Inhibition of telomerase limits the growth of human cancer cells

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Telomerase is a ribonucleoprotein enzyme that maintains the protective structures at the ends of eukaryotic chromosomes, called telomeres. In most human somatic cells, telomerase expression is repressed, and telomeres shorten progressively with each cell division. In contrast, most human tumors express telomerase, resulting in stabilized telomere length. These observations indicate that telomere maintenance is essential to the proliferation of tumor cells. We show here that expression of a mutant catalytic subunit of human telomerase results in complete inhibition of telomerase activity, reduction in telomere length and death of tumor cells. Moreover, expression of this mutant telomerase eliminated tumorigenicity *in vivo*. These observations demonstrate that disruption of telomere maintenance limits cellular lifespan in human cancer cells, thus validating human telomerase reverse transcriptase as an important target for the development of anti-neoplastic therapies.

In culture, normal human cells have a finite lifespan, ultimately ceasing to proliferate in a process called replicative senescence[1]. Introduction of certain viral oncoproteins[2] or ablation of tumor suppressor gene function[3] permits human cells to bypass senescence; however, such post-senescent cells eventually reach crisis, a period of widespread cell death. The limited replicative lifespan of human cells has been postulated to serve as an important barrier to malignant transformation[4], indicating that cancer cells must overcome this obstacle and achieve replicative immortality before they can form malignant neoplasms.

Several observations indicate that telomeres, DNA–protein structures located at the ends of eukaryotic chromosomes, are important in the immortalization process[5]. In most normal human cells, telomeric DNA is progressively lost with each round of cell division[6,7]. Eventually, telomeres shorten to a critical length and lose their ability to protect the ends of chromosomal DNA (refs. 8–10). As a consequence, widespread chromosomal fusion and degradation occur; these karyotypic changes correspond in time to the growth arrest found in cultured cells[11,12]. In contrast, telomere length is stable in immortalized cells including tumor cells[10], indicating that their replicative immortality is attained through stabilization of telomere length.

In most tumors, this stabilization seems to be achieved through the expression of telomerase, which maintains and elongates telomeres by the *de novo* synthesis of telomeric DNA. The telomerase holoenzyme[13] is composed minimally of a constitutively expressed, template-containing RNA subunit[14] and a catalytic protein subunit[15–19] (human telomerase reverse transcriptase; hTERT). The level of hTERT expression is the rate-limiting component of this complex; most normal human somatic cells do not have detectable telomerase activity and lack expression of hTERT, whereas most immortalized cells have readily detectable telomerase activity and express hTERT (refs. 15,16,19–21). Ectopic expression of hTERT in telomerase-negative pre-senescent[22,23] and pre-crisis[24–26] cells results in telomerase activity and stabilization of telomere length and permits these cells to bypass senescence and crisis, respectively. Moreover, hTERT cooperates with the simian virus 40 large T antigen and oncogenic *ras* to convert normal human cells into transformed, tumorigenic cells[27].

These observations and the resulting model indicate that the telomerase expression found in 80–90% of human cancers[20,28] is essential for the continued growth of malignant cells, rather than being a secondary marker of the transformed state. One prediction of this model is that inhibition of telomerase function may alter the growth properties of malignant cells and, if

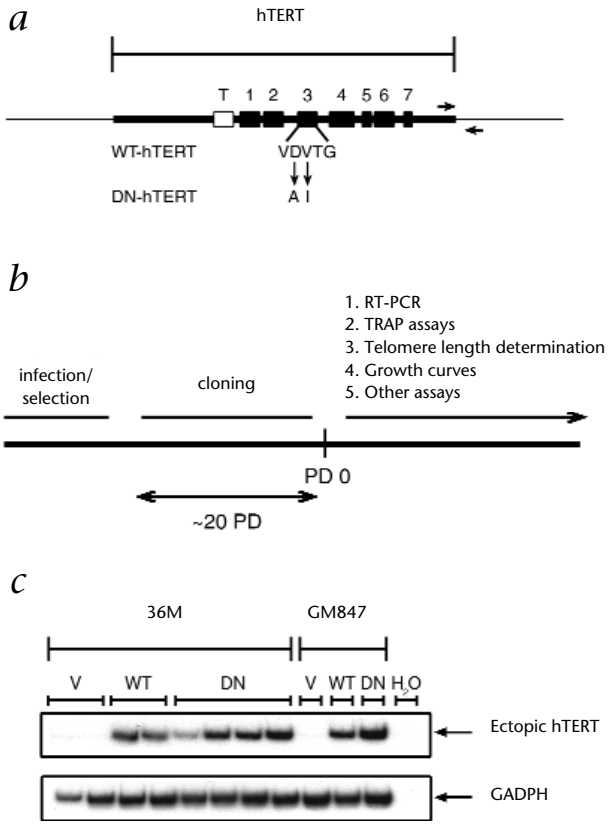

Fig. 1 Expression of WT-hTERT and DN-TERT in immortalized cell lines. ***a***, Retroviral constructs used to express WT-hTERT and DN-hTERT. Arrows, primers used for RT–PCR. Motifs labeled as described[16]; motif 3 has also been called motif A (ref. 15). T, region conserved among the TERTs but not among other reverse transcriptases[29]. ***b***, Experimental design and reference time frame. After infection, cells were selected in puromycin, grown to confluence (infection/selection, cloning), and cloned by limiting dilution and ring cloning (cloning). Population doubling (PD) 0 was defined after this point. The process of obtaining clonal isolates required approximately 20 population doublings before the designation of PD 0. ***c***, Expression of virally expressed WT-hTERT and DN-hTERT (ectoptic hTERT) analyzed by RT–PCR. Total RNA was analyzed from 36M (single-cell clones) and GM847 immortal cell lines. Above, WT-hTERT (WT) and DN-hTERT (DN) expression. V, control vector. GADPH (below), amplified to confirm that an equal amount of mRNA was present in each sample. No contaminating DNA was present in the samples (data not shown).

