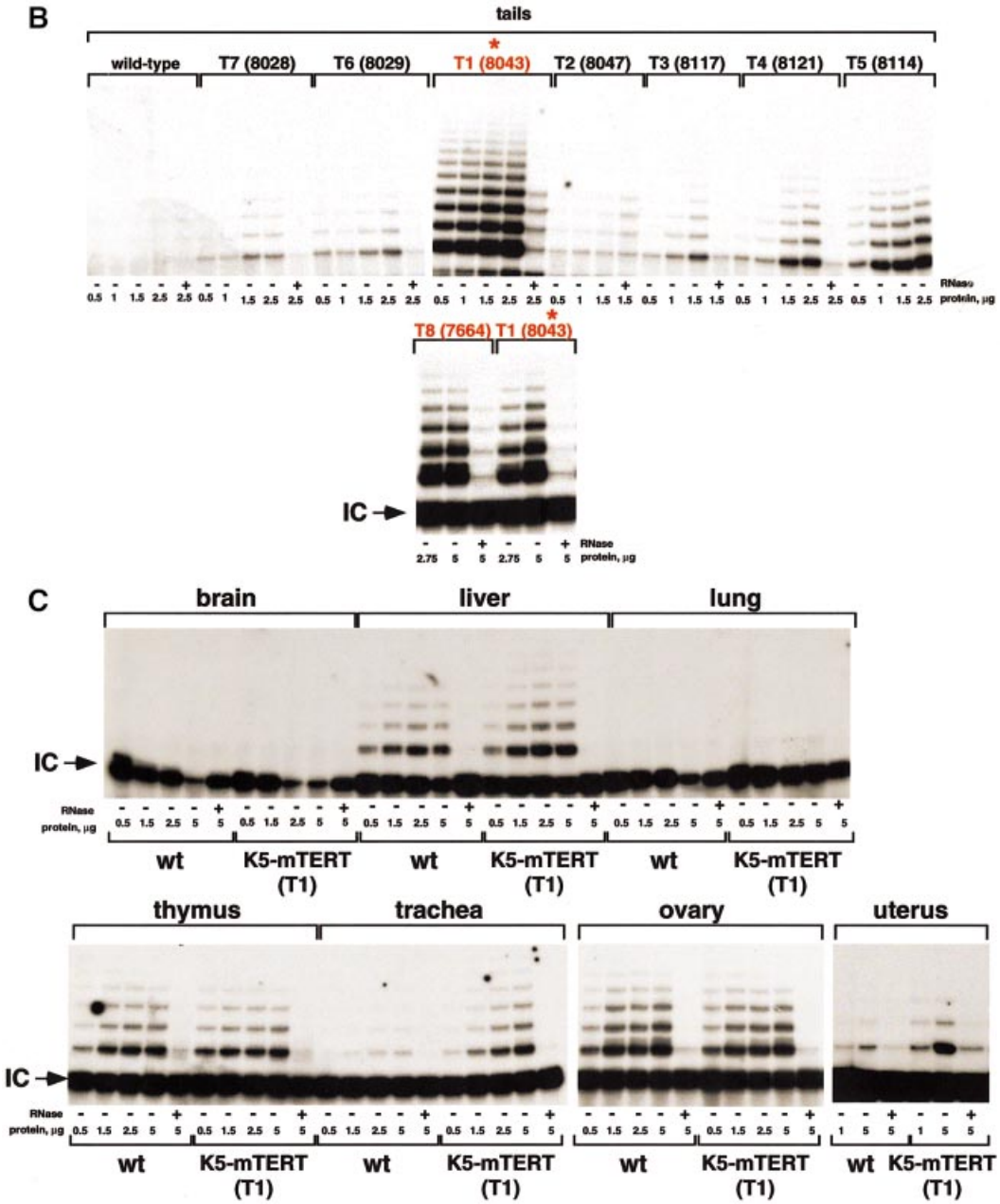


Fig. 1. Generation of K5-mTERT mice. (**A**) A scheme of the K5-mTERT transgene construct. The functional elements include a *Sal*I–*Nru*I fragment with the bovine K5 regulatory sequences (K5 promoter box), the rabbit β-globin intron 2 (G1 box), the coding sequence of the mTERT gene (mTERT box) and the SV40 early gene poly(A) addition signal (PA box). (**B**) Telomerase TRAP activity in the tail of wild-type and K5-mTERT founders (T1–T8). The two founders chosen for the study (T1 and T8) are highlighted in red. The asterisk highlights founder mouse T1, which was used for most of the experiments described here. (**C**) Telomerase TRAP activity in wild-type and K5-mTERT (T1) tissues. The indicated protein concentrations of the S-100 extract were used. Extracts were pre-treated (+) or not (–) with RNase A.

