Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis

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Inhibition of telomerase is proposed to limit the growth of cancer cells by triggering telomere shortening and cell death[1–9]. Telomere maintenance by telomerase is sufficient, in some cell types, to allow immortal growth[1–5]. Telomerase has been shown to cooperate with oncogenes in transforming cultured primary human cells into neoplastic cells, suggesting that telomerase activation contributes to malignant transformation[6]. Moreover, telomerase inhibition in human tumour cell lines using dominant-negative versions of TERT leads to telomere shortening and cell death[7,8]. These findings have led to the proposition that telomerase inhibition may result in cessation of tumour growth[9]. The absence of telomerase from most normal cells supports the potential efficacy of anti-telomerase drugs for tumour therapy, as its inhibition is unlikely to have toxic effects. Mice deficient for *Terc* RNA (encoding telomerase) lack telomerase activity, and constitute a model for evaluating the role of telomerase and telomeres in tumourigenesis[10]. Late-generation *Terc*$^{-/-}$ mice show defects in proliferative tissues[10–15] and a moderate increase in the incidence of spontaneous tumours in highly proliferative cell types (lymphomas, teratocarcinomas[12]). The appearance of these tumours is thought to be a consequence of chromosomal instability in these mice[10,12,16,17]. These observations have challenged the expected effectiveness of anti-telomerase–based cancer therapies[18]. Different cell types may nonetheless vary in their sensitivity to the chromosomal instability produced by telomere loss or to the activation of telomere-rescue mechanisms. Here we show that late-generation *Terc*$^{-/-}$ mice, which have short telomeres and are telomerase-deficient[10], are resistant to tumour development in multi-stage skin carcinogenesis. Our results predict that an anti-telomerase–based tumour therapy may be effective in epithelial tumours.

The stages of initiation, promotion and tumour progression in the skin carcinogenesis model are well characterized[19,20]. In normal mice, initiation using 7,12-dimethylbenz(a)anthracene (DMBA) and promotion with 12-O-tetradecanoylphorbol 13-acetate (TPA) provoke papillomas that are hyperplastic, well-differentiated skin lesions. *H-RAS* activation and telomerase upregulation are reported to occur in most DMBA-initiated papillomas[21–23]. After a latency period, a percentage of papillomas progress to carcinoma.

To test the impact of telomerase deficiency in skin tumour progression, we gave wild-type, first-generation (G1) *Terc*$^{-/-}$ or fifth-generation (G5) *Terc*$^{-/-}$ mice a single DMBA treatment and TPA-acetone treatment twice a week for 15 weeks. Seven weeks after DMBA treatment, papillomas appeared in both wild-type and G1 *Terc*$^{-/-}$ animals; these papillomas continued to grow throughout treatment (Fig. 1*a*,*b*). All carcinogen-treated wild-type and G1 *Terc*$^{-/-}$ mice developed several papillomas, a total of approximately 110 and approximately 80 papillomas in wild-type and G1 *Terc*$^{-/-}$ mice at week 16, respectively (Fig. 1*a*). In G1 *Terc*$^{-/-}$ mice, the number, size and growth rate of papillomas was reduced compared with wild-type mice (30% less; Fig. 1*a*,*b*). This decrease may be attributed to the absence of telomerase activity *per se*[23] or to the fact that G1 *Terc*$^{-/-}$ telomeres are on average 3–5 kb shorter than those of wild type[10]. Histological analysis of carcinogen-treated wild-type and G1 *Terc*$^{-/-}$ skin showed that papillomas consisted of skin folds integrated by a core of connective tissue and lined by an acanthotic, hyperkeratotic, stratified squamous epithelium. We found moderately dysplastic changes, such as altered polarity in basal cells and mitosis in the suprabasal layer (Fig. 2). Papilloma number was reduced in G5 *Terc*$^{-/-}$ mice compared with wild-type and G1 *Terc*$^{-/-}$ animals (Fig. 1*a*,*b*). We detected five papillomas in the carcinogen-treated G5 *Terc*$^{-/-}$ mice at the end of TPA treatment (compare with ~110 and ~80 papillomas in wild-type and G1 *Terc*$^{-/-}$ mice,

