

Ectopic Expression of a COOH-terminal Fragment of the Human Telomerase Reverse Transcriptase Leads to Telomere Dysfunction and Reduction of Growth and Tumorigenicity in HeLa Cells¹

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ABSTRACT

The COOH-terminus of telomerase reverse transcriptase (hTERT) has been shown to participate in the nuclear translocation of TERT. Here, we constructed plasmids expressing the COOH-terminal M_r 27,000 polypeptide of hTERT (hTERTC27) with the telomerase RNA-binding domains and the reverse transcriptase domains deleted. We showed that ectopic overexpression of this polypeptide caused a defect in telomere maintenance in hTERT-positive HeLa cells, which led to senescence-like growth arrest and apoptosis. The hTERTC27 appears to work by inducing telomere dysfunction, exemplified by significantly increased anaphase chromosome end-to-end fusion events in transfected cells. Significantly, it had no effect on the cellular telomerase enzymatic activity or telomere length. The *in vivo* effect was further demonstrated as HeLa cells stably expressing hTERTC27 have significantly lower growth rate and reduced tumorigenicity in nude mice xenografts. Results from this study revealed an important function for the COOH terminus of hTERT in maintaining the integrity of telomere structure and chromosome ends, as well as in cell senescence and apoptosis. Furthermore, hTERTC27 provides a new strategy for cancer therapy by inducing telomere dysfunction in cancer cells without affecting the telomerase enzymatic activity.

INTRODUCTION

Telomeres are special DNA protein complexes at the end of chromosomes, whose major function is to protect chromosomes from degradation and end-to-end fusion; therefore, they are essential for genome stability and cell survival (1, 2). Telomere lengths in normal cells shorten with each cell division, and progressive telomere shortening eventually results in cell crisis or senescence attributable to telomere dysfunction, characterized by mass genome instability (*e.g.*, widespread chromosome end-to-end fusions), senescence-like growth arrest, and apoptosis (1, 3, 4). The telomerase plays a pivotal role in the maintenance of telomeres and cell proliferation during embryonic development. In adult tissues, although telomerase activity is absent in most somatic cells, in >85% of all human cancer cells, telomerase is reactivated (5, 6), and the activation of telomerase activity has been shown to prevent telomere shortening, thereby immortalizing these cancer cells (7, 8). The expression of telomerase is also required both for the malignant transformation of normal human cells (9–11) and the continued proliferation of cancer cells (12, 13).

Telomerase is an RNA complex containing the TR⁴ component and

the catalytic protein subunit TERT, which are conserved among all species (14–16). TERT reverse transcribes the TR template into telomeric DNA sequence and elongates the telomere length, and it is the rate-limiting factor for telomerase activity (17–21).

Because telomere stabilization through TERT activation is critical for the long-term survival of cancer cells, the disruption of this event represents an excellent anticancer therapeutic target. Previous strategies to destroy the RNA template (TR; Refs. 22–24) or inhibit the catalytic activity of TERT (12, 24) have effectively led to telomerase suppression, telomere shortening, telomere dysfunction, and ultimately reduced cancer cell growth both *in vitro* and *in vivo*. Nevertheless, inhibiting telomerase activity may induce cancer cells to undergo a process to elicit the ALT mechanism (25), which is a telomerase-independent pathway normally suppressed by telomerase activity (26, 27). This ALT mechanism uses the recombination-based pathway for telomere maintenance, and the activation of this pathway has been demonstrated in human cancer cells that are telomerase negative (25, 28, 29).

hTERT protein contains the hTR-binding domain and the conserved reverse transcriptase motifs, which are located at its NH₂ terminus and the central regions of the protein, respectively (20, 30–33). Both domains are required for the enzymatic activity. hTERT also needs to be translocated to the nucleus and targeted to the telomere ends to be functional. Nuclear translocation has been shown to be regulated by the interactions between the hTERT COOH terminus and the protein 14-3-3 (34). However, the mechanism for the telomere end targeting of hTERT is not understood.

It was reported previously that ectopic overexpression of hTERT is sufficient to allow T-Ag-transformed human embryonic kidney cells to bypass crisis; however, when the COOH terminus of hTERT is attached with an HA epitope tag (hTERT-HA), it loses this ability. The hTERT-HA-transfected cells continued to lose telomeric DNA and ceased to proliferate (13, 35), thus suggesting a specific biological function for the COOH terminus of hTERT in telomere maintenance and cell proliferation.