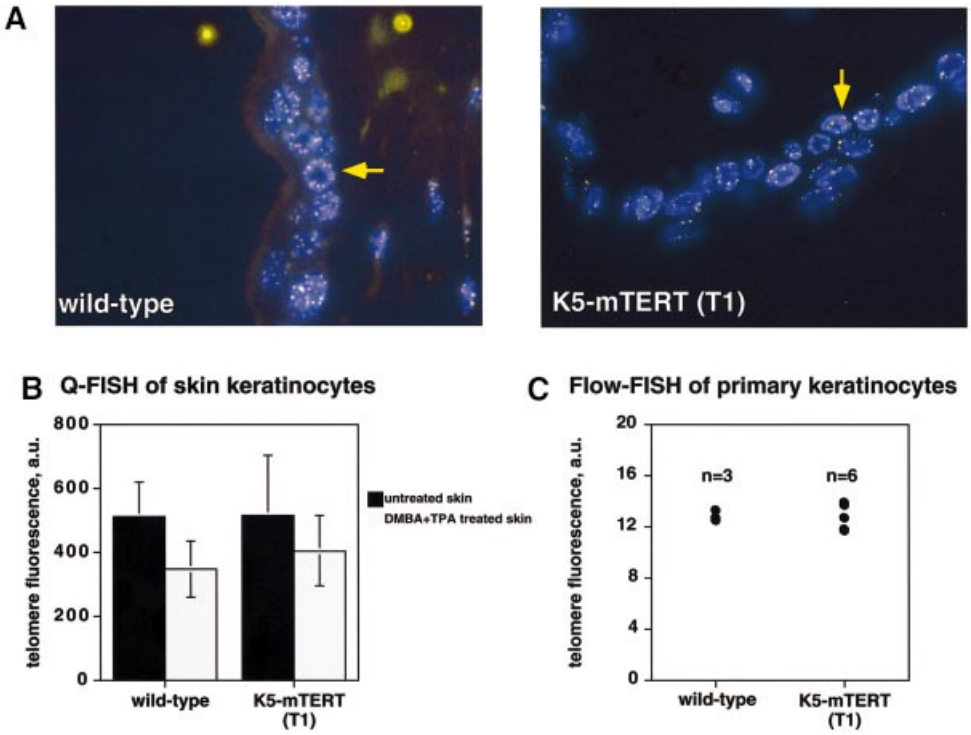


Fig. 2. Telomere fluorescence in skin sections. (**A**) Illustrative images showing telomere fluorescence in wild-type and K5-mTERT (T1) skin sections. The basal layer of skin keratinocytes is indicated (yellow arrow). (**B**) Quantification of telomere fluorescence on untreated skin sections or on skin sections treated with DMBA + TPA, as indicated. More than 50 keratinocyte nuclei of each genotype were analyzed by Q-FISH. (**C**) Average telomere fluorescence as determined by Flow-FISH of three wild-type and six K5-mTERT primary keratinocyte cultures. Between 2000 and 3000 nuclei were analyzed by flow cytometry for each culture.

maintaining the difference in total number of papillomas between wild-type and K5-mTERT (T1) littermates. In particular, 6.4 and 7.2% of the papillomas at week 15 progressed to lesions >1 cm at week 30, in wild-type and K5-mTERT (T1) mice, respectively (Figure 4A). Despite the difference in total number of papillomas, no histological differences were found between wild-type and K5-mTERT (T1) papillomas (Figure 6A; papilloma in wild-type and K5-mTERT T1 mice).

The increased susceptibility of transgenic K5-mTERT (T1) skin to develop papillomas due to chemical carcinogenesis was confirmed using K5-mTERT mice derived from a different founder, T8 (Figure 1B). K5-mTERT (T8) transgenics also showed an increased number of papillomas upon DMBA + TPA treatment as compared with the corresponding wild-type littermates (Figure 5).

Strikingly, 80% of the DMBA + TPA-treated K5-mTERT (T1) mice (eight out of 10) died before week 60 after the start of treatment, whereas 90% of the similarly treated wild-type cohorts survived during this time (nine out of 10; Figure 4C). Death of the DMBA + TPA-treated K5-mTERT (T1) mice was associated with several pathologies (see Table I for quantifications) including the following. (i) Severe gastric hemorrhages resulting from an abnormal forestomach epithelium, which showed a marked hyperplasia and hyperkeratosis (Figure 6A; compare the normal forestomach epithelium in DMBA + TPA-treated wild-type mice with the abnormal forestomach in DMBA + TPA-treated K5-mTERT mice). This observation suggests that other K5-mTERT (T1) stratified epithelia besides the skin are prone to tumorigenesis upon DMBA + TPA treatment. (ii) Benign epidermal lesions: numerous keratoacanthomas and blue nevi (melanocyte accumulations in the skin). (iii) Premalignant epidermal lesions: severe dysplastic papillomas. (iv) Malignant epidermal tumors: basal cell carcinomas, malignant keratoacanthomas. (v) Tumors of non-epithelial origin: subcutaneous fibrosarcoma, uterus histiocytic sarcoma, liver histiocytic sarcoma, cecum lymphoma, as well as infiltrations of tumoral lymphoid cells in various tissues (multicentric lymphomas).

Hence, the increased mortality of K5-mTERT mice upon DMBA + TPA carcinogenesis coincides with a higher susceptibility of these mice to develop neoplasias.

Telomeric Q-FISH analysis on skin sections from DMBA + TPA-treated mice showed that telomeres were similar or slightly elongated in K5-mTERT (T1) mice as compared with wild-type controls (Figure 2B; gray bars).

K5-mTERT skins are more sensitive to the mitogenic effects of phorbol esters

Curiously, whereas wild-type mice showed areas of histologically normal skin surrounding papillomas 7 weeks after termination of DMBA + TPA treatment (Figure 6A; normal skin), the DMBA + TPA-treated K5-mTERT (T1) mice maintained a marked hyperplasia of the skin, with regions showing up to 10 layers of keratinocytes (Figure 6A; hyperplastic skin). These observations suggested an increased proliferative response of K5-mTERT (T1) basal keratinocytes to repetitive TPA treatment. To study this further, wild-type and K5-mTERT (T1) mice

