Mutations in the promoter of the telomerase gene \textit{TERT} contribute to tumorigenesis by a two-step mechanism

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Abstract

\textit{TERT} promoter mutations (TPMs) are the most common non-coding mutations in cancer. The timing and consequences of TPMs have not been fully established. Here we show that TPMs acquired at the transition from benign nevus to malignant melanoma do not support telomere maintenance. \textit{In vitro} experiments revealed that TPMs do not prevent telomere attrition, resulting in cells with critically short and unprotected telomeres. Immortalization by TPMs requires a gradual upregulation of telomerase, coinciding with telomere fusions. These data suggest that TPMs contribute to tumorigenesis by promoting immortalization and genomic instability in two phases. In an initial phase, TPMs do not prevent bulk telomere shortening but extend cellular life-span by healing the shortest telomeres. In the second phase, the critically short telomeres lead to genome instability and telomerase is further upregulated to sustain cell proliferation.
constitutionally shorter telomeres have a decreased cancer risk (15–17). The current model of telomerase reactivation during crisis fails to explain these contradictory observations. Moreover, it is not understood why tumors with TPMs are associated with a poor prognosis (8, 18).

To determine the role of TPMs in telomere maintenance during tumor formation, we followed the intratumoral changes in telomere length in tumors with TPMs. TPMs occur with high frequency (60–85%) in cutaneous melanoma (3, 4, 19). We took advantage of a subset of human melanomas, which arose from adjacent pre-neoplastic lesions such as nevi (7). This allowed us to compare genetic alterations and telomere length in four melanomas that acquired TPMs as they arose from nevi (Fig. 1 and Fig. S1). Using structured illumination microscopy, we acquired large, high-resolution telomere and centromere fluorescence in situ hybridization images of melanoma and nevus in the same tissue section and quantitatively analyzed telomere length (Fig. 1A, 1B and Fig. S1). Multiple iterations of random sampling of normalized telomeric signals showed that in all four cases telomeres were significantly shorter in the melanoma than in the nevus (reduced normalized signal intensity in melanoma compared to the nevus, Fig. 1C and 1D, Fig. S1D). These data highlight that in vivo, TPMs do not induce TERT expression levels sufficiently to counteract or reverse telomere shortening.

To explore the long-term effects of TPMs on telomere length and genomic stability in a genetically defined setting, we engineered the three most frequent TPMs de novo into human embryonic stem cells (hESCs) using CAS9 mediated genome editing (20) (Fig. 2A and Fig. S2A–D). We started the “experimental clock” by differentiating the parental cell lines into fibroblasts, which are normally telomerase-negative. We assayed the proliferative capacity of hESC-derived fibroblasts with and without a single TPM using three distinct conditions: (i) fibroblasts transduced with Simian virus 40 large T antigen (SV40 TAg, inactivating pRb and p53 signaling) (Fig. 2B, S2B), (ii) fibroblasts with intact cell cycle and DNA damage checkpoints (Fig. 2C), and (iii) fibroblasts with inactivated cell cycle and DNA damage checkpoints, achieved by deletion of p14/p16 function (CDKN2AΔ/Δ) (Fig. 2D, Fig. S2C). In all cell lines the TPMs extended the proliferative capacity significantly past the proliferative barrier of wild-type cells. Cells with TPMs proliferated without signs of crisis or a strong decrease in doubling rate at the time when wild-type cells arrested in a telomere length dependent manner (21) (Fig. 2B–D, Fig. S2E and S2F). Overall, these data demonstrate that TPMs promote the immortalization of bulk cell populations.

Telomere maintenance is essential for cancer cell immortalization (22). To investigate whether TPMs prevented telomere shortening, we measured telomere length over time in the fibroblast model system (Fig. 2E–G and Fig. S3A and S3B). In wild-type cells, telomeres shortened progressively. Surprisingly, telomeres in cells with TPMs initially also shortened and stabilized only after approximately 70 population doublings (PD) at a length of 2.5–4.5 kb. The stabilized mean telomere length in the TPM cells was shorter than telomere length in wild-type control cells at the time of crisis (Fig. 2E–G). The analysis of the telomere length distribution of cells with TPMs revealed that the mean length shifted from long to short telomeres and then stabilized (Fig. 2H and Fig. S3C), suggesting that short telomeres were preferentially maintained, while long telomeres continued to shorten.
Unprotected short telomeres can lead to genomic instability through telomere fusion events, which have been detected in a variety of tumor samples and cell lines (11–13, 23–25). To assess whether the chromosome ends are protected in cells with TPMs, we used a PCR-based method to quantify telomere-telomere fusion events over the course of a year after cell differentiation (23). We detected fusions in SV40 TAg cells with the -124 and -146 TPMs when telomere length stabilized (Fig. 3A and 3B and Fig. S4A and S4B). These fusion events were suppressed by overexpression of TERT (Fig. S4C), showing that they were driven by telomere attrition. Cells with the -57 TPM showed fewer fusions, presumably due to their slightly longer telomeres (Fig. 2E). Thus, TPMs not only promote cancer cell immortalization but might also drive cancer cell evolution via dysregulation of telomere protection.