In this study, we investigated the function of hTERT COOH terminus and its potential application in cancer therapy by using a truncated hTERT (hTERTC27) that encodes the hTERT COOH terminus M_r 27,000 polypeptide. In this polypeptide, most of the conserved reverse transcriptase motifs and the hTR-binding domains were deleted. The telomerase-positive HeLa cells stably expressing hTERTC27 under the control of tetracycline (Tet-off expression system) progressed rapidly into a senescence-like growth arrest and apoptosis. The expression of hTERTC27 also inhibited solid tumor formation and tumor growth *in vivo* in nude mice xenografts. The expressed hTERTC27 is capable of nuclear translocation/telomere targeting and induction of acute chromosome end-to-end fusions,

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⁴The abbreviations used are: TR, telomerase RNA; TERT, telomerase reverse transcriptase; hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA;

ALT, alternative lengthening of telomere; EGFP, enhanced green fluorescence protein; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment; SA, senescence-associated; PI, propidium iodide.

characteristic of a telomere dysfunction (1, 3, 4, 36). Moreover, this occurs without affecting cellular telomerase enzymatic activity of the transfected cells. To our knowledge, this is the first report demonstrating that a hTERT COOH-terminal polypeptide fragment alone can induce telomere dysfunction and exhibit antiproliferation activities, indicating an important function for the COOH terminus of hTERT in telomere maintenance. Our results also suggested a new strategy for the induction of telomere dysfunction in cancer cells, which may serve as a novel approach for cancer therapy.

MATERIALS AND METHODS

Construction of the hTERT27 Expression Plasmids. The pCI-neo-hTERT plasmid containing cDNA encoding the wild-type hTERT was kindly provided by Dr. R. A. Weinberg at the Massachusetts Institute of Technology. A DNA fragment encoding the M_r 27,000 hTERT COOH-terminal polypeptide (the hTERT amino acid 882-1132, termed hTERTC27) was generated by PCR from pCI-neo-hTERT, and the sequence was confirmed by DNA sequencing. The pLEGFP-hTERTC27 plasmid contains the hTERTC27 fragment inserted in-frame into the *EcoRI* and *Sall* sites of the retroviral expression vector pLEGFP-C1 (Clontech). The fragment encoding EGFP-hTERTC27 was then isolated from pLEGFP-hTERTC27 by *NcoI* (blunt) and *Sall* digestions and cloned into the *BamHI* (blunt) and *Sall* sites of the expression vector pRevTRE (Clontech), creating the expression plasmid pTetEGFP-hTERTC27, under the control of a tetracycline-regulated promoter.

Generation of Stable Clones of HeLa Cells Expressing EGFP-hTERTC27. The pLEGFP-hTERTC27 and the control pLEGFP-C1 vectors were transfected into the packaging cell lines PT67 (Clontech) to generate the recombinant retrovirus to infect HeLa cells by the procedure described in the "retroviral gene transfer and expression user manual" (Clontech). After infection, cells were selected in growth medium containing 400 $\mu\text{g/ml}$ G-418 (Life Technologies, Inc.) for 5 days. Cells surviving from G418 selection were pooled for use. In addition, the pTetEGFP-hTERTC27 and the control pRevTRE vectors were transfected into the HeLa Tet-off cells (Clontech) by calcium phosphate coprecipitation and selected under doxycycline (100 ng/ml) and hygromycin (200 $\mu\text{g/ml}$). Hygromycin-resistant clones were isolated by ring cloning and tested for expression of hTERTC27 polypeptides after the induction by withdrawal of doxycycline from the culture media.

Detection of the hTERTC27 and Visualization of Chromosomal DNA. The expression of the hTERTC27 protein was determined by Western blotting using antibody raised against the hTERT COOH-terminal fragment (Santa Cruz Biotechnology; sc-7212). To determine the subcellular localization of the ectopic expressed EGFP-hTERTC27 fusion protein in live cells, cells were grown on 60-mm culture plates, and the EGFP fluorescent protein was visualized directly under a Zeiss Axioplan inverse microscope. For chromosomal DNA observation, cells were grown on coverslip under the experimental conditions indicated, fixed with methanol, and then stained with PI. Micrographs were recorded with a Kodak DCS200 digital camera. Images were noise filtered, corrected for background, and merged using Adobe PhotoShop.

Cell Growth, Senescence, and Apoptosis. Cells were plated into cell culture plates at the density of 2×10^5 cells/60-mm dish 24 h before the initiation of an experiment. For growth rate determination, cells were washed three times with medium containing hygromycin (100 $\mu\text{g/ml}$) in the presence or absence of doxycycline (100 ng/ml) and treated for varied times as indicated in the text. At the end of the experiments, cells were harvested, and the number of viable cells was counted by trypan blue staining. For senescence assays, cells were induced to express hTERTC27 and stained for SA β -galactosidase as described previously (36, 37). For quantitative analysis of apoptosis, cells were harvested at indicated time points, washed once in ice-cold PBS, incubated with annexin V-fluorescein/PI (Boehringer Mannheim) in calcium-containing HEPES buffer, and then immediately analyzed with a FACScan machine (Becton Dickinson).

Telomerase Activity and Telomere-length Assays. Telomerase activity was determined by a highly sensitive, PCR-based TRAP-ELISA assay, using a Telomerase PCR ELISA Kit (Roche) following the manufacturer's instructions. Briefly, cells were lysed, and cellular protein in the supernatant was prepared by centrifugation. Cell protein (3 mg) was incubated first with the biotin-labeled primers, then the telomeric repeats were added onto the ends of

the synthetic primers by the cellular telomerase. To quantitate the cellular telomerase activity, the resulted telomere DNA was PCR amplified, denatured, hybridized with the digoxigenin-labeled telomeric repeat-specific probe, quantitated using peroxidase-conjugated antibodies against digoxigenin, and visualized at 450 nm using an ELISA reader. Telomerase activity was determined in triplicate. Negative controls were prepared by inactivating the telomerase activity present in the HeLa cell extracts with high temperature (95°C, 10 min) before the incubation.