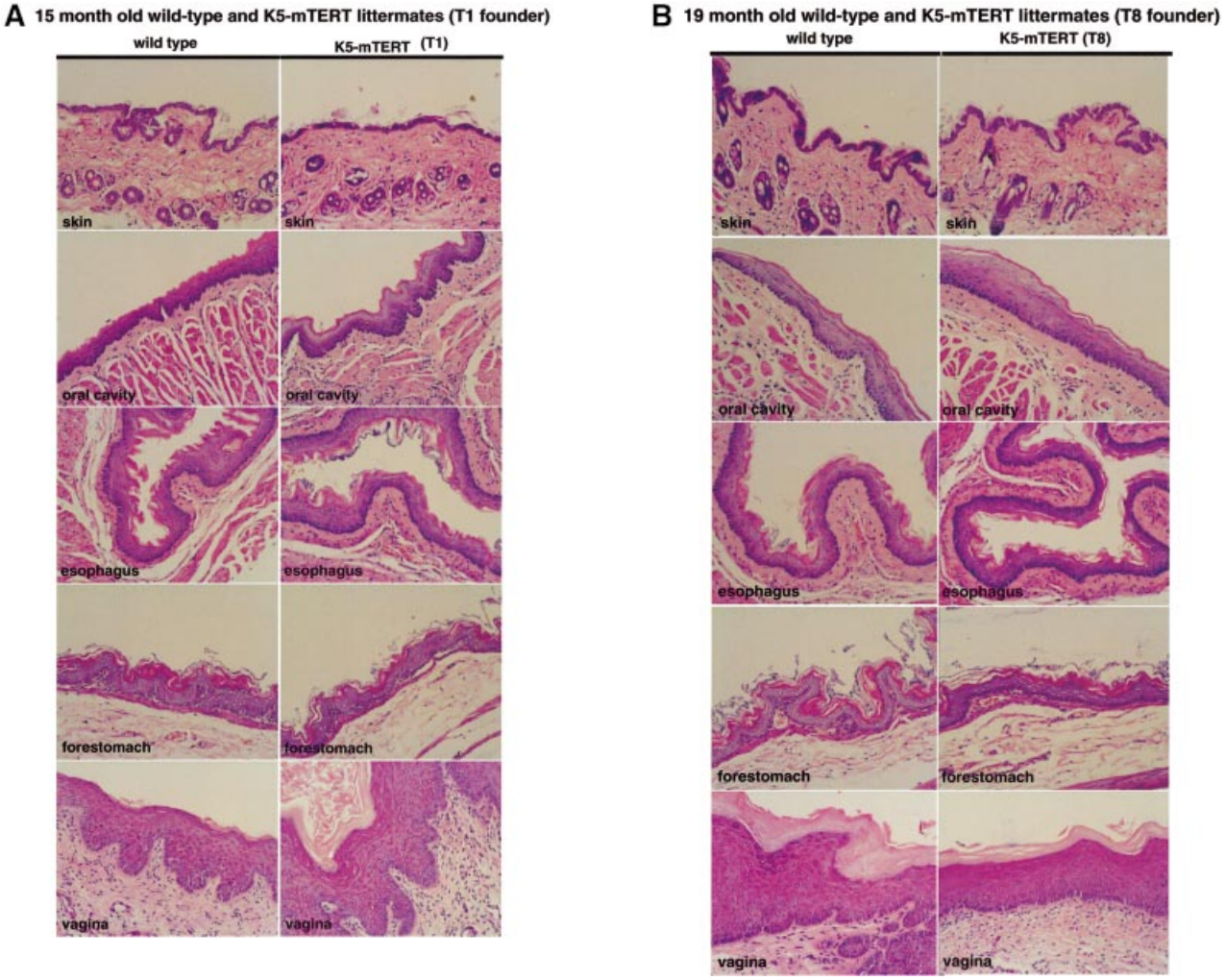


Fig. 3. (**A** and **B**) Histology of stratified epithelia in littermate 15- to 19-month-old wild-type and K5-mTERT (T1 or T8, as indicated) transgenics. The epithelia studied were skin, oral cavity, esophagus, forestomach and vagina. No significant histological differences between genotypes were observed. Magnification ×20.

were treated weekly with TPA (5 μg of TPA in 200 μl of acetone) in the absence of previous treatment with the carcinogen DMBA. After 3 weeks of repetitive TPA exposure, K5-mTERT (T1) mice showed a more marked hyperplasia of the skin than similarly treated wild-type mice (Figure 6B; hyperplastic skin at week 3 of TPA treatment). Strikingly, after prolonged (3 and 7 weeks) repetitive TPA exposure, the K5-mTERT (T1) mice showed incipient papilloma lesions in the skin, which were never detected in similarly treated wild-type mice (Figure 6B; incipient papillomas at weeks 3 and 7 of TPA treatment). Some of these incipient papilloma lesions in the K5-mTERT (T1) transgenics progressed to macroscopic papillomas upon further TPA treatment; again, this was never observed in the similarly treated wild-type littermates (not shown). Furthermore, probably due to licking of the skin-treated region, TPA-treated K5-mTERT mice also showed areas of focal hyperplasia in the esophagus and forestomach epithelia, which were absent in similarly treated wild-type mice (Figure 6B; hyperplastic esophagus at week 7 of TPA treatment).

Taken together, these results suggest that constitutive telomerase activity in stratified epithelia results in increased proliferation of basal keratinocytes in response to TPA treatment. This could be a way in which constitutive telomerase expression helps to promote tumor formation and progression in the K5-mTERT transgenic mice subjected to chemical carcinogenesis of the skin.

Increased wound healing in K5-mTERT epidermis as compared with wild-type epidermis

As an independent way to assay the functionality of K5-mTERT (T1) trangenic epithelia, we carried out wound-healing experiments in the skin of 2- to 4-month-old littermate wild-type and K5-mTERT (T1) transgenics. Wound healing is a complex process involving cellular proliferation, growth factor production and an immune system reaction. Two consecutive 13 mm^2 (4 mm diameter) circular punch biopsies were performed on the back skin of six wild-type and six K5-mTERT (T1) littermate mice. The rate of wound healing was monitored as the