so, may represent a new strategy for anti-neoplastic therapies. hTERT is a particularly attractive target, as it shares considerable sequence similarity with reverse transcriptases[29]. Replacement of an aspartic acid residue (D530) located in the reverse transcriptase-like catalytic cleft of the yeast TERT completely abolishes its catalytic activity[30,31] and acts as a partial dominant negative allele when overexpressed in yeast[31]. Similar mutations in the sequences encoding the catalytic core of hTERT abrogated the catalytic activity of hTERT in *in vitro* reconstitution assays[17,32].

To determine whether disruption of hTERT catalytic function would limit the growth of normal and malignant cells, we created a catalytically inactive, dominant negative form of hTERT. We expressed this mutant (DN-hTERT) ectopically in human immortalized cells and cancer cells to assess the biochemical and physiological effects of telomerase inhibition on cellular immortality and tumorigenicity.

Effects of DN-hTERT on telomerase activity

To create DN-hTERT, we substituted the aspartic acid and valine residues at positions 710 and 711 in the third RT motif of hTERT with alanine and isoleucine, respectively (Fig. 1*a*). We introduced amphotropic retroviral vectors encoding DN-hTERT, wild-type hTERT (WT-hTERT), or a control vector expressing only a drug resistance marker into various cell lines (Fig. 1*b*). The infected cell lines included the human cancer cell lines 36M (ovarian), SW613 (breast), LoVo (colon) and SKBR3 (breast) as well as spontaneously immortalized, telomerase-positive human embryonic kidney cells[33] (HA-1). To ascertain the specific effects of DN-hTERT and WT-hTERT, we also introduced these two alleles of *hTERT* into the immortal human cell line GM847, which maintains telomere length by an as-yet uncharacterized alternative mechanism[34]. After drug selection, the successfully infected cells were cloned and the expression of the introduced WT- and DN-hTERT was confirmed using RT–PCR with primers specific for the introduced genes (Fig. 1*a* and c).

We analyzed telomerase activity in clonal isolates of each of the human cell lines that had acquired the control retroviral vector, WT-hTERT or DN-hTERT. Expression of DN-hTERT in telomerase-negative

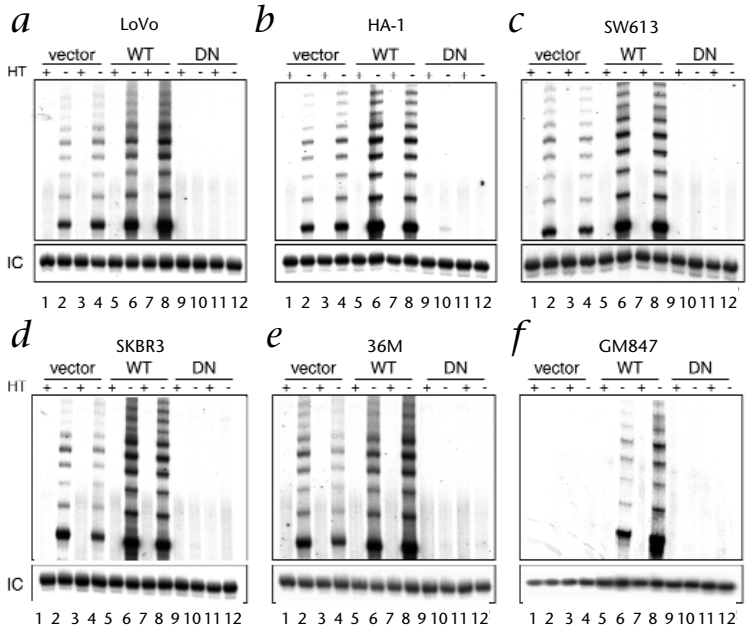

Fig. 2 Effects of WT-hTERT and DN-hTERT on telomerase activity. After infection and cloning, telomerase activity was determined in cell lines LoVo ***a***, HA-1 ***b***, SW613 ***c***, SKBR3 ***d*** and 36M ***e***. Telomerase activity was also determined in the telomerase-negative cell line GM847 ***f***. For each cell, 200 ng cellular lysate was analyzed for telomerase activity using the TRAP assay. Analysis of up to 1 μg lysate gave identical results (data not shown). Lanes 1–4, two clones expressing the control retrovirus; lanes 5–8, two clones expressing WT-hTERT; lanes 9–12, two clones expressing DN-hTERT. HT +, heat treatment of samples before the TRAP assay; HT –, no heat treatment; IC, internal control PCR product, demonstrating the absence of PCR inhibitors in the lysates. Percentage of completely telomerase-negative clones isolated from each cell line: LoVo, 70%; HA-1, 79%; SW613, 30%; SKBR3, 71%; 36M, 71%.