Telomere length was analyzed by the TRF length assay using a Telo TTAGGG Telomere Length Assay Kit (Roche). Briefly, genomic DNA was isolated from cells. The DNA was analyzed by Southern blots probed with a telomere-specific probe to visualize the TRF.

Nude Mice Xenograft Experiments. Pooled HeLa cells stably expressing EGFP-hTERTC27 or EGFP were generated by pLEGFP-hTERTC27 or pLEGFP infection followed by G418 selection as mentioned before. Cells were then harvested in log phase growth, washed twice in PBS, resuspended in DMEM medium (1×10^7 cells/ml), and injected (0.2 ml) s.c. at the base of the right flank of a nude mouse (aged between 5 and 6 weeks) at 2×10^6 cells/mouse. Tumor growth was monitored weekly for 4 weeks. Tumor size was determined by the product of two perpendicular diameters and the height above the skin surface.

Statistical Analysis. Values are presented as means \pm SD. Data were analyzed using one-way ANOVA, and significant differences between means were evaluated by the Bonferroni post-hoc test.

RESULTS

The Design of hTERTC27 and Its Expression in HeLa Cells.

The wild-type hTERT is a M_r 127,000 protein consisting of 1,132 amino acid residues (20). It contains an hTR-binding domain (Fig. 1A, top panel, shaded boxes: T and T2 are the two conserved motifs) located in the NH₂ terminus (30–32), and a reverse transcriptase domain (Fig. 1A, top panel, shaded boxes: 1, 2, A, B', C, D, and E represent seven conserved short motifs) located in the central region (20, 33). We designed an EGFP-hTERTC27 fusion protein containing the M_r 27,000 COOH-terminal amino acid residues 882-1132 of the

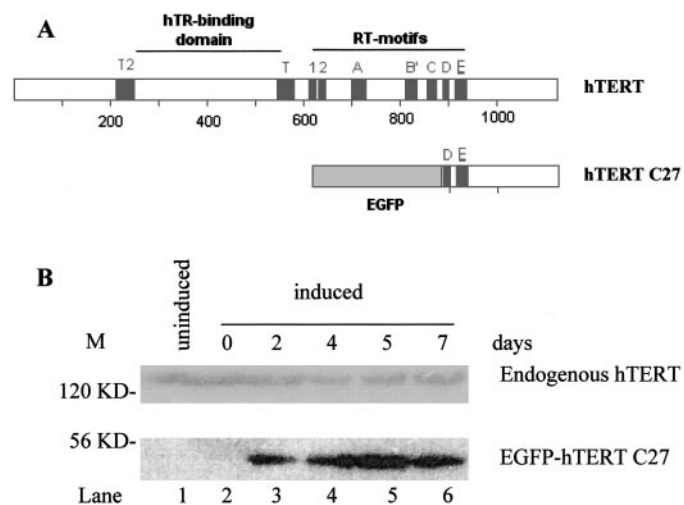


Fig. 1. The design and the expression of a truncated COOH-terminal hTERT M_r 27,000 polypeptide. A, the structure of the full-length wild-type hTERT (top panel) and the truncated mutant hTERTC27 (bottom panel). To facilitate the detection of its expression and cellular localization, the truncated hTERTC27 is linked to a NH₂-terminal EGFP tag. B, Western blotting analysis of the regulated expression of the hTERTC27 polypeptides. Whole cell extracts were prepared from a clone of HeLa Tet-off cells stably expressing the pTetEGFP-hTERTC27 plasmid. The cells were maintained in medium supplemented with doxycycline. At the beginning of the experiments, doxycycline was withdrawn from the medium to induce the expression of EGFP-hTERTC27 for the indicated amount of time. In each lane, 40 μg of protein were loaded. Western analysis was performed with specific antibody against the hTERT COOH-terminal polypeptide (Santa Cruz Biotechnology; sc-7212). The endogenous hTERT (M_r 127,000) was detected using longer exposure time. The data shown are obtained from a representative experiment using the C8 clone of hTERTC27-expressing cells.

hTERT, fused at the NH₂ terminal with EGFP so that its expression and cellular localization can be traced in live cells (Fig. 1A, bottom panel). The nuclear export signal (residues 970–981) and the 14-3-3-binding amphipathic helix (residues 1030–1047) responsible for the nuclear translocation are included (34). The deleted region includes all of the TR-binding domain and most of the conserved reverse transcriptase domains.

The cDNA of this fusion protein was subcloned into the pTetEGFP-hTERTC27 expression plasmid and transfected into the HeLa Tet-off cell line. Clones of cells stably expressing EGFP-hTERTC27 under the control of tetracycline were isolated. Among 56 such clones, 2 clones (C8 and G11) showed highest levels of inducible expression of EGFP-hTERTC27 (by doxycyclin withdrawal) as judged by Western blotting analysis (Fig. 1B, bottom panel, with the predicted size of M_r 54,000). These two clones were therefore selected for additional studies. For the sake of simplicity, we presented the data from clone C8, as similar results were obtained from clone G11. We found that the induction of EGFP-hTERTC27 expression did not change the level of endogenous hTERT protein expression (Fig. 1B, top panel, with the predicted size of M_r 120,000).