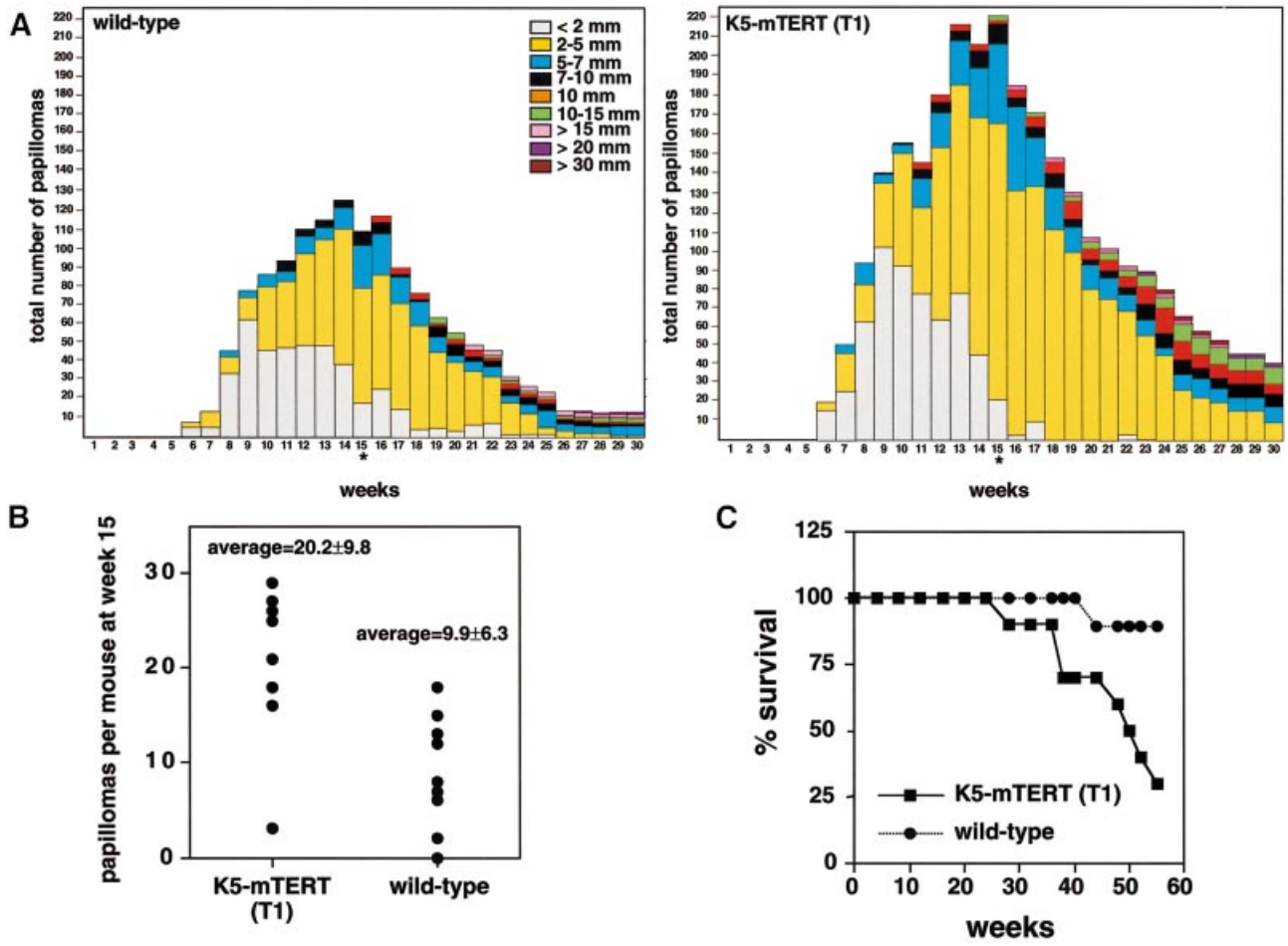


Fig. 4. (**A**) The total numbers of papillomas are plotted versus the number of weeks after the start of carcinogen treatment. Termination of TPA treatment (week 15) is indicated by an asterisk. (**B**) Average number of papillomas per mouse at week 15 after the start of carcinogen treatment. Wild-type and K5-mTERT (T1) transgenics showed an average of 9.9 ± 6.3 and 20.0 ± 9.8 papillomas per mouse, respectively. (**C**) Survival of DMBA + TPA-treated wild-type and K5-mTERT (T1) mice during the multistage chemical carcinogenesis experiment.

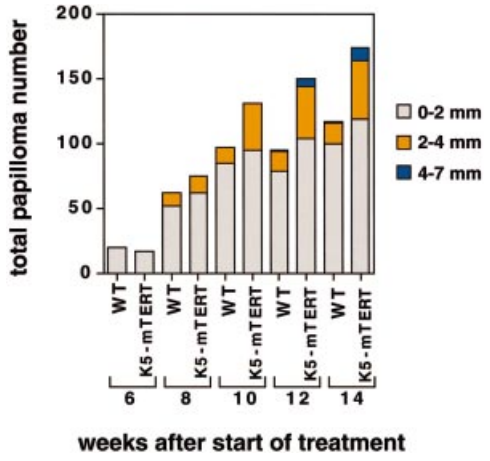


Fig. 5. The total numbers of papillomas of different sizes are plotted versus the number of weeks after the start of DMBA + TPA treatment in wild-type and K5-mTERT (T8) transgenics. The total number of mice is nine each for wild-type and K5-mTERT genotypes (T8).

percentage of the initial wound area left open with time after the punch was made (see Materials and methods). One wound was made 2 days after the other and the animals were killed 7 days after the first wound was made. K5-mTERT (T1) transgenics showed faster wound healing compared with wild-type littermates (Figure 7A–C). Figure 7A shows the percentage of the initial wound area open in wild-type and K5-mTERT (T1) mice (six mice in each group) 3 and 4 days after each wound was created. The average wound areas at day 3 were 82.85 ± 12.38 and 46.95 ± 8.3% for wild-type and K5-mTERT (T1) mice, respectively (Student's *t*-tests $P = 0.001$); and 75.45 ± 17.11 and 30.8 ± 15.5% for wild-type and K5-mTERT (T1) mice, respectively, at day 4 (Student's *t*-tests $P = 0.0031$) (Figure 7A). These differences in wound healing between genotypes were highly significant, as indicated by the Student's *t*-test values ($P < 0.01$). As examples, Figure 7B and C shows the rate of healing of the two wounds (wound 1 and 2) in two representative pairs of wild-type and K5-mTERT (T1) littermates. Figure 7D shows representative images of wild-type and K5-mTERT (T1) wounds 4 days after the first wound was made (1 and 2 in Figure 7D correspond to the first and second wound, respectively); the two wild-type wounds remained opened whereas the two K5-mTERT (T1) wounds were already closed. As a standard for wound size, fresh punch biopsies were also made prior