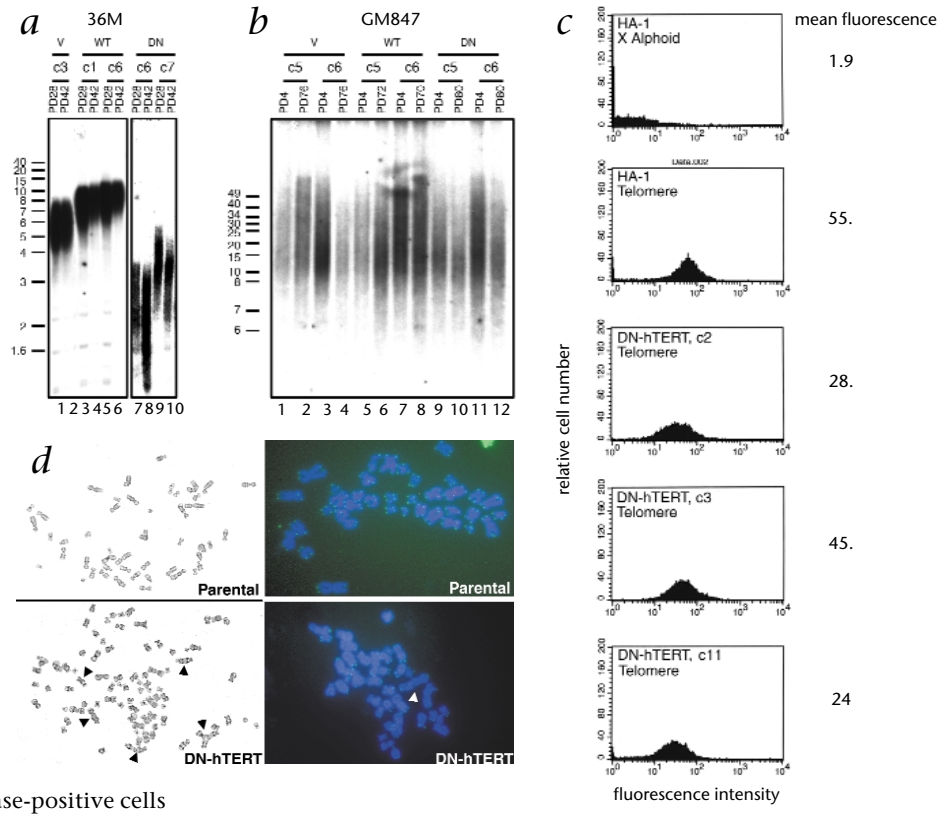

Fig. 3 Effects of DN-hTERT expression on telomere length. ***a*** and ***b***, 36M (*a*) or GM847 (*b*) single-cell clones expressing a drug resistance marker alone (V), WT-hTERT (WT) or DN-hTERT (DN) are the same as those analyzed in Fig. 2*e* and *f*. Total genomic DNA was assessed for telomere restriction fragment size by Southern blot analysis with a telomeric probe. *a*, Conventional electrophoresis. *b*, Pulse-field electrophoresis. PD, population doubling; c, clone number. Left margin, molecular size markers (kb). ***c***, Clonal isolates of HA-1 cells expressing DN-hTERT were analyzed by flow–FISH at late passage, and their telomere length was compared with that of parental HA-1 cells. Horizontal axis, logarithmic scale. Right, Mean fluorescence (geometric mean). c, clone number. Telomere and X Alphoid probes used for flow-FISH. ***d***, Telomere dysfunction in cells expressing DN-hTERT. Metaphase chromosomes obtained from 36M cells were banded with trypsin and stained with Giemsa (left) or were analyzed by FISH using a telomere-specific peptide-nucleic acid probe (right, green). Top row, Parental (uninfected) cells; bottom row, cells expressing DN-hTERT. Arrowheads, chromosomal fusions resulting in dicentric chromosomes.

GM847 cells did not result in telomerase activity, confirming that this mutant is catalytically inactive (Fig. 2*f*, lanes 9–12)(refs. 17,32). Expression of DN-hTERT in previously telomerase-positive cells produced multiple cell clones that lacked detectable telomerase activity (Fig. 2*a–e*, lanes 9–12). In contrast, expression of WT-hTERT slightly increased the overall telomerase activity in telomerase-positive cells (LoVo, HA-1, SW613, SKBR3, 36M; Fig. 2*a–e*, lanes 5–8) and induced enzyme activity in the telomerase-negative cell line GM847 (Fig. 2*f*, lanes 5–8), confirming that ectopic expression of hTERT was not cytotoxic. Thus, expression of a catalytically inactive mutant of hTERT results in disruption of existing telomerase activity.

Effects of DN-hTERT on telomere length and function

We next sought to determine whether inhibition of telomerase activity influenced telomere length. We assessed mean telomere length in 36M ovarian cancer cell clones expressing either WT- or DN-hTERT. The telomere lengths in this cell line are ordinarily maintained in the range of 5–7 kb (Fig. 3*a*). As 36M cell clones expressing DN-hTERT were passaged, gradual telomere shortening occurred (Fig. 3*a*, lanes 7–10). We estimate that these cells lost 3–5 kb of telomere length from the time of infection. As the process of isolating cell clones expends approximately 20 population doublings (Fig. 1*b*), the loss of telomere sequences is consistent with prior measurements of telomere loss in telomerase-negative cells[6]. In contrast, cells expressing a control retrovirus maintained stable telomere length (Fig. 3*a*, lanes 1 and 2) and cells expressing WT-hTERT showed stable telomere maintenance at a slightly longer length (Fig. 3*a*, lanes 3–6). Similar results were obtained with SW613 breast cancer cells expressing WT- or DN-hTERT (data not shown). Analysis of telomerase-negative GM847 cell clones expressing DN-hTERT also demonstrated that their telomere length was stable over nearly 100 population doublings (Fig 3*b*), indicating that the mutant enzyme had no effects on the telomeres of cells that maintain their telomeres through a telomerase-independent mechanism.