Ectopic Expression of hTERTC27 Caused Senescence-like Growth Arrest and Apoptosis in HeLa Cells. Induction of hTERTC27 expression caused a time-dependent decrease of the cell proliferation rate (Fig. 2A). Significant decrease was noticed from day 3 onward. Cell growth was completely halted 7 days after doxycyclin withdrawal. Using the trypan blue exclusion assay, we found that hTERTC27 caused a significant increase of the number of nonviable cells (from ~5% of the total cells in the non-hTERTC27-expressing cells to 25% in the hTERTC27-expressing cells after a 4-day induction). To determine whether this is attributable to apoptosis, we measured the percentage of apoptotic cells using flow cytometry analysis of cells doubly labeled with annexin V and PI. The annexin V binding is a marker for early apoptotic cells (38), whereas PI staining reflects the late stage of cell death. Thus, annexin V and PI double labeling allows a further distinction of early apoptotic (annexin V+/PI-, shown in Fig. 2, B–D as the H4 section) and late apoptotic/necrotic (V+/PI+, shown in Fig. 2, B–D as the H2 section) cells. hTERT appears to cause a time-dependent increase in the percentage of apoptotic cells in both the early and late stages. Before the induction of hTERTC27 expression, basal content of apoptotic cells was low (Fig. 2B, 1.8% annexin V+/PI-, H4 section and 1% annexin V+/PI+, H2 section). At day 4 (Fig. 2, C and E), a significant increase of both early and late apoptotic cells (6.3% annexin V+/PI- and 4% annexin V+/PI+ cells) was detected. At day 5, 14.2 and 6.3% of cells were in the early and late apoptotic states, respectively (Fig. 2E). At day 7, massive apoptosis was observed (Fig. 2, D and E, 21.7% annexin V+/PI- cells and 12.7% annexin V+/PI+ cells). Meanwhile, the control noninduced group contains only 3–5% of apoptotic cells during the whole time course of the study.

In addition, the expression of hTERTC27 also caused marked changes in cell morphology (Fig. 3, A–D). At day 4 postinduction, although the control-noninduced cells exhibited normal morphology (Fig. 3A), a noticeable number of the hTERTC27 expressing cells became enlarged and flattened with a vacuolated cytoplasm. Most of these enlarged cells showed positive staining of SA β -galactosidase (Fig. 3B), a phenotype reminiscent to that of the primary human cells undergoing senescence (37). The percentage of cells that became senescence like continues to increase, and on the 7th-day postinduction, most of the cells became senescent (Fig. 3C). Consistent with the senescence phenotype, this morphological change was irreversible, as replenishing the media with doxycycline to suppress hTERTC27 expression on day-6 postinduction for 7 days could not reverse the changes in cell morphology or the rate of cell proliferation. These

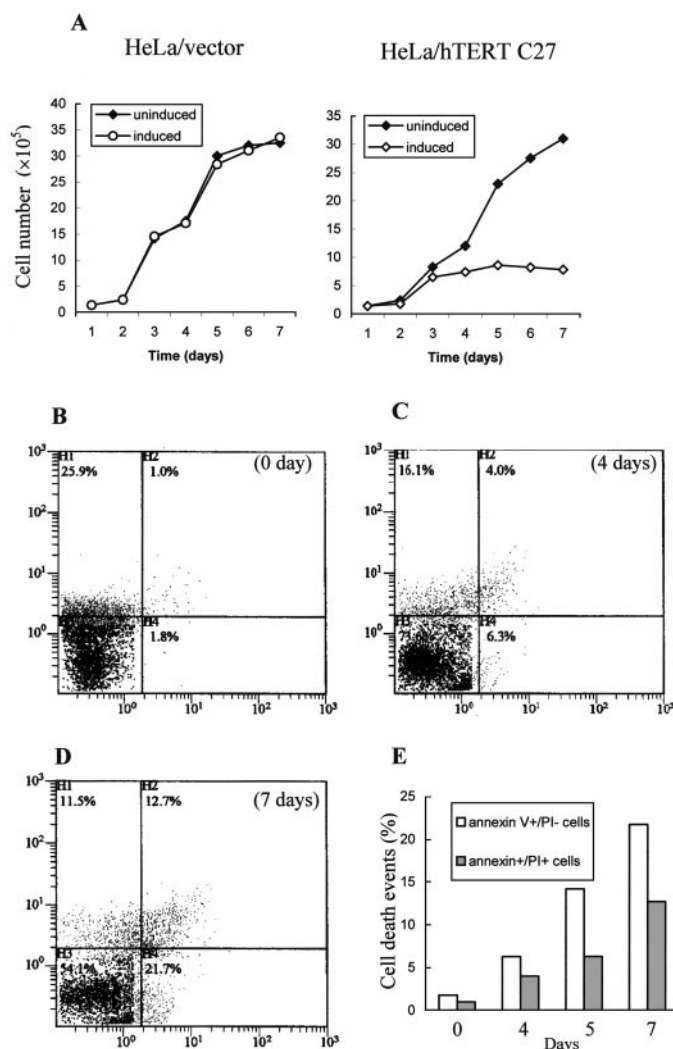


Fig. 2. Effect of hTERT C27 overexpression on cell growth inhibition and apoptosis. A, growth curve showing the effect of either the control (left panel) or EGFP-hTERTC27 (right panel) expression on the growth of HeLa cells. Cell numbers were counted based on the trypan blue assay. B–D, flow cytometry analysis of cells double labeled with annexin V and PI. Cells were harvested at indicated time points postinduction. E, summary of the data from the flow cytometry analysis.