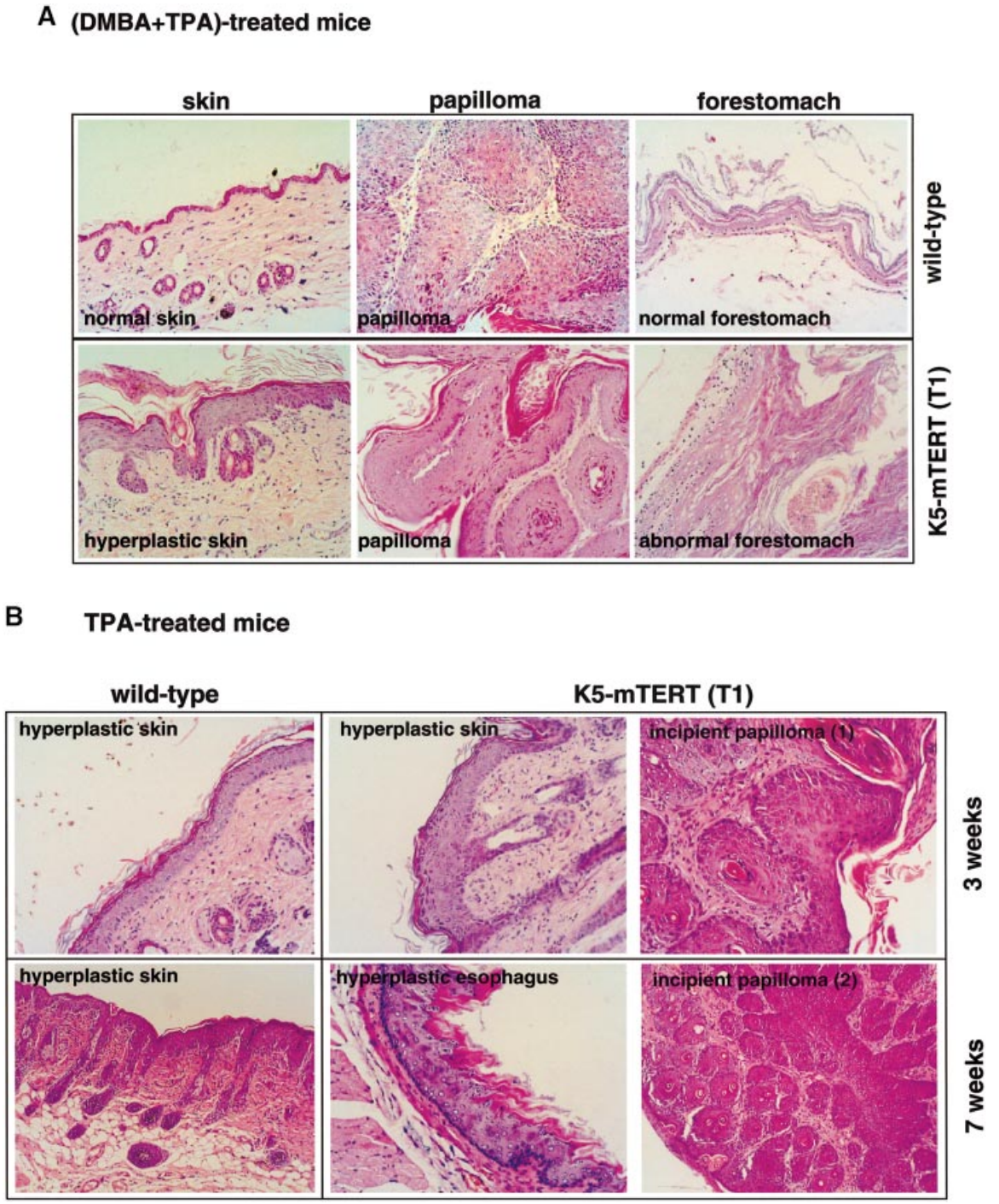


Fig. 6. Histopathology of skin lesions. (**A**) Both DMBA + TPA-treated wild-type and K5-mTERT (T1) mice show typical papilloma lesions in the skin (see 'papilloma'). Seven weeks after termination of DMBA + TPA treatment, the DMBA + TPA-treated K5-mTERT (T1) mice show skin with severe hyperplasia (see 'hyperplastic skin'); this is not observed in the skin of similarly treated wild-type mice (see 'normal skin'). A forestomach lesion showing marked hyperplasia and hyperkeratosis corresponding to a DMBA + TPA-treated K5-mTERT (T1) mouse is shown (see 'abnormal forestomach'); these lesions were never detected in similary treated wild-type mice (see 'normal forestomach'). Magnification ×20. (**B**) Skin lesions in wild-type and K5-mTERT (T1) trangenics after repeated weekly exposure to TPA (no DMBA). Incipient papilloma lesions are shown for TPA-treated K5-mTERT (T1) mice (see 'incipient papilloma'); these lesions were never detected in similarly treated wild-type mice. A hyperplastic esophagus lesion is shown for TPA-treated K5-mTERT (T1) mice (see 'hyperplastic esophagus'); these lesions were never found in similarly treated wild-type mice. Magnification ×20.

to sacrifice of the mice (marked with a 3 in Figure 7D). Histology of the wounds confirmed a faster rate of re-epithelilization of the K5-mTERT (T1) wounds as compared with wild-type littermates (not shown).

Taken together, these results indicate that the skin of K5-mTERT (T1) mice has a faster wound-healing rate than that of the corresponding wild-type littermates. This may reflect a proliferative advantage of telomerase-