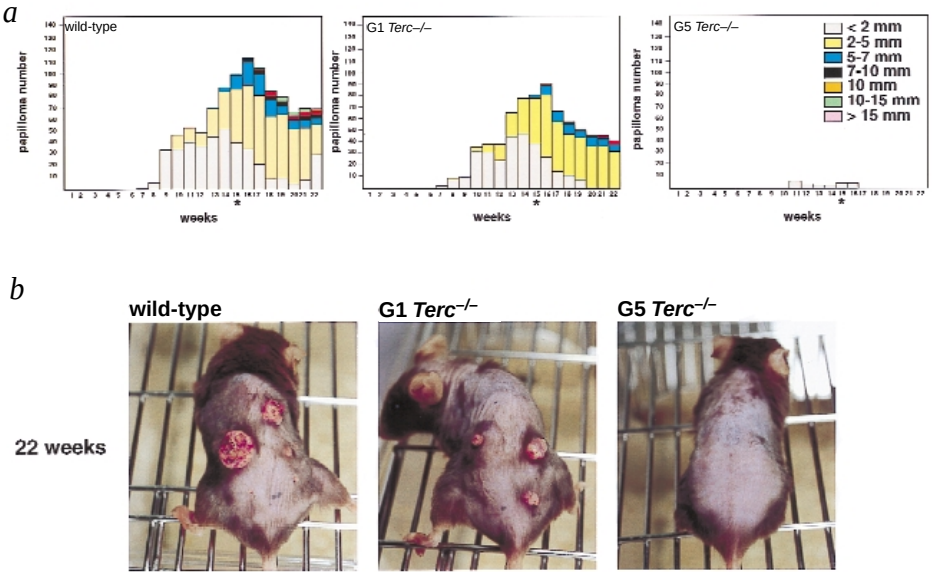

Fig. 1 Papillomas in wild-type and *Terc*$^{-/-}$ mice. ***a***, Total numbers of papillomas of different sizes are plotted versus the number of weeks after the start of carcinogen treatment. The end of TPA treatment (week 15) is indicated by an asterisk. ***b***, Examples of skin lesions in wild-type and *Terc*$^{-/-}$ mice at week 22 after the start of treatment.

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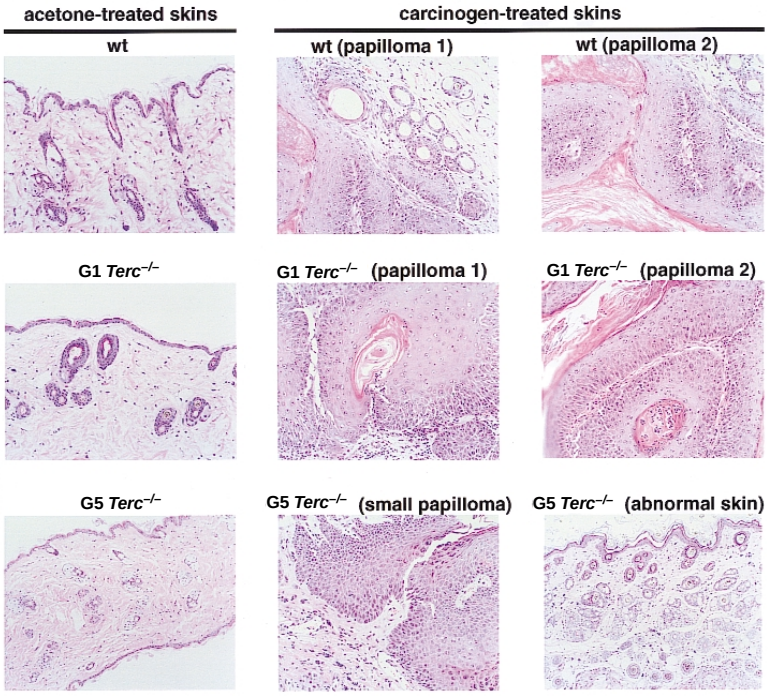

Fig. 2 Histopathology of skin lesions. The wild-type and G1 papillomas shown are of similar sizes. In the case of carcinogen-treated G5 *Terc*$^{-/-}$ skin, a small papilloma and abnormal skin showing chronic dermatitis are shown.