We next analyzed spontaneously immortalized HA-1 cells, which maintain telomere length in the range of 3–4 kb (ref. 35). Introduction and subsequent selection of clonal isolates expressing DN-hTERT in these cells yielded only small numbers of transduced cells, forcing us to analyze telomere length by the flow cytometric–fluorescent *in situ* hybridization (flow–FISH) technique[36]. This assay permits the examination of telomere length in a small number of cells. Expression of DN-hTERT in these cells resulted in loss of substantial telomeric sequences compared with those of the parental cells (Fig. 3*c*).

To determine whether the telomere loss induced by DN-hTERT led to telomere dysfunction, we analyzed chromosomal metaphase spreads derived from the 36M ovarian cancer cell clones expressing DN-hTERT. Confirming the Southern blot analysis results (Fig. 3*a*), loss of telomeric sequences in cells expressing DN-hTERT was easily detected using the FISH technique with a telomere-specific probe (Fig. 3*d*, right). Furthermore, in metaphase cells expressing DN-hTERT, we identified the presence of dicentric chromosomes and chromosomal fusions (in 14 of 14 cells analyzed; Fig. 3*d*, left). Such fusions occurred both in cells with normal numbers of chromosomes (1–3 fusions/metaphase) as well as in aneuploid cells (3–17 fusions/metaphase) and seemed to occur between chromosome ends that lacked telomeric sequences detectable by the FISH technique (Fig. 3*d*, right). In contrast, we did not identify any such structures in parental 36M cells (0 of 20 cells analyzed; Fig. 3*d*) or in 36M cells expressing WT-hTERT (0 of 12 cells analyzed), even though these cells were also aneuploid (data not shown). These results confirm that inhibition of enzyme activity by DN-hTERT disrupts telomere maintenance and ultimately results in telomere dysfunction.

Effects of DN-hTERT on cell proliferation

We characterized the growth properties of cells expressing either WT-hTERT or DN-hTERT. The growth kinetics of cells ex-

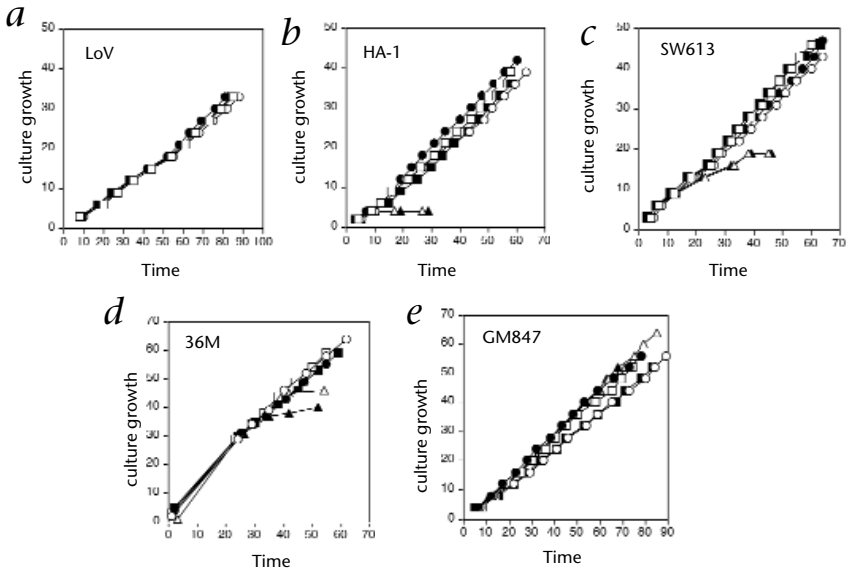

Fig. 4 Effects of DN-hTERT on cell proliferation. Clonal isolates analyzed are from cell lines LoVo ***a***, HA-1 ***b***, SW613 ***c***, 36M ***d*** and GM847 cells ***e***; in order of initial telomere length from shortest (LoVo) to longest (GM847). For each cell line, two clones are shown expressing the control retrovirus (□, ■), WT-hTERT (○, ●) or DN-hTERT (△, ▲).

pressing WT-hTERT did not differ substantially from those of cells carrying a control retrovirus vector that encodes only a drug resistance marker (Fig. 4*a–d*). In addition, expression of either WT- or DN-hTERT in the immortal, telomerase-negative cell line GM847 had no effect on the growth kinetics of these cells (Fig. 4*e*).

In contrast to the lack of response seen in GM847 cells after the introduction of DN-hTERT, cells that were initially telomerase-positive and received the DN-hTERT at levels sufficient to inhibit telomerase activity showed slowed growth and eventually stopped proliferating (Fig. 4*a-d*). The onset of cellular arrest in each cell line was related to their initial telomere length. For example, the mean telomere length in the colon cancer cell line LoVo is 2–3 kb (data not shown). After acquiring DN-hTERT, clonal isolates of LoVo cells did not continue to proliferate long enough to reach confluence (population doubling 0), whereas parallel cultures of these cells expressing either WT-hTERT or the control vector showed no change in growth (Fig. 4*a*).

Introduction of DN-hTERT into HA-1 and SW613 cells, which normally maintain longer initial telomere lengths of 3–4 kb and 4–5 kb, respectively (data not shown), did not immediately cause a growth arrest. Instead, these cells continued to proliferate for 10 days and 20 days, respectively, before growth arrest (Fig. 4*b* and *c*). In addition, 36M cells (initial telomere length of 5–7 kb; Fig 3*a*, lanes 1 and 2) expressing DN-hTERT showed no changes in growth rate until 30–40 days had elapsed (Fig. 4*d*), indicating that the rapidity of response to the introduced DN-hTERT gene depended on the initial length of the telomeres. The correlation between telomere length and time to growth arrest provided further evidence that DN-hTERT does not have direct cytostatic or cytotoxic effects on rapidly proliferating cells.