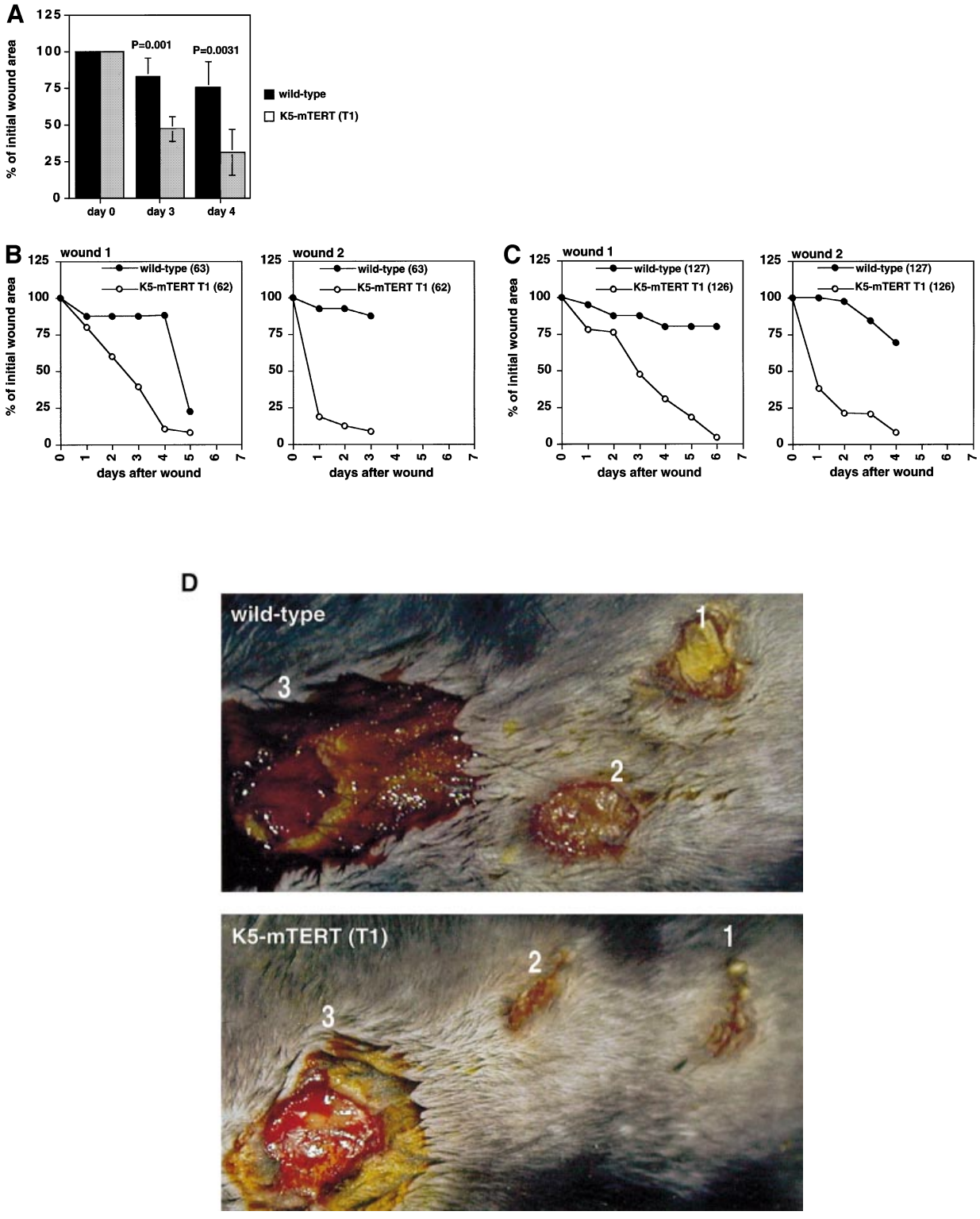


Fig. 7. Wound healing of wild-type and K5-mTERT (T1) skin. (**A**) Percentage of initial wound area left 3 and 4 days after the punch was created in a group of six wild-type and six K5-mTERT (T1) mice. Statistical analysis showing that the healing rate is significantly different between genotypes is also shown (*P*-values). (**B** and **C**) Rate of wound healing in two wild-type and K5-TERT (T1) littermate pairs; the results are presented as the percentage of open wound area versus time after wound creation. Closed circles, wild-type mice; open circles, K5-mTERT (T1) transgenics. (**D**) Gross appearance of healing wounds in a wild-type and a K5-mTERT (T1) mouse 5 and 2 days, for wounds 1 and 2, respectively, after wound creation. Just prior to sacrifice of the mice, a third wound (wound 3) was created as an indicator of initial wound areas.

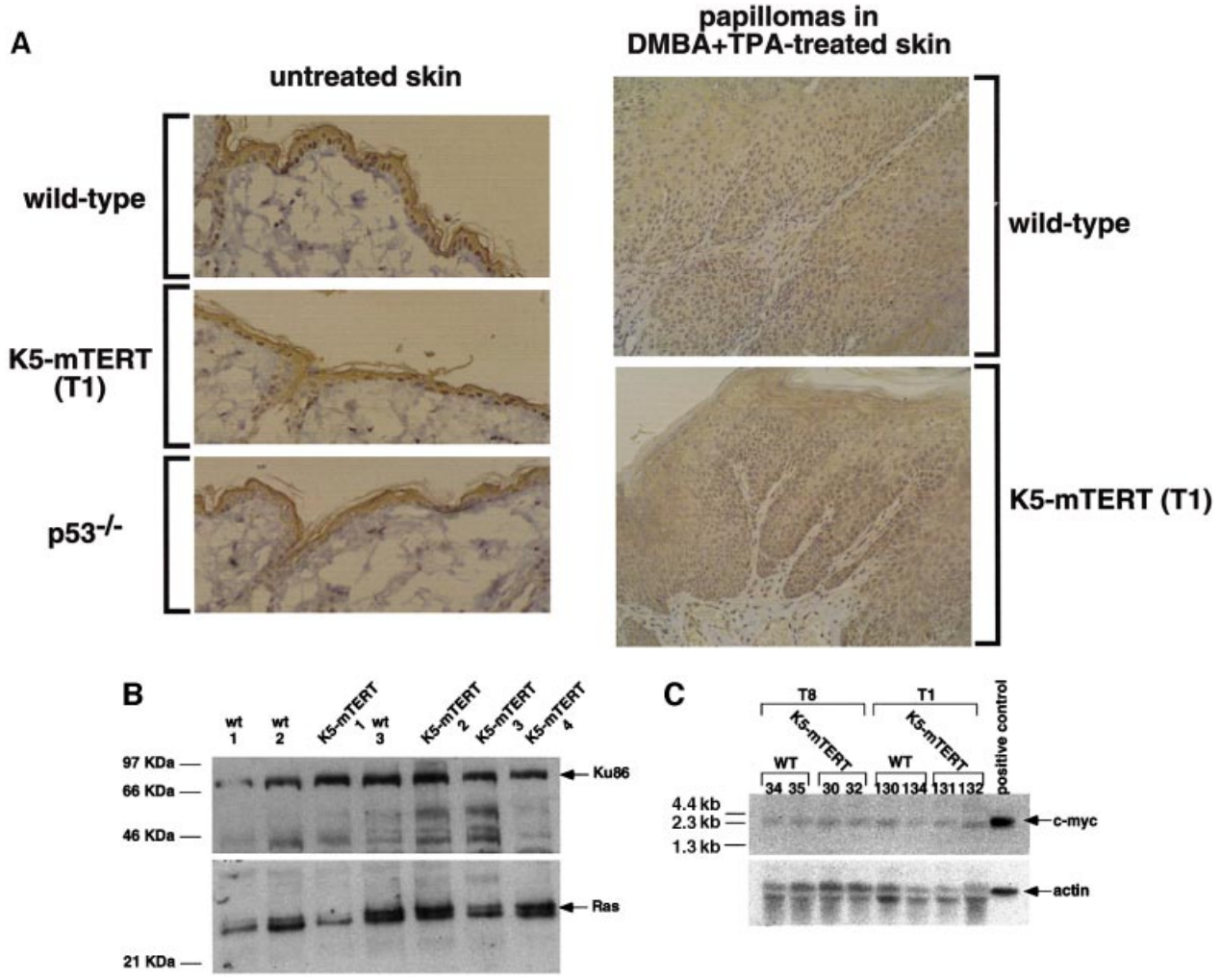


Fig. 8. (**A**) p53 levels in wild-type and K5-mTERT (T1) skin keratinocytes. Immunohistochemistry with p53 antibody of untreated and DMBA + TPA-treated wild-type and K5-mTERT (T1) skin. The control untreated wild-type and K5-mTERT (T1) skin show similar p53 positivity in basal keratinocyte nuclei; $p53^{-/-}$ mouse skin is shown as a negative control. Wild-type and K5-mTERT (T1) papillomas show decreased p53 staining of basal keratinocytes compared with wild-type skin. Magnification ×20. (**B**) Ras protein levels detected by western blot in wild-type and K5-mTERT (T1) primary keratinocytes. No significant differences in Ras protein levels were detected between genotypes. As a control for loading, nuclear protein Ku70 was also detected. Different numbers indicate different primary keratinocyte cultures. The arrows indicate the positions of theRas- and Ku86-specific bands. (**C**) c-*myc* mRNA levels detected by northern blot in the skin of four K5-mTERT (T1 or T8, as indicated) and the corresponding wild-type littermate skins. Mice 30, 32, 34 and 35 are littermates derived from founder T8. Mice 130, 131, 132 and 134 are littermates derived from founder T1. As a positive control for c-*myc* detection, total RNA from interleukin-2-stimulated Ba/FO3 cells was used (Hatakeyama *et al*., 1989). As control for loading, actin was also detected in the blots. Table II shows quantification of c-*myc* levels.

transgenic skins as compared with controls in response to proliferative signals associated with wound healing, in agreement with the greater hyperplasia seen in K5-mTERT epithelia as compared with wild-type littermates upon treatment with the mitogen TPA.