respectively), and none was larger than 2 mm (Fig. 1*a*). All G5 *Terc*$^{-/-}$ papillomas regressed within one week after termination of TPA treatment (Fig. 1*a*). Moreover, all wild-type and G1 *Terc*$^{-/-}$ mice developed papillomas, whereas only 37% of the carcinogen-treated G5 *Terc*$^{-/-}$ mice ever developed a papilloma. An incipient papilloma in a carcinogen-treated G5 *Terc*$^{-/-}$ mouse is shown (Fig. 2). Most G5 *Terc*$^{-/-}$ mice (acetone- and TPA-treated), but none of the wild-type or the G1 *Terc*$^{-/-}$ mice, developed areas of thickened skin in the neck and anterior part of the dorsal surface, which did not coincide with the area to which carcinogens had been applied. Histology of abnormal G5 *Terc*$^{-/-}$ skin showed chronic hyperplastic dermatitis with moderate acanthosis, hyperkeratosis and follicular infundibulum thickening, and a interstitial inflammatory infiltrate consisting of mast cells (Fig. 2, G5 *Terc*$^{-/-}$ abnormal skin). These inflammatory lesions in G5 *Terc*$^{-/-}$ skin may be triggered by repeated acetone exposure; indeed, the lesions regressed at the termination of TPA treatment.

The resistance of G5 *Terc*$^{-/-}$ skin to carcinogen-induced tumourigenesis may be a direct consequence of shorter telomeres in G5 *Terc*$^{-/-}$ skin. We measured average telomere length in wild-type, G1 and G5 *Terc*$^{-/-}$ skin by quantitative fluorescence *in situ* hybridization (Q–FISH) on skin sections (Fig. 3*a*). Quantification of fluorescence intensity of telomeric dots showed that average telomere fluorescence in G5 *Terc*$^{-/-}$ skin is 41% that of the wild-type skin (Fig. 3*b*). G5 *Terc*$^{-/-}$ telomeres were also shorter than those of wild-type and G1 *Terc*$^{-/-}$ cohorts when we measured telomere length in several primary keratinocytes cultures using flow cytometry FISH (FLOW FISH; Fig. 3*b*). These results demonstrate that G5 *Terc*$^{-/-}$ skin keratinocytes have shorter telomeres than those of wild-type and G1 *Terc*$^{-/-}$ mice.

Mouse embryonic fibroblasts and testis from late-generation *Terc*$^{-/-}$ mice show induction of p53 protein concentrations with respect to wild type, consistent with a role for p53 in the response to telomere loss[16]. To determine whether p53 is elevated in G5 *Terc*$^{-/-}$ skin compared with wild-type skin, we analysed normal skin and papillomas by immunohistochemistry using an anti-p53 antibody. Western-blot analysis was used to detect p21 concentrations in wild-type, G1 and G5 *Terc*$^{-/-}$ keratinocytes. We found similar p53 reactivity in basal keratinocyte nuclei of all genotypes (Fig. 4*a*), suggesting that p53 concentrations are not elevated in G5 *Terc*$^{-/-}$ skin compared with wild-type skin. As a negative control, we examined the skin from a *Trp53*$^{-/-}$ mouse. p21 concentrations are also similar in nuclear extracts from wild-type, G1 and G5 primary keratinocytes (Fig. 4*b*). p53 concentrations decreased in wild-type and G1 *Terc*$^{-/-}$ papillomas compared with the corresponding normal skin (Fig. 4*a*). A small G5 *Terc*$^{-/-}$ papilloma showed higher p53 reactivity in basal layer nuclei than did wild-type and G1 *Terc*$^{-/-}$ papillomas; this G5 *Terc*$^{-/-}$ papilloma also showed p53-positive nuclei in the suprabasal layer (Fig. 4*a*). The higher p53 concentrations detected in this papilloma may be the consequence of short telomeres and may prevent further tumour development.