To determine the effect of TPMs on telomerase levels, we assayed enzymatic activity and TERT mRNA expression over time (Fig. 3C, Fig. S4D and S4E). Shortly after differentiation, telomerase activity and TERT mRNA expression levels were higher in cells with TPMs than in wild-type cells, consistent with previous reports (20). However, telomerase levels rapidly fell below the detection limit of the assay in both cell types (Fig. 3C and Fig. S4D). Nevertheless, cells with the TPM proliferated at a similar rate as cells overexpressing TERT (Fig. S5A–C), demonstrating that TERT expression levels were not limiting for proliferation when telomeres were long. To examine whether TERT was fully transcriptionally silenced, we overexpressed POT1ΔOB, an allele sensitizing cells to low telomerase levels (26). The expression of the allele induced significant telomere elongation specifically in cells with TPMs (Fig. S5D–G). Therefore telomerase was not fully transcriptionally silenced but TPMs initially lead to telomerase activity below the detection threshold. At the time when telomere length in TPM cells had stabilized, telomerase expression became detectable again and activity rose as high as 5–10% of that found in hESCs and established cancer cell lines (Fig. 3C and Fig. S6). To exclude the possibility that this upregulation of telomerase was the result of an outgrowth of a small subpopulation of cells with preexisting high telomerase activity, we performed sub-cloning experiments. Individual cell clones recapitulated the behavior of the bulk population (Fig. S7). Like the bulk cell population, individual clones also first had low telomerase levels, which became elevated with increasing PDs. This confirmed that TERT upregulation was the consequence of an additional event occurring when telomeres became critically short.

At the time point of the acquisition of a TPM telomeres did not immediately stop shortening. The time lag until telomerase activity increased sufficiently to maintain all telomeres could provide a rationale for the cancer-protective function of constitutionally short telomeres, and explain how the cellular context influences the immortalization process. We hypothesize that: (i) telomere length at the time when a cell acquires a TPM determines the number of cell divisions a cell can undergo before telomeres become critically short and (ii) once telomeres have become critically short the ability to upregulate telomerase determines whether a cell with a TPM becomes immortalized or ceases to proliferate. To test these hypotheses, we generated hESCs with shorter telomeres by modulating TERT expression. We attenuated the effect of TPMs by mutating the three endogenous ETS-factor binding sites in the TERT promoter (E3), which facilitate binding of the GABPα as a heterodimer with GABPβ (27, 28) (Fig. 4A), and which we show to be essential in hESCs (Fig. S8A.
and S8B). hESCs with a TPM and the E3 mutation (E3-124 and E3-146) had lower TERT expression and stably maintained shorter telomeres in comparison to hESCs with only a TPM (-124 and -146) (Fig. 4B, 4C and S8C–F). From this allelic series we generated SV40 Tag-transduced fibroblasts and monitored proliferation, telomere length, and frequency of telomere fusions (Fig. 4B, and 4D–E). Fibroblasts without the TPM, but with the three ETS mutations (wt-E3), had the shortest telomeres and died shortly after differentiation. In accordance with our first hypothesis, -124-E3 and -146-E3 cells with constitutionally shorter telomeres showed a high frequency of fusion events, much earlier than -124/-146 cells (Fig. 4D and S8G). Both -124-E3 and -146-E3 cells initially proliferated despite their short telomeres (Fig. 4E), arguing that they retained low levels of telomerase activity. Although they harbored TPMs, both -124-E3 and -146-E3 cells upregulated telomerase to only low levels; these cells entered crisis and did not become immortalized, confirming our second hypothesis.

In summary, we propose that TPMs drive cell immortalization in two phases (Fig. 4F). TPMs that arise in early phases of tumorigenesis result in telomerase activity that is insufficient to prevent bulk telomere shortening, preferentially acting on critically short telomeres and thereby delaying replicative senescence (Phase 1). During subsequent divisions, the number of critically short telomeres increases, and telomerase activity becomes rate-limiting, causing telomere-driven genomic instability and selective pressure for telomerase upregulation by yet to be characterized factors (Phase 2). In contrast to crisis, where genomic instability arises as an acute and pronounced phenomenon, the genomic instability in cells with TPMs is reduced and persists over a longer period. Thus, TPMs play a dual role in tumorigenesis by enabling not only cancer cell immortalization via upregulation of TERT but also by promoting genome instability.