Normal p53, Ras and c-Myc levels in K5-mTERT skin

p53 loss of function and H-*ras* upregulation are described as occurring in multistage chemical carcinogenesis of the skin, and are thought to favor the appearance of tumors (Balmain *et al*., 1984, 1992; Quintanilla *et al*., 1986). To determine whether constitutive telomerase expression induces changes associated with transformation in the skin, we studied several tumor markers such as p53, Ras and c-Myc in the skin of wild-type and littermate K5-mTERT (T1) mice. To analyze p53 status in skin keratinocytes, we performed immunohistochemistry of the skin with anti-p53 antibody, as previously described (González-Suárez *et al*., 2000) (see Materials and methods). There is no significant variation in p53 levels between wild-type and K5-mTERT (T1) untreated skin or DMBA + TPA-induced papillomas (Figure 8A), suggesting that transgenic telomerase expression does not affect normal p53 levels; as a control, we show the skin of a p53-deficient mouse (Figure 8A). Furthermore, there was no significant difference in Ras protein levels in nuclear extracts prepared from wild-type and K5-mTERT (T1)

Table I. Non-papilloma lesions detected in DMBA + TPA-treated wild-type and K5-mTERT transgenics that died before week 60 after the start of treatment

	Total no. of lesions	No. of mice[a]	Weeks after start of treatment[b]
Non-papilloma epithelial lesions[c]			
keratoacanthoma	9	4	38–50
blue nevus	17	6	38–58
hyperplastic forestomach	3	3	28–38
basal cell tumor	3	3	38–58
severely dysplastic papilloma	1	1	28–50
malignant keratoacanthoma	3	1	50
Non-epithelial lesions[c]			
uterus histiocytic sarcoma	1	1	48
liver histiocytic sarcoma	1	1	52
lymphoma of cecum	1	1	38
multicentric lymphoma	1	1	50
subcutaneus fibrosarcoma	1	1	48
lymphoid hyperplasia	1	1	44
Non-epithelial lesions[d]			
subcutaneus fibrosarcoma	1	1	44

[a]Number of mice that showed the lesion at the time of death before week 60 after the start of DMBA + TPA treatment. The lesions were counted in eight K5-mTERT transgenics and one wild-type littermate that died after the start of the DMBA + TPA treatment.
[b]Time at which lesions appeared (in weeks) after the start of DMBA + TPA treatment.
[c]K5-mTERT transgenics.
[d]Wild-type mice.

Table II. Quantification of c-*myc* mRNA levels in the skin of K5-mTERT (T1 and T8, as indicated) and corresponding wild-type littermates

	c-*myc* (AU)	Actin (AU)	Relative c-*myc* abundance (%)[a]
Wild-type 34 (T8)	57 050.67	409 571.97	127
Wild-type 35 (T8)	72 780.37	558 146.15	118
K5-mTERT 30 (T8)	73 277,28	661 005.82	101
K5-mTERT 32 (T8)	80 397.53	622 336.42	117
Wild-type 130 (T1)	84 549.74	675 757.15	114
Wild-type 134 (T1)	53 872.73	491 477.81	**100**
K5-mTERT 131 (T1)	61 120.25	478 277,03	116
K5-mTERT 132 (T1)	83 926.68	595 155,99	128
Control c-*myc*	200 557.77	503 331.87	363

AU, arbitrary units after quantification with the phosphoimager; T8, mice derived from the T8 founder; T1, mice derived from the T1 founder. Mice 30,32, 34 and 35 are littermates, and mice 130, 131, 132 and 134 are littermates.
[a]Relative abundance of c-*myc* corrected by actin levels and expressed as a percentage. Levels were considered 100% in one of the wild-type mice showing the lowest c-*myc* levels (indicated in bold).

primary keratinocyte cultures, as determined by western blot (Figure 8B). Detection of Ku70 nuclear protein was used as a loading control (Figure 8B).

It has been shown recently that TERT-driven cell proliferation in cultured cells can result in the activation of the c-*myc* oncogene (Wang *et al*., 2000). We studied c-*myc* mRNA levels in the skin from several K5-mTERT (T1 and T8, as indicated) mice and corresponding wild-type littermate controls. No significant differences in the c-*myc* mRNA levels were observed between genotypes (see Materials and methods; Figure 8C and Table II for quantitation of c-Myc levels using a phosphoimager), suggesting that transgenic mTERT expression in the skin does not result in c-*myc* upregulation.

Conclusions

We show here that constitutive high levels of telomerase activity in stratified epithelia do not alter the normal epithelium structure and are not associated with changes in p53, Ras or c-Myc levels, in agreement with previous reports showing that TERT immortalized human cultured cells do not show changes associated with transformation (Jiang *et al*., 1999; Morales *et al*., 1999). However, following DMBA + TPA treatment of the skin, K5-mTERT (T1 and T8) mice are significantly more susceptible to the development of neoplasias than the corresponding wild-type littermates. This increased tumor susceptibility of K5-mTERT transgenic skin is accompanied by a greater proliferation of basal keratinocytes upon TPA treatment, as well as by an increased would-healing rate of the K5-mTERT skin compared with wild-type mice. All these observations are in agreement with a direct or indirect role for telomerase in signaling proliferation under mitogenic conditions. These results agree with previous observations that indicated that first generation *Terc*$^{-/-}$ mice, which lack telomerase activity but still have long telomeres, show a lower incidence of papillomas than wild-type mice (González-Suárez *et al*., 2000). These findings unravel the potential risks of gene therapy for age-associated diseases based on telomerase upregulation, and indicate that telomerase upregulation, which occurs in the vast

majority of mouse and human tumors, actively promotes tumor growth even in the presence of long telomeres. Since the average length of telomeres in wild-type and K5-mTERT skin was similar, it is tempting to speculate that telomere conformation must be different whether or not telomerase activity is present in the cell, and that telomeres that are being 'maintained' by telomerase could be signaling proliferation and/or survival under mitogenic conditions.