cells remain enlarged and flattened (Fig. 3D). The number of enlarged, SA β -gal-positive cells per optical field in hTERTC27-expressing culture increased from an average of 12 cells at day 4 to an average of 128 cells at day 7 postinduction. In contrast, the uninduced cells have <1 senescence positive cell/optical field. Therefore, we conclude that hTERTC27 inhibited HeLa cell proliferation by inducing both cell senescence and apoptosis.

hTERTC27 Caused Chromosome End-to-end Fusions. The most important biological function of telomerase and hTERT is to maintain the integrity of the telomere structure at the chromosome ends. Therefore, we examined whether and how hTERTC27 affected telomere structure. Cells were induced to express EGFP-hTERTC27 for an indicated time and then fixed by methanol for analysis of aberrant chromosome events. The subcellular localization of hTERTC27 was examined under fluorescent microscope after the green fluorescent signal provided by EGFP tracer. We found that hTERTC27 alone contains a sufficient signal for its nuclear localization at the chromosome ends. The fusion protein accumulated in the nucleus, as all green fluorescence signals were clearly seen in the nucleus with a punctate localization pattern, whereas no signal could

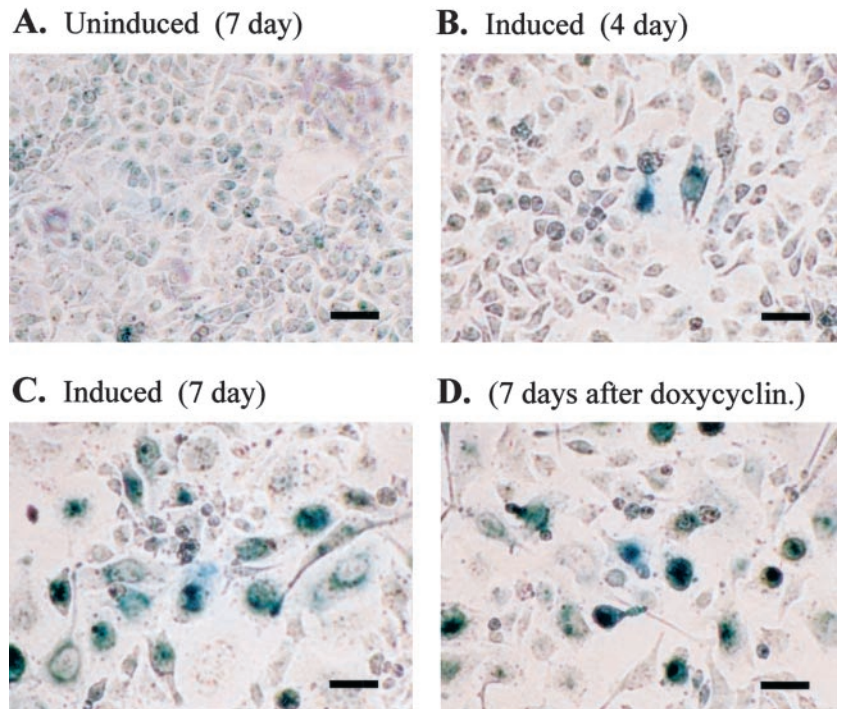


Fig. 3. Induction of cell senescence by hTERTC27. Representative data obtained from the HeLa Tet-off clone C8 were shown. Cell senescence was monitored by the morphological changes of noninduced (A), induced to express the hTERTC27 for 4 days (B), induced to express hTERTC27 for 7 days (C), and induced to express hTERTC27 for 6 days followed by the suppression of hTERT C27 expression for 7 days (D). Cells were stained for β -galactosidase activity at pH 6.0 and photographed using a phase-contrast microscope. The magnification is $\times 200$.

be detected in the cytosol (Fig. 4A, green color for EGFP). When the chromosomal DNA was stained with PI, the expressed hTERT C27 fusion proteins were found to be localized on both the interphase (Fig. 4B) and mitotic (Fig. 4C) chromosomes. At 4-days postinduction, significantly increased chromosome end-to-end fusion events were observed in hTERTC27-expressing cells (Table 1), which is exemplified with increased anaphase bridges (Fig. 4, D–G). This special nuclear localization pattern was endowed by the hTERTC27 polypeptide only, as control wild-type EGFP proteins were found only in the cytoplasm (data not shown). We also prepared a modified version of hTERTC27 by adding an HA tag to the COOH terminus of the EGFP-hTERTC27 (EGFP-hTERTC27-HA). HeLa cells transiently transfected with EGFP-hTERTC27-HA showed a diffused nuclear expression pattern with a small fraction of the protein expressed in the cytosol. There was no punctate localization pattern, indicating that the modified fusion protein was not located at a particular point of the chromosome (Fig. 4H). Therefore, the addition of the HA tag at the COOH terminus of hTERTC27 significantly decreased its ability

for nuclear translocation and totally abolished its ability to bind to the chromosome end. This indicates that an unhindered COOH terminus is essential for the nuclear translocation and binding to the chromosome ends.