DN-hTERT expression and apoptosis

When cells expressing DN-hTERT stopped proliferating, they showed the morphological characteristics associated with crisis (Fig. 5*a*). Large cells with a flattened appearance predominated at first in these cultures; later, widespread cell death was apparent. As apoptosis is a common mechanism of cellular death, we investigated whether these cells had the phenotypic hallmarks of apoptosis. Flow cytometric analysis of DNA content in HA-1 cells expressing DN-hTERT demonstrated the appearance of a sub-G1 peak at a time corresponding to the appearance of shortened telomeres and morphological changes (Table 1). Similar results were obtained by TUNEL assay[37] of DNA fragmentation in both HA-1 (Table 1) and 36M cells (Fig. 5*b*). These results indicate that shortening of telomeres in these cells eventually results in the induction of apoptosis.

A mutant form of the telomere-binding protein telomere restriction fragment-2 can induce changes in telomere structure and a subsequent p53-dependent induction of apoptosis[38]. Here, we analyzed HA-1 cells in which the p53 protein has been inactivated by large T antigen[33], and 36M cells that contain defective p53 (ref. 39). In contrast to human embryonic kidney cells lacking large T antigen, HA-1 (Table 1) and 36M cells[39] were refractory to arrest at the G1 checkpoint induced by γ-irradiation, confirming that the p53 pathway is indeed nonfunctional[40]. Nonetheless, these cells underwent apoptosis after telomere shortening (Table 1 and Fig. 5*b*), indicating that telomere loss and the resulting induction of apoptosis can be mediated by a p53-independent pathway.

Effect of DN-hTERT on tumorigenicity

Inhibition of cell growth and induction of apoptosis *in vitro* indicated that inhibition of telomerase activity would reduce the tumorigenicity of cells *in vivo*. To confirm this, we injected late-passage clonal isolates from 36M cells expressing control retrovirus, WT-hTERT or DN-hTERT vectors subcutaneously into immunodeficient nude mice. Cells expressing control retrovirus or WT-hTERT rapidly produced tumors in this assay

Table 1 Expression of DN-hTERT leads to apoptosis in HA-1 cells

	% Apoptosis	G1/G2
HEK	ND	1.1
vector	6.3	0.2
WT-hTERT	10.1	0.8
DN-hTERT, c1	92.4	ND
DN-hTERT, c4	91.1	ND
DN-hTERT, c8	41.0	0.2
DN-hTERT, c10	81.6	0.3
WT-hTERT	8.9	ND
DN-hTERT, c1	42.1	ND
DN-hTERT, c10-1	65.0	ND
DN-hTERT, c11	25.5	ND
DN-hTERT, c15	43.2	ND

Top: % Apoptosis, percentage of cells in a sub-G1 peak, quantified by flow cytometric DNA analysis; G1/G2, percentage of cells arrested in G1 and G2 after treatment with γ-radiation (3,000 rad), quantified by flow cytometry, to confirm p53 was non-functional. HEK, parental human embryonic kidney cells, lacking large T antigen. Bottom, TUNEL analysis showing percentage of cells staining positive for TdT. ND, not determined; c, clone number.

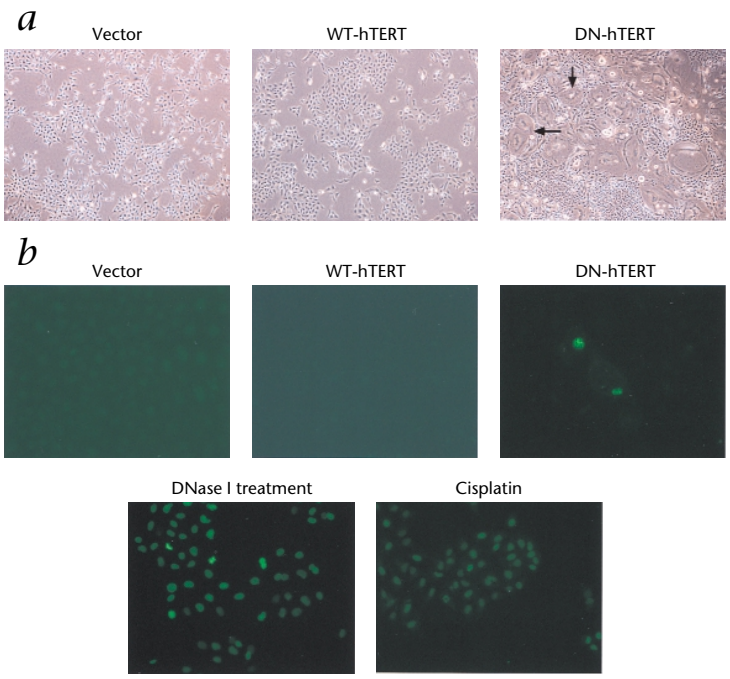

Fig. 5 Expression of DN-hTERT induces apoptosis. ***a***, Phase contrast micrographs demonstrating cellular morphology of 36M cells expressing control vector, WT-hTERT or DN-hTERT. Arrows indicate large, flat cells reminiscent of cells in crisis. These large cells constituted most of the later cultures expressing DN-hTERT, but were not found in cultures expressing control vector or WT-hTERT. ***b***, 36M cells expressing control vector, WT-hTERT or DN-hTERT, analyzed by the TUNEL assay for apoptosis. Bottom row, Parental 36M cells treated with DNase I or cisplatin (controls). The exposure time for vector and WT-hTERT samples was three times longer than that for DN-hTERT.