The finding that TPMs can become selected at the transition from pre-neoplastic to cancerous lesions indicates that telomere shortening acts as a critical barrier early during the neoplastic transformation of some cancers (7). Together with our findings that TPMs are necessary but not sufficient to maintain telomere length this might explain why individuals with constitutionally short telomeres have a decreased rate of melanoma incidence (15–17). Variation in constitutional telomere length likely limits the numeric expansion of early neoplastic cells at a precancerous stage. By characterizing the TPM-driven immortalization process over extended periods of time, we were able to reconcile the seemingly contradictory observations that short telomeres can initially protect against cancer formation (29, 30), yet have a cancer promoting effect and can be associated with a poor prognosis (8, 18) by later fueling genomic instability (11, 12, 24).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We would like to thank G. Steven Martin, Robert Tjian and the members of the Hockemeyer lab for critical comments on the manuscript. K.C. is supported by a Nakajima foundation fellowship. D.H. is a Pew-Stewart Scholar for Cancer Research supported by the Pew Charitable Trusts and the Alexander and Margaret Stewart Trust.
D.H. supported by the Siebel Stem Cell Institute and NIH R01-CA196884. A.H.S is supported by NIH 5T32 CA177555. B.C.B is supported by R01 - CA142873. X.D. is supported by the California Institute for Regenerative Medicine grant LA1-08013. StellarVision is an imaging instrument developed by Optical Biosystems, and A.O. and J.R. are full time employees by this company. MATLAB code used for image analysis is available under https://gitlab.com/tjian-darzacq-lab. Patient sequencing data is available within the Short Read Archive with the accession number SRP113411.

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Fig. 1. Telomeres shorten during progression from nevus to melanoma despite acquisition of a TPM

(A) Schematic overview of workflow. Melanoma progression samples from four patients were imaged using structured illumination microscopy. Acquired images were stitched and regions of interest were annotated. Telomeric and centromeric repeat FISH signals were detected and mapped back on pre-defined regions of interest. The relative telomere length was calculated by normalization to the closest centromeric signals. (B) The genetic evolution of each progression case, rooted at the germline shows how each case evolves from a wild-type nevus to a melanoma with TPM (nevus in blue, melanoma in red). Adjacent is the H&E staining of the analyzed sections of each case (Scale bar 1mm). (C) Magnification of the FISH image resolving individual telomere and centromere spots in nevus/melanoma area (DAPI = blue, telomere = green, centromere = magenta). TPM status is noted as wild-type (WT) or mutant (mut) (Scale bar 20 μm). (D) Violin plots of normalized telomere signal (telomere/centromere) with an inset boxplot indicating the median and quartiles of the signal. Histograms show the fold change of telomere spot intensity after random sampling (fold change melanoma/nevus).
Fig. 2. TPMs support cellular immortalization in vitro but do not prevent telomere shortening

(A) Experimental overview: Isogenic hESCs with the TPMs were differentiated into fibroblasts. To inactivate cell cycle and DNA damage checkpoints, either CDKN2A function was deleted in hESCs prior differentiation or fibroblasts were infected with SV40 TAg. (B-D) Growth curves of cumulative PDs over days after differentiation. (B) SV40 TAg fibroblasts with (red, -57, -124, -146) or without (blue, wt) TPMs (C) DNA damage checkpoint proficient cells with (red, -124) or without (blue, wt) TPM. (1), (2) indicate two independent experiments. (D) CDKN2AΔ/Δ cells with (red, -124) or without (blue, wt) TPM (E-G) Quantification of mean telomere length over time after differentiation. (H) Accumulation of shorter telomeres over time shown by visualization of telomere length distribution of images shown in Fig. S3A. Quantification of the normalized pixel intensity over molecular weight per lane for the indicated time points after differentiation.
Fig. 3. TPMs do not fully protect against genomic instability and mutant cells gradually increase telomerase expression
(A) Detection of interchromosomal telomere fusions over time in SV40 TAg fibroblasts by PCR of specific subtelomeric regions. Fusions were detected by probes against the 17p subtelomeric regions (23). DNA from cells in crisis served as a positive control (P.C.: TERT, p53 and p16 triple knockout fibroblasts cultured into crisis). (B) Quantification of fusion events shown in Fig. 3A. (C) Telomerase activity assay of the indicated cell lines over time. IC: internal control. * indicates time points when telomerase activity became detectable.
Fig. 4. Short telomeres and low telomerase levels protect cells from immortalization by TPMs

(A) Schematic overview of allelic series of cells carrying TPMs (red) and/or endogenous ETS site mutations (E3: mutated TC>GA, blue) in the TERT promoter. E3 refers to the simultaneous mutations of all three endogenous ETS sites (-191, -97 and -92 from the translational start site: ATG). (B) Telomere length analysis for hESCs and fibroblasts with TPMs and ETS mutation combinations. (C) TERT expression normalized to GAPDH relative to wt hESC. Error bars: SEM, two-sided student t-test, n = 3. (D) Telomere fusions detected in -124 and -124 E3 fibroblasts with 17p. (E) Growth curve of fibroblasts with or without TPMs and/or ETS mutations. (F) Telomere length (top) and TERT expression (bottom) in a revised model for immortalization by TPMs. In the classic crisis model, TERT expression is acquired during crisis as a single event (left panel). In the biphasic model (right panel), TPMs can be acquired in a telomere length independent manner at any time (Phase 1). In phase 2 cells are required to gradually upregulate telomerase. Due to unprotected telomeres cells can acquire genomic instability.