Results from the cytogenetic analysis were combined and presented in Table 1. The expression of hTERTC27 caused a significant increase in the frequency of the aberrant chromosomes, such as anaphase bridges, evident for a chromosome end-to-end fusion. In the cells transfected with control EGFP vector, no change in the frequency of chromosomal alteration was observed. These results strongly suggest that the binding of hTERTC27 to the chromosome ends, presumably at the telomeres, leads to telomere dysfunction, which is one of the major causes triggering subsequent activation of cellular senescence and apoptotic pathways.

Ectopic Expression of hTERTC27 Did Not Affect Telomerase Activity. The phenomenon of telomere dysfunction and cell growth inhibition may be explained if hTERTC27 acts in a dominant negative manner to inhibit telomerase activity and, hence, result in telomere

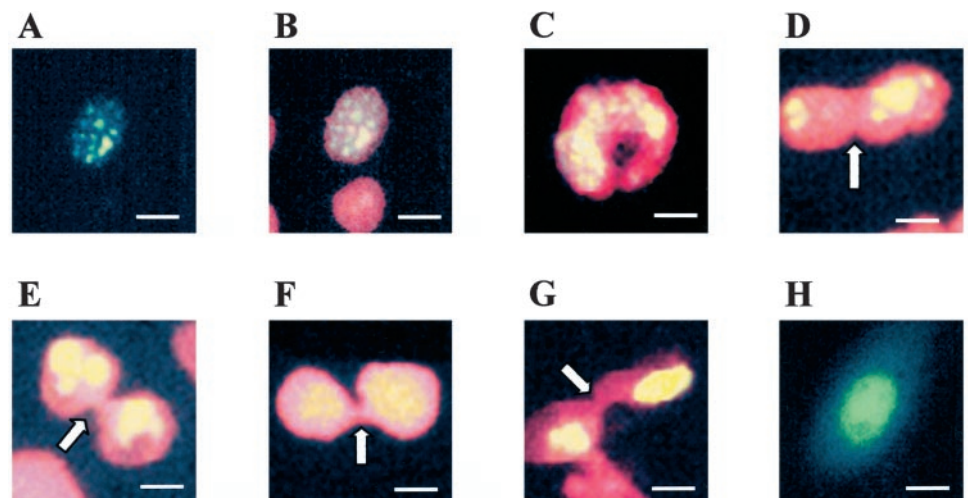


Fig. 4. Nuclear translocation and induction of chromosome end-to-end fusions. C8 clone was induced to express hTERTC27 for 4 days. In A, the hTERTC27 (using the EGFP tag as a tracer) was found to be localized in nucleus and showed the punctate pattern (green) in live HeLa cells. B–G, the chromosomal DNA (stained with PI and shown as red) and the overlapped images of the EGFP signal for hTERTC27 (shown as yellow). Localization of the ectopic expressed hTERTC27 in an interphase nucleus (B) and a mitotic chromosome (C). D–G, hTERTC27 induced anaphase bridge chromosome end-to-end fusion (arrows). H, HeLa cells transiently transfected with the modified EGFP-hTERTC27-HA plasmid.

Table 1 Induction of anaphase chromosome end-to-end fusions by hTERTC27

Cell line	Induction	No. of cells examined	Fraction with fusion event ^a (%)
HeLa Tet-off	-	50	12 ± 2
HeLa Tet-off	+	50	12 ± 3
HeLa-hTERTC27	-	100	18 ± 3
HeLa-hTERTC27	+	100	35 ± 5 ^b

^a Percentage of cells that exhibit chromosome end fusions were determined by calculating the ratio of aberrant mitosis events showing either chromosome bridges to the total mitosis events observed at day-4 postinduction. Value represents the mean ± SD of three separate experiments.

^b $P < 0.05$, significantly different from the control uninduced HeLa-hTERTC27 and HeLa Tet-off groups.