Finally, K5-mTERT mice constitute a new experimental model to study the direct impact of transgenic telomerase expression in both skin tumorigenesis and aging *in vivo*. Tissue-specific telomerase-transgenic mice are essential to study the impact of potential use of telomerase for gene therapy of age-related diseases. K5-mTERT mice also constitute a new animal model system to study the effectiveness of anti-telomerase therapies in cancer.

Materials and methods

Generation and genotyping of transgenic K5-mTERT mice

An *Eco*RI fragment containing the full-length mTERT cDNA and 3′-untranslated sequences was cloned 3′ downstream of 5 kb of bovine keratin K5 regulatory sequences (see Figure 1A) (Ramírez *et al*., 1994; Martín-Rivera *et al*., 1998). The transgene was digested with *Not*I, purified using Genclean (Bio 101 Inc.), adjusted to a final concentration of ~2 μg/ml and microinjected into (C57BL/6 × DBA/2) mouse embryos, as described (Hogan *et al*., 1986). Eight different founder mice were identified by Southern blot analysis of tail DNA, using the 5 kb fragment corresponding to the bovine keratin K5 promoter as a probe (not shown). The experiments described here were carried out with founder T1 and were reproduced, as indicated, with founder T8, which shows a similar upregulation of telomerase activity (T1 = 8043 and T8 = 7664; highlighted in red in Figure 1B). The other founders showing lower reconstitution of telomerase activity in the skin were not included in this study.

Handling of mice

Mice were housed in our 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 FELASA recommendations (Federation of European Laboratory Animal Science Associations).

Telomerase assays

Telomerase activity was measured with a modified telomeric repeat amplification protocol (TRAP), as described (Blasco *et al*., 1997).

Tumor induction experiments

Ten and 12 age-matched (8- to 12-week-old) mice of each genotype, wild-type and K5-mTERT (T1), respectively, were shaved and treated with a single dose of DMBA (0.1 μg/μl in acetone; Sigma). Nine age-matched (8-week-old) mice of each genotype, wild-type and K5-mTERT (T8), were shaved and treated with a single dose of DMBA (0.1 μg/μl in acetone; Sigma). In all cases, subsequently mice were treated twice weekly with TPA (12.5 μg in 200 μl acetone each treatment; Sigma), for 15 weeks. Four control mice of each genotype were treated with acetone only. In samples analyzed after weekly cumulative TPA doses, the TPA concentration used was 12.3 μg in 200 μl of acetone.

Histopathological analyses and immunochemistry

Papilloma, skin, esophagus, forestomach, oral mucosa and vagina sections from 15- to 19-month-old wild-type and K5-mTERT (T1 or T8, as indicated) mice were fixed in 10% buffered formalin and stained with hematoxylin–eosin. Images were captured with an Olympus-Vanox microscope at a 20× magnification.

For the DMBA + TPA-treated mice that died, necropsy of animals was performed and all organs were examined macroscopically. Tumoral and normal tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections of 4 μm were stained with hematoxylin–eosin. Images were captured with an Olympus-Vanox microscope at a 20× magnification.

Immunohistochemistry for p53 was performed on deparaffinated sections (Vectabond slides) after pressure cooker processing. For p53 detection, we used a streptavidin–biotin complex technique, with a polyclonal rabbit anti-mouse p53 antibody, CM5 (Novocastra Laboratory Ltd; 1:1500 dilution, overnight, 4°C), and a biotinylated anti-rabbit IgG (Vector Laboratory; 1:400 dilution, 30 min, room temperature), followed by streptavidin peroxidase (Zymed Laboratory, Inc.; 1:20 dilution, 30 min, room temperature); the chromogen was developed with diaminobenzidine, and slides were counterstained with hematoxylin. Control slides were obtained by replacing the primary antibody with TBS (not shown) (see also González-Suárez *et al*., 2000).

Wound-healing experiments

Two to three full-thickness punch biopsies extending through the epidermis and dermis (punch diameter 4 mm; PFM, Köln, Germany) were performed in six wild-type and six K5-mTERT mice (2–4 months of age) after depilation. Mice were anesthesized prior to wound creation. The wound-healing rate was calculated as the percentage of initial wound area with time. Wound areas were calculated with the formula (area = πr^2; where r is the ratio of the wound).

Telomere length measurements

For Q-FISH, paraffin-embedded skin sections (untreated or treated with DMBA + TPA) were hybridized with a PNA-tel probe, and telomere length was determined as described (Zijlmans *et al*., 1997; González-Suárez *et al*., 2000). Slides were deparaffinated in three xylene washes (3 min each), then treated for 3 min with a 100, 95 and 70% ethanol series. More than 50 keratinocyte nuclei from each genotype were captured at a 100× magnification and the telomere fluorescence was integrated using spot IOD analysis in the TFL-TELO program. For Flow-FISH, primary mouse keratinocytes (10^5) were prepared (Hennings, 1994) and hybridized using the Flow-FISH protocol (Rufer *et al*., 1998). Telomere fluorescence of 2000–3000 nuclei gated at the G_1–G_0 cell cycle stage was measured using a Coulter EPICS XL flow cytometer with SYSTEM 2 software. The same cells were hybridized in parallel without the PNA–fluorescein isothiocyanate (FITC) probe to obtain background fluorescence values, which were subtracted from the fluorescence intensity of each sample. We used three wild-type and six K5-mTERT (T1) primary keratinocyte cultures.

Western blots

Nuclear extracts were prepared from three primary wild-type and four K5-mTERT (T1) keratinocyte cultures (Hennings, 1994), and western blots were performed as previously described (González-Suárez *et al*., 2000). The protein concentration per well was 20 μg. For Ras detection, pan-ras antibody (Ab-3; Calbiochem) at a 1:1000 dilution was used. For Ku86 detection, anti-human p70 (Hamiya Biomedical Company, 1:000 dilution) was used. The percentage of the acrylamide gel was 15%.

Northern blots

Total RNA was extracted from the skin of a total of four wild-type and four K5-mTERT (T1 or T8, as indicated) littermate mice essentially as described (Chomczynski and Sacchi, 1987). A 50 μg aliquot of RNA was used per lane in the northern blot. A 1500 nucleotide fragment of the human c-*myc* gene was used as probe for murine c-*myc* mRNA detection; as a positive control, total RNA from murine lymphocyte cell line Ba/FO3 upon interleukin-2 stimulation was used (Hatakeyama *et al*., 1989). As control for loading, actin was also detected in the blots.

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