(Table 2). In contrast, multiple clones expressing DN-hTERT did not form tumors (Table 2). Thus, inhibition of hTERT not only limited the growth of these cells *in vitro* but also limited their tumorigenic capacity *in vivo*.

Discussion

Several lines of evidence now indicate that activation of telomerase and subsequent telomere stabilization are important and necessary steps in tumorigenesis. The results presented here show that inhibition of the catalytic subunit of telomerase, hTERT, results in telomere loss and limits the growth of several human cancer cell lines. Furthermore, these cells undergo apoptosis when their telomeres reach a critically short length, and such cells are no longer tumorigenic in immunodeficient mice. These results indicate that continuous hTERT activity is required for the maintenance of the malignant growth phenotype of many tumor cells. Moreover, they validate hTERT as an attractive target for the development of new anti-neoplastic therapeutics.

Strategies that target the RNA subunit of telomerase with antisense RNA (refs. 14,41–43) or peptide nucleic acids[44] diminish cellular telomerase activity and induce some changes in cellular growth. Those approaches, however, did not consistently lead to complete inhibition of telomerase activity, and the specific effects of such agents on both telomere shortening and cell death were difficult to assess. Here we have shown that inhibition hTERT reproducibly results not only in telomere shortening but also in growth arrest and death in many histologically distinct immortal and malignant cells. These results demonstrate that complete inhibition of telomerase can be achieved by targeting the active site of hTERT.

Although most human cancers express telomerase, some tumors maintain telomere length through an undefined alternative mechanism[34]. In *Saccharomyces cerevisiae*, the telomerase-independent mechanism to maintain telomere length involves recombination[45], and in *Schizosaccharomyces pombe*, cells that lack telomerase maintain telomeres either by recombination or by circularization of their chromosomes[46]. Cells from late-generation mice in which the telomerase RNA subunit has been deleted can still be transformed[8]; however, analysis of the telomeres in such immortalized cells shows that they are maintained at a shortened but stable length, probably through a mechanism related to the alternative mechanism[9]. Although we have not yet identified cells that survive the period of crisis induced by DN-hTERT, these observations indicate that tumor cells subjected to anti-telomerase therapies may acquire resistance through the development of other mechanisms to maintain telomeres, such as the alternative mechanism.

Mutations of the tumor suppressor gene p53 are found in at least 50% of human cancers[47]. As p53 is important in directing the cellular response to DNA damage, such mutations may be essential during the process of malignant transformation. In addition, loss of p53 function may explain in part the common problem of resistance to chemotherapy and radiotherapy[48,49]. Furthermore, p53 has been suggested to be one participant in the cellular response to telomere disruption and loss[38,50]. Here we have shown that telomere shortening induced by DN-hTERT leads to apoptosis in a p53-independent manner, indicating that anti-neoplastic therapies based on inhibition of hTERT will still be effective in limiting cancer cell growth even in cells that lack functional p53.

Unlike most conventional chemotherapeutic agents, agents that target telomerase may not induce cytotoxicity immediately after administration. Although many tumors maintain short telomeres[51], complete inhibition of tumor cell proliferation will require continued cell division until their telomeres reach a critically short length. This lag in therapeutic response will permit continued tumor cell growth in the presence of therapy for a period of time depending on the telomere length in the tumor cells at the time that therapy was initiated and thus may allow clini-

Table 2 Effects of DN-hTERT on tumorigenicity in 36M ovarian cancer cells

	Number of tumors/ Number of injections	Population doubling	Mean telomere length (kb)
Vector, c3	6/6	32	5–7
WT-hTERT, c6	6/6	33	6–8
DN-hTERT, c2	0/6	30	ND
DN-hTERT, c6	0/6	34	2–3.5
DN-hTERT, c7	0/6	32	2.5–4.5

Population doubling and mean telomere lengths of cells at the time of injection. ND, not determined; c, clone number.

cally important tumor cell growth. Moreover, the success of such strategies will require that telomerase inhibition be maintained until this telomere length is achieved. Thus, such anti-telomerase therapies will probably need to be coupled with other therapeutic modalities, particularly those that result in the prior debulking of the tumor mass.

Analysis of the hTERT protein has demonstrated that it is evolutionarily related to other reverse transcriptases. The successful and widespread use of reverse transcriptase inhibitors such as the nucleoside analogs used in HIV-1-infected individuals makes the development of small molecule inhibitors of hTERT an achievable prospect. Ideally, such inhibitors should be specific for hTERT and spare other polymerases in the cell. Indeed, some but not all nucleoside analogs can inhibit telomerase activity[52]. The results presented here indicate that specific, potent inhibitors of the hTERT enzyme are likely to be very effective in limiting the growth of many types of human cancer cells.

Methods

Generation of retroviruses and cell lines. The breast cancer cell lines SKBR3 and SW613 and the colon cancer cell line LoVo were obtained from the American Type Culture Collection (Rockville, Maryland). The ovarian cell line 36M was a gift from S. Cannistra, and the GM847 cell line was a gift from O. Pereira-Smith. DN-hTERT was created by substituting the aspartic acid and alanine residues at positions 710 and 711 with valine and isoleucine residues, respectively, by site-directed mutagenesis of pCI-neo-hTERT-HA using the oligonucleotides 5′–ATCACGGGCGCGTACGACACCATCCCCCA–3′ and 5′–CGCGACCTTGACAAAGTACAGCTCAGGCG–3′. The influenza virus hemagglutinin epitope tag was removed as described[24]. The resulting mutant was sequenced completely and subcloned into the vector pBABE-puro[53]. Amphotropic retroviruses were created with pBABE-puro, pBABE-puro–DN-hTERT or pBABE-puro–hTERT (ref. 24) as described[27]. Cells were selected continuously in puromycin (2 μg/ml). In all cases, the time at which a culture reached confluence in a 10-cm culture dish after viral infection, drug selection and ring cloning was called PD (population doubling) 0 (Fig 1*b*).