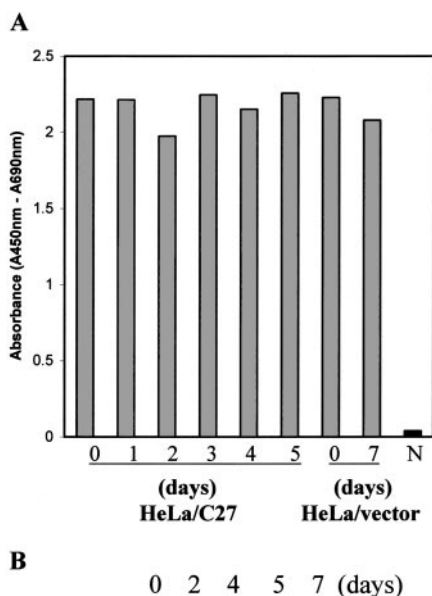


Fig. 5. Effects of ectopic expression of hTERTC27 on telomerase activity and telomere lengths. In A, whole cell extracts were prepared from HeLa Tet-off cells at indicated time points after the induction to express hTERTC27. Telomerase activity was detected by the TRAP-ELISA assay with a telomerase PCR ELISA Kit (Roche). The data represent the average values of three independent experiments for each sample. In B, genomic DNA was prepared from cells at indicated time points after the induction for hTERTC27 expression. For each sample, 2 μ g of genomic DNA were digested with the *RsaI* and *HinfI*. Telomere lengths were detected by the TRF assay with a Telo TTAGGG telomere-length assay kit (Roche).

shortening. To address this possibility, we analyzed the effect of hTERTC27 expression on cellular telomerase activity by the TRAP-ELISA assay. HeLa cells were transfected with pTetEGFP-hTERTC27 and induced to express the protein. The telomerase activity measurements showed that hTERTC27 expression had no effect on cellular telomerase activity (Fig. 5A). To further support this finding, genomic DNA from hTERTC27-expressing HeLa cells was isolated, and the telomere length was determined by TRF assay. Again, events of telomere shortening were not observed (Fig. 5B). Both of these data confirmed that hTERTC27-induced cell growth inhibition is independent of the telomerase activity.

Expression of hTERTC27 Reduced the Tumorigenicity of HeLa Cells *in Vivo* in Nude Mice Xenografts. To evaluate the potential anticell-proliferation effect of hTERTC27 *in vivo*, pooled clones of HeLa cells stably expressing either the EGFP-hTERTC27 or the control EGFP were generated by retrovirus transfection followed by G418 selection. These cells were then xenografted into nude mice ($n =$ four per group), and the solid tumor formation and tumor growth were analyzed. As shown in Fig. 6, at 4-weeks postxenografting, only one of the four mice receiving EGFP-hTERTC27-expressing HeLa cells developed tumors. The tumor mass was first noticed at day 17 with a slower growth rate and smaller size of tumor mass at the end of the experiment (Fig. 6A and Table 2). In contrast, all of the four mice xenografted with the control EGFP-expressing HeLa cells had noticeable tumors appear on the injected sites before day-13 post-xenografting. The tumors grew rapidly, and large tumor masses were observed at the end of the experiments (Fig. 6B and Table 2). These results are consistent with that obtained from the *in vitro* cell culture studies and indicate that hTERTC27 overexpression resulted in a reduced tumorigenicity of the cultured HeLa cells in the nude mice xenograft model.

DISCUSSION

In this study, we showed that ectopic expression of the hTERT COOH-terminal M_r 27,000 polypeptide in the telomerase-positive HeLa cells induced a rapid senescence-like growth arrest and apoptosis *in vitro*. Furthermore, hTERTC27 also reduced HeLa cell's tumorigenicity *in vivo* in the nude mice xenograft model.

All these tumor suppressing properties are likely caused by hTERTC27-induced telomere dysfunction, as evident by the nuclear/telomere localization of hTERTC27 and the chromosome end-to-end

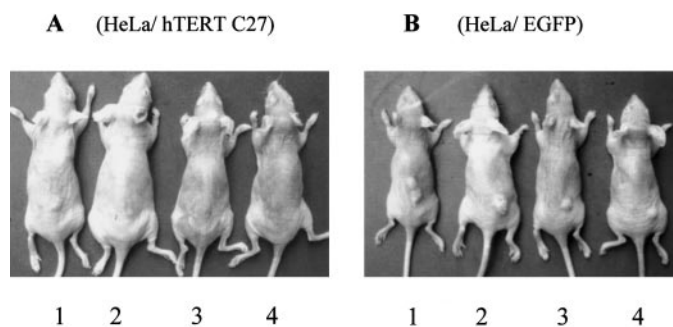


Fig. 6. hTERTC27 reduced HeLa cell tumorigenicity in the xenografted nude mice model. Photographs of nude mice ($n =$ four per group) injected s.c. with HeLa cells (2×10^6 cells/mouse). A, hTERTC27-expressing HeLa cells; B, control EGFP-expressing HeLa cells. The growth rates of the solid tumors were monitored weekly for 4 weeks. At the end of the experiments, mice were sacrificed, and photographs were taken to view the solid tumor formations. The experiments were repeated twice, and the representative results are shown.

Table 2 Inhibition of tumor formation in nude mice by hTERTC27^a

Cell line	Incidence of tumor formed ^b	Mean tumor size at 4 weeks (mm ³) ^c
A.		
HeLa EGFP	4/4	1298 ± 560
HeLa EGFP-hTERT C27	1/4	34 ± 68 ^d
B.		
HeLa EGFP	4/4	1304 ± 870
HeLa EGFP-hTERT C27	1/4	88 ± 175 ^d

^a Nude mice were injected s.c. with 2×10^6 cells of the indicated cell line and incidence of tumor formed/tumor size at 4 weeks after xenograft tumors were measured.