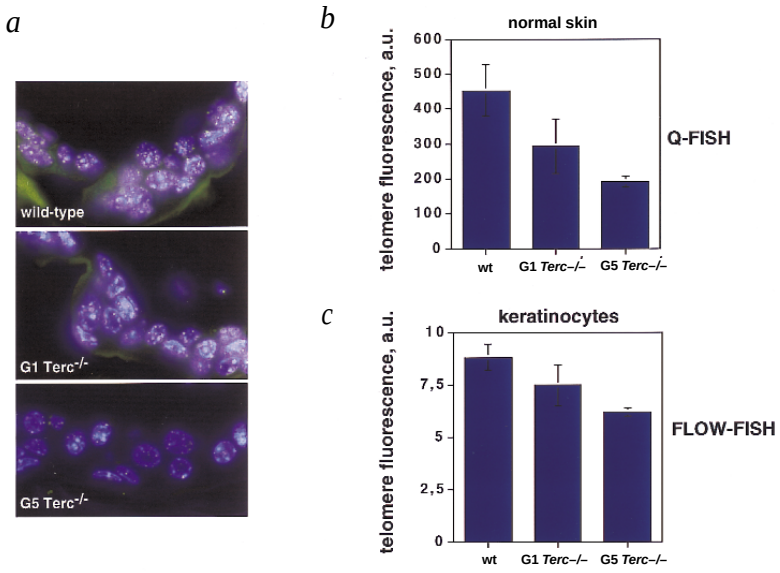

Fig. 3 Telomere fluorescence in skin sections. ***a***, Basal keratinocyte telomere fluorescence in wild-type, G1 and G5 *Terc*$^{-/-}$ skin sections. ***b***, Quantification of telomere fluorescence in skin sections. More than 50 basal keratinocyte nuclei of each genotype were analysed by Q–FISH. ***c***, Average telomere fluorescence as determined by FLOW FISH of six wild-type, six G1 and six G5 *Terc*$^{-/-}$ primary keratinocyte cultures. Between 2,000 and 3,000 nuclei were analysed for each individual culture.