Telomerase assays, RT–PCR and telomere analysis. Cellular extracts assayed for telomerase activity using a PCR-based telomeric repeat amplification protocol (TRAP) assay[54]. For RT–PCR, total cellular RNA was prepared from cells using RNazol (TelTest B, Friendswood, Texas), and RT–PCR was done as described[27]. Telomere length was measured either by hybridizing a ^{32}P-labeled telomeric $(CCCTAA)_3$ probe to genomic DNA digested with *Hin*fI and *Rsa*I (ref. 10) separated by conventional or pulse-field electrophoresis, or by flow–FISH (ref. 36).

Analysis of chromosome structure. Metaphase chromosomes were prepared by treatment of cells with 0.1 μg/ml colcemid for 3.5 h, followed by hypotonic lysis in 0.075 M KCl, and fixation according to standard methods. Chromosomes were then analyzed by GTG banding[55] or by FISH (ref. 56).

Tumorigenicity assays. The ability of human cells to form tumors in immunodeficient mice (Balb/c-ByJ-Hfh11^{nu}; Jackson Laboratory, Bar Harbor, Maine) was determined as described[27].

Apoptosis assays. Apoptosis was assessed by flow cytometric DNA analysis and by using the TdT *in situ* Apoptosis Detection Kit (Genzyme, Cambridge, Massachusetts). Both floating and attached cells were collected from each sample. DNA was analyzed by flow cytometry by exposing cells to a solution of hypotonic propidum iodide and quantifying the fraction of cells containing a sub-G1 peak[37,57]. For the TUNEL assay, HA-1 cells were collected as described above, fixed for 10 min at room temperature in 4% paraformaldehyde (diluted in PBS and methanol-free), washed with PBS containing 1% BSA, and permeabilized with Cytopore following the manufacturer's instructions (Genzyme, Cambridge, Massachusetts). 36M cells were grown on coverslips and then fixed and permeabilized as described above for TdT analysis. The TdT assay was done following the manufacturer's instructions (Genzyme, Cambridge, Massachusetts). After the TdT assay, cells were counterstained with 0.06 μg/ml propidum iodide and 10 μg/ml RNAase A to analyze the cellular DNA content. To assess p53 function, we analyzed DNA content by flow cytometry 24 h after cells were exposed to γ-radiation (4,000 rad).

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1. Hayflick, L. & Moorhead, P.S. The serial cultivation of human diploid cell strains. *Exp. Cell. Res.* **25**, 585–621 (1961).
2. Shay, J.W., Wright, W.E. & Werbin, H. Defining the molecular mechanisms of human cell immortalization. *Biochim. Biophys. Acta* **1072**, 1–7 (1991).
3. Brown, J.P., Wei, W. & Sedivy, J.M. Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. *Science* **277**, 831–834 (1997).
4. Sager, R. Senescence as a mode of tumor suppression. *Environ. Health Perspect.* **93**, 59–62 (1991).
5. Harley, C.B. *et al.* Telomerase, cell immortality, and cancer. *Cold Spring Harb. Symp. Quant. Biol.* **59**, 307–315 (1994).
6. Harley, C.B., Futcher, A.B. & Greider, C.W. Telomeres shorten during ageing of human fibroblasts. *Nature* **345**, 458–460 (1990).
7. Hastie, N.D. *et al.* Telomere reduction in human colorectal carcinoma and with ageing. *Nature* **346**, 866–868 (1990).
8. Blasco, M.A. *et al.* Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* **91**, 25–34 (1997).
9. Hande, M.P., Samper, E., Lansdorp, P. & Blasco, M.A. Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice. *J. Cell. Biol.* **144**, 589–601 (1999).
10. Counter, C.M. *et al.* Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* **11**, 1921–1929 (1992).
11. Counter, C.M., Hirte, H.W., Bacchetti, S. & Harley, C.B. Telomerase activity in human ovarian carcinoma. *Proc. Natl. Acad. Sci. USA* **91**, 2900–2904 (1994).
12. Morales, C.P. *et al.* Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nature Genet.* **21**, 115–118 (1999).
13. Greider, C.W. in *Telomeres* (eds. Blackburn, E.H. & Greider, C.W.) 35–68 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1995).
14. Feng, J. *et al.* The RNA component of human telomerase. *Science* **269**, 1236–1241 (1995).
15. Nakamura, T.M. *et al.* Telomerase catalytic subunit homologs from fission yeast and human. *Science* **277**, 955–959 (1997).
16. Meyerson, M. *et al. hEST2*, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* 90, 785–795 (1997).
17. Harrington, L. *et al.* Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev.* **11**, 3109–3115 (1997).
18. Kilian, A. *et al.* Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum. Mol. Genet.* **6**, 2011–2019 (1997).
19. Nakayama, J. *et al.* Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. *Nature Genet.* **18**, 65–68 (1998).
20. Kim, N.W. *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015 (1994).
21. Ramakrishnan, S., Eppenberger, U., Mueller, H., Shinkai, Y. & Narayanan, R. Expression profile of the putative catalytic subunit of the telomerase gene. *Cancer Res.* **58**, 622–625 (1998).
22. Bodnar, A.G. *et al.* Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349–352 (1998).
23. Vaziri, H. & Benchimol, S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr. Biol.* **8**, 279–282 (1998).
24. Counter, C.M. *et al.* Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. *Proc. Natl. Acad. Sci. USA* **95**, 14723–14728 (1998).
25. Halvorsen, T.L., Leibowitz, G. & Levine, F. Telomerase activity is sufficient to allow transformed cells to escape from crisis. *Mol. Cell. Biol.* **19**, 1864–1870 (1999).