^b Data represent the number of mice with tumor/total number of mice in two separate experiments (A and B) each with four mice per group.

^c Values are mean ± SD.

^d $P < 0.01$ significantly different from the control HeLa EGFP group.

fusion events, which precede cell growth arrest. This mechanism is different from that of the previously reported hTERT antisense or dominant negative hTERT mutants, as the previous strategies all cause telomere dysfunction by inhibition of telomerase activity, thus causing progressive shortening of the telomere lengths and damage to the telomere structure (12, 24). In contrast, hTERTC27 does not inhibit telomerase activity, thus, reducing the possibility of activating ALT. Therefore, hTERTC27 may provide a more favorable clinical outcome.

The molecular basis of hTERTC27-induced telomere dysfunction is not known. We noticed that the phenotypes induced by hTERTC27 are very similar to that of a dominant negative mutant of the telomere-binding protein TRF2 (DN-TRF2). DN-TRF2 acts by disrupting the binding of TRF2 to telomere and rapidly induces telomere dysfunction and cell growth inhibition without affecting the telomerase enzymatic activity (36). Thus, it is possible that hTERTC27 binds TRF2, and this binding allows for precise homing and disruption of the telomere cap at the chromosome end. At present, the mechanism for recruiting hTERT to telomere end is not well understood. In yeast, the telomerase-associated protein Est1 and the telomeric single-strand DNA-binding protein Cdc13 are thought to participate in this process (39–42). Even though the human counter part for Est1 or Cdc13 has not been identified, it remains possible that other components of the telomere-binding proteins, such as pot 1 or the human counterpart of the yeast Est1/Cdc13, is the target and is responsible for the activity of hTERTC27.

Therefore, we propose a model for hTERT in telomere maintenance. In this model, the NH₂-terminal/central regions of hTERT are responsible for the telomerase enzymatic activity, and the COOH terminus of hTERT plays a pivotal role not only in nuclear translocation but also in telomere end targeting and the protection of the telomere structure. This model is consistent with several lines of evidence: (a) data obtained from the present study showed that hTERTC27 is capable of finding its way to the nucleus and the telomere ends and causing telomere dysfunction, presumably through disrupting the normal function of the COOH terminus of endogenous hTERT; (b) although normal somatic cells enter senescence or crisis when their telomere length shortens to ~5–8 kb, most TERT-positive cancer cells can survive with telomere length < 5–7 kb or shorter (43, 44), suggesting that TERT provided additional protection of the telomere structure in cancer cells; and (c) when telomerase are activated by hTERT expression in human diploid cells transformed with an activated oncogene, these cells can endure further telomere shortening without encountering cell senescence or cell crisis. Therefore, activation of hTERT may stabilize the telomere structure specifically on the content of shorter telomeric DNA lengths seen in cancer cells and thus prevent telomere dysfunction, which is critical for cancer cell immortalization (35, 45).

It is worth to note that the COOH-terminal regions of TERT are highly divergent among different species. In fact, no apparent amino acid sequence similarity could be found in this short fragment (30, 31, 46). Although this diversion may have been emerged from evolutionary selection to endowing species-specific regulations or functions, this diversion also makes it difficult to predict the potential functional motifs, which is essential for the maintenance of telomere structure, as well as cell immortalization.

Recently, the COOH terminus of hTERT is shown to be responsible for the nuclear localization of hTERT, as the hTERT COOH-terminal domain binds to 14-3-3 protein, and this binding prevents hTERT from being exported out of the nucleus. The nuclear import mechanism is not well understood at present. Results from our study indicated that hTERTC27 contains all of the domains sufficient for nuclear import, telomere-end targeting, as well as remaining in the

nucleus through binding to 14-3-3. Therefore, it appears possible that hTERTC27 expression may inhibit the nuclear localization of hTERT so that the full-length endogenous hTERT would remain in the cytosol. If this is the case, one may predict that hTERTC27 should also block hTERT-associated telomerase activity and cellular telomere-length maintenance. We found that ectopic expression of the hTERTC27 in HeLa cells did not affect telomerase activity and cellular telomere length. Therefore, our data do not support the assumption that the nuclear transport of the endogenous full-length hTERT was inhibited, or the hTERT should remain in the cytosol in cells expressing hTERTC27. One possible explanation for these observations is that 14-3-3 and the machinery responsible for hTERT nuclear import are in abundant supply; thus, hTERTC27 expression does not block 14-3-3 binding to hTERT in live cells. Furthermore, we did not observe a significant increase of aneuploid events in these affected HeLa cells. As the HeLa is a highly aneuploid cell line and the expression of C27 rapidly induced senescence, therefore, it is expected that these transfected cells will not divide further after the induction of C27 expression.

Additional research aimed at identifying the functional motifs, elucidating the molecular targets and mechanisms for hTERTC27-induced telomere dysfunction, will certainly shed a light in our understanding regarding the role of hTERT in cancer carcinogenesis. In view of the critical role of hTERT in the survival of cancer cells, as well as our findings that hTERTC27 polypeptide is sufficient to induce telomere dysfunction and cell senescence on its own, we propose that the clinical application of hTERTC27 in cancer therapy be further explored.

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