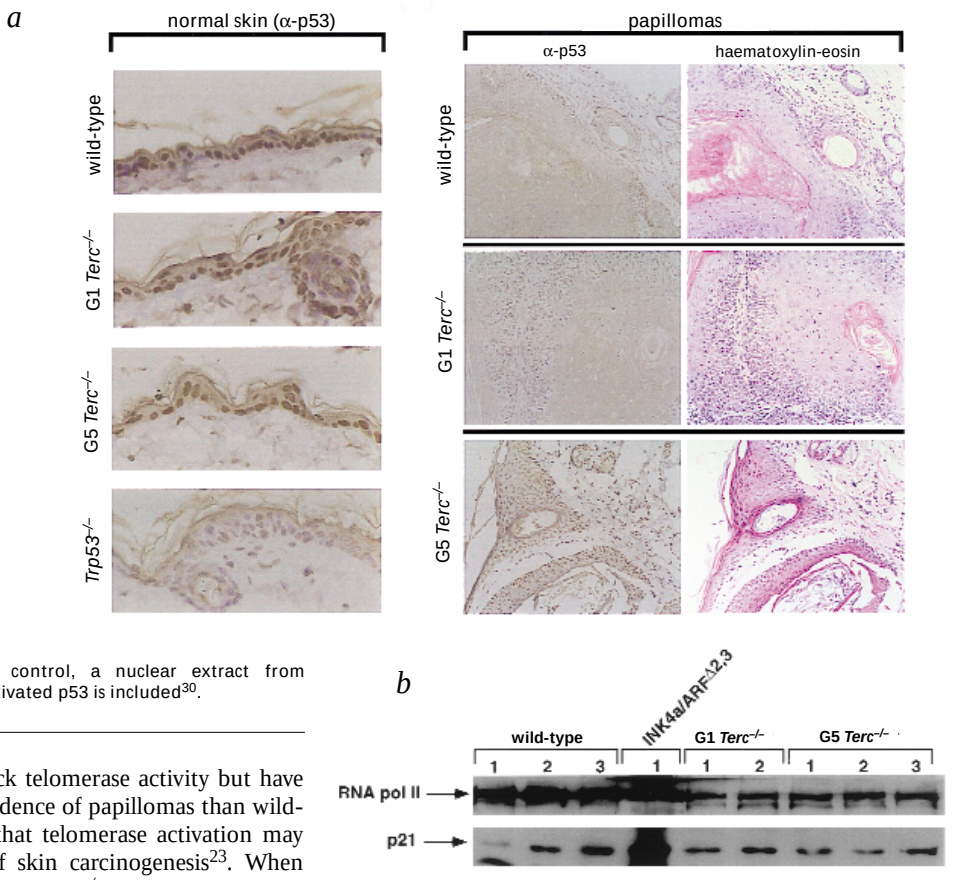

Fig. 4 p53 and p21 concentrations in wild-type and *Terc*$^{-/-}$ skin keratinocytes. ***a***, Immunohistochemistry and histopathology of acetone-treated and carcinogen-treated wild-type, G1 and G5 *Terc*$^{-/-}$ mouse skins. The control acetone-treated skins show a high p53 positivity in the nucleus of basal keratinocytes; as a negative control, the skin of a *TRp53*$^{-/-}$ mouse is shown. Wild-type and G1 *Terc*$^{-/-}$ papillomas show decreased p53 staining of basal keratinocytes compared with wild-type skin. p53 immunostaining of a G5 *Terc*$^{-/-}$ papilloma shows strong, homogeneous p53 reactivity in both basal and suprabasal keratinocyte layers. All sections were processed in parallel for p53 staining. ***b***, Extracts were prepared from three primary wild-type, two G1 and three G5 *Terc*$^{-/-}$ keratinocyte cultures, and western blots performed as described[27,29,30]. p21 and RNA pol II (loading control) specific bands are indicated with arrows. No differences in p21 concentrations were detected, in agreement with comparable p53 concentrations detected by immunohistochemistry in the different skin samples. As a positive control, a nuclear extract from INK4a/ARFΔ2,3 MEFs overexpressing activated p53 is included[30].

The G1 *Terc*$^{-/-}$ mice, which lack telomerase activity but have long telomeres, show a lower incidence of papillomas than wild-type mice (Fig. 1*a*), suggesting that telomerase activation may contribute to the progression of skin carcinogenesis[23]. When telomeres are critically short, as in G5 *Terc*$^{-/-}$ mice, the absence of telomerase has an impact on skin tumourigenesis, suggesting that tumour formation in skin requires telomere maintenance above a threshold length and that anti-telomerase–based tumour therapy may be effective in epithelial tumours. The increased concentrations of p53 in G5 *Terc*$^{-/-}$ papillomas suggest that p53 may be sensing short telomeres and contributing to cessation of growth. Study of skin tumorigenesis in mice deficient for both p53 and telomerase[16] would be of great relevance. Finally, the apparently opposite impact of telomerase absence and short telomeres in distinct cell types[12] may be caused by differential activation of telomerase-independent telomere maintenance mechanisms[24,25].

Methods

Mice. We generated wild-type and *Terc*$^{-/-}$ mice as described[10]. The genetic background was 60% C57BL6, 37.5% 129Sv, 2.5% SJL (ref. 10).

Tumour-induction experiments and histopathological analyses. We shaved 8 mice of each genotype, wild-type, G1 *Terc*$^{-/-}$ and G5 *Terc*$^{-/-}$, and treated them with DMBA (20 μg in 200 μl; Sigma) 48 h after shaving. Mice were subsequently treated twice weekly with TPA (10^{-4} in acetone; 200 μl; Sigma) for 16 weeks. Two control mice of each genotype were treated with acetone alone. Papilloma and normal skin sections from wild-type and different-generation *Terc*$^{-/-}$ mice were fixed in 10% buffered formalin and stained with haematoxylin and eosin. Images were captured at a ×20 magnification with an Olympus-Vanox microscope.

Immunohistochemistry was performed on deparaffinized sections (Vectabond slides) after pressure cooker processing. We used a streptavidin-biotin complex technique with a polyclonal rabbit anti-mouse p53 antibody, CM5 (Novocastra Laboratory; dilution 1:1,500, overnight, 4 °C) and a biotinylated anti-rabbit IgG (Vector Laboratory; dilution 1:400, 30 min, RT), followed by streptavidin peroxidase (Zymed Laboratory; dilution 1:20, 30 min, RT); the chromogen was developed with diaminobenzidine, and slides were haematoxylin counterstained. Control slides were obtained by replacing the primary antibody with TBS (data not shown).

Measurements of telomere length. For Q-FISH, paraffin-embedded skin sections were hybridized with a PNA-tel probe as described[26]. Slides were deparaffinized in 3 xylene washes (3 min each), then treated for 3 min with a 100%, 95% and 70% ethanol series. More than 50 keratynocyte 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[27] and hybridized using the FLOW FISH protocol[28]. Telomere fluorescence of 2,000–3,000 nuclei gated at G1-G0 cell cycle stage was measured using a Coulter EPICS XL flow cytometer with the SYSTEM 2 software. The same cells were hybridized in parallel without the PNA-FITC probe to obtain background fluorescence values that were subtracted from the fluorescence intensity of each sample. We used six wild type, six G1 and six G5 *Terc*$^{-/-}$ primary keratinocyte cultures.

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