26. Zhu, J., Wang, H., Bishop, J.M. & Blackburn, E.H. Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening. *Proc. Natl. Acad. Sci. USA* **96**, 3723–3728 (1999).
27. Hahn, W.C. *et al.* Creation of human tumor cells with defined genetic elements. *Nature* **400**; 464–468 (1999).
28. Shay, J.W. & Bacchetti, S. A survey of telomerase activity in human cancer. *Eur. J. Cancer* **33**, 787–791 (1997).
29. Nakamura, T.M. & Cech, T.R. Reversing time: origin of telomerase. *Cell* **92**, 587–590 (1998).
30. Counter, C.M., Meyerson, M., Eaton, E.N. & Weinberg, R.A. The catalytic subunit of yeast telomerase. *Proc. Natl. Acad. Sci. USA* **94**, 9202–9207 (1997).
31. Lingner, J. *et al.* Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* **276**, 561–567 (1997).
32. Weinrich, S.L. *et al.* Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nature Genet.* **17**, 498–502 (1997).
33. Stewart, N. & Bacchetti, S. Expression of SV40 large T antigen, but not small t antigen, is required for the induction of chromosomal aberrations in transformed human cells. *Virology* **180**, 49–57 (1991).
34. Reddel, R.R., Bryan, T.M. & Murnane, J.P. Immortalized cells with no detectable telomerase activity. A review. *Biochemistry (Mosc.)* **62**, 1254–1262 (1997).
35. Counter, C.M., Botelho, F.M., Wang, P., Harley, C.B. & Bacchetti, S. Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B lymphocytes. *J. Virol.* **68**, 3410–3414 (1994).
36. Rufer, N., Dragowska, W., Thornbury, G., Roosnek, E. & Lansdorp, P.M. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. *Nature Biotechnol.* **16**, 743–747 (1998).
37. Goi, K. *et al.* DNA damage-associated dysregulation of the cell cycle and apoptosis control in cells with germ-line p53 mutation. *Cancer Res* **57**, 1895–1902 (1997).
38. Karlseder, J., Broccoli, D., Dai, Y., Hardy, S. & de Lange, T. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science* **283**, 1321–1325 (1999).
39. Strobel, T., Tai, Y.-T., Korsmeyer, S. & Cannistra, S.A. BAD partly reverses paclitaxel resistance in human ovarian carcinoma cells. *Oncogene* **17**, 2419–2427 (1998).
40. Kastan, M.B. *et al.* A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* **71**, 587–597 (1992).
41. Glukhov, A.I., Zimnik, O.V., Gordeev, S.A. & Severin, S.E. Inhibition of telomerase activity of melanoma cells *in vitro* by antisense oligonucleotides. *Biochem. Biophys. Res. Commun.* **248**, 368–371 (1998).
42. Bisoffi, M. *et al.* Inhibition of human telomerase by a retrovirus expressing telomeric antisense RNA. *Eur. J. Cancer* **34**, 1242–1249 (1998).
43. Kondo, S. *et al.* Antisense telomerase treatment: induction of two distinct pathways, apoptosis and differentiation. *FASEB J.* **12**, 801–811 (1998).
44. Norton, J.C., Piatyszek, M.A., Wright, W.E., Shay, J.W. & Corey, D.R. Inhibition of human telomerase activity by peptide nucleic acids. *Nature Biotechnol.* **14**, 615–619 (1996).
45. Lundblad, V. & Blackburn, E.H. An alternative pathway for yeast telomere maintenance rescues est1- senescence. *Cell* **73**, 347–360 (1993).
46. Nakamura, T.M., Cooper, J.P. & Cech, T.R. Two modes of survival of fission yeast without telomerase. *Science* **282**, 493–496 (1998).
47. Levine, A.J. p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323–331 (1997).
48. Aas, T. *et al.* Specific p53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nature Med.* **2**, 811–814 (1996).
49. Lowe, S.W., Ruley, H.E., Jacks, T. & Houseman, S.E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74**, 957–968 (1993).
50. Chin, L. *et al.* p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell* **97**, 527–538 (1999).
51. de Lange, T. *et al.* Structure and variability of human chromosome ends. *Mol. Cell. Biol.* **10**, 518–527 (1990).
52. Strahl, C. & Blackburn, E.H. Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. *Mol. Cell. Biol.* **16**, 53–65 (1996).
53. Morgenstern, J.P. & Land, H. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18**, 3587–3596 (1990).
54. Kim, N.W. & Wu, F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res.* **25**, 2595–2597 (1997).
55. Seabright, M. A rapid banding technique for human chromosomes. *Lancet* **2**, 971–972 (1971).
56. Landsdorp, P.M. *et al.* Heterogeneity in telomere length of human chromosomes. *Hum. Mol. Genet.* **5**, 685–691 (1996).
57. Schmid, I., Uittenbogaart, C.H. & Giorgi, J.V. A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification. *Cytometry* **12**, 279–285 (1991).