Limited Proliferation and Telomere Dysfunction following Telomerase Inhibition in Immortal Murine Fibroblasts

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ABSTRACT

Telomerase is a ribonucleoprotein enzyme that functions to maintain telomeres, the terminal DNA that protects chromosomal integrity, regulating cellular replicative life span. Telomerase is not expressed in most normal human somatic cells but is active in stabilizing telomeres of certain self-renewing cell populations and most malignant cells, making the enzyme an appealing target for anticancer therapy. We describe here a novel cross-species approach to telomerase inhibition. Ectopic expression of the human telomerase catalytic reverse transcriptase component in murine cells inhibited endogenous murine telomerase activity. Using this approach, telomerase inhibition in immortal murine fibroblasts resulted in critical telomere shortening, leading to slowed proliferation, abnormal morphology, altered cell cycle, and telomere dysfunction with cytogenetic instability, followed by apoptotic cell death. Subpopulations of two telomerase-inhibited clones escaped widespread apoptosis, showing proliferative recovery in culture despite persistently inhibited telomerase activity with progressive telomere shortening and dysfunction. This study, by targeting immortal murine cells for telomerase inhibition, demonstrates the importance of telomerase to murine cell immortalization and telomere maintenance. Moreover, the murine model used here should prove useful in further evaluating telomerase inhibition as an anticancer therapy.

INTRODUCTION

Telomerase is a ribonucleoprotein enzyme containing a catalytic reverse transcriptase component, hTERT or mTERT, and a RNA template component, hTR or mTR. Telomerase functions to maintain telomeres, the terminal DNA composed of TTAGGG repeats in both human and mouse cells, which protect eukaryotic chromosomal integrity (1). In addition to catalyzing the synthesis of telomeric DNA, telomerase may also have additional roles and interactions within the telomeric complex (2–5). In most normal human somatic cells, where telomerase is not expressed, telomeres progressively shorten with each cell division until a critically short length leads to cell senescence in culture (6, 7). Telomerase is expressed in certain normal self-renewing cells, such as germ cells and hematopoietic progenitor cells, where levels down-regulate with differentiation (8, 9). Most immortal cells, including 80–90% of human cancers, have high telomerase activity, enabling them to bypass the limitations on cell replicative life span (10).

Both the hTERT and mTERT genes have been cloned (11–14). Gene transfer of hTERT, the rate-limiting component of the telomerase complex, into such normal human cells as fibroblasts, retinal epithelium, and vascular endothelium allows them to bypass senescence and has led to apparent immortalization without evidence of neoplasia (15–18).

Activation of certain oncogenes or ablation of tumor suppressor gene function may allow cells to bypass the senescence experienced by normal cells (19, 20). Eventually, these cells will reach crisis, a period associated with critically short telomeres and characterized by cytogenetic instability and widespread cell death. If telomerase is activated, these cells will emerge from crisis to be immortalized (21). Retroviral transfer of hTERT into postmesencephalic SV40 large T antigen-containing human embryonic kidney cells or fibroblasts allows the cells to bypass crisis (22). When hTERT is combined with two oncogenes, SV40 large T antigen and H-rasV12, in normal human epithelial and fibroblast cells, oncogenic transformation occurs (23). Thus, cell immortalization via telomerase activation appears to be an important step in the oncogenic transformation pathway.

In view of its role in cell immortalization and lack of expression in most normal somatic cells, telomerase is an appealing target for anticancer therapy. Several approaches have been tried, including the use of antisense oligomers to the RNA component, small molecular weight compounds, and reverse transcriptase inhibitors (24, 25). Most recently, two groups have demonstrated telomerase inhibition leading to growth inhibition and apoptosis after gene transfer of a dominant negative mutant of hTERT into human cancer cells (26, 27). Many questions remain about the clinical viability of telomerase inhibition therapy. Will cells up-regulate telomerase expression to overcome inhibition or maintain telomere length by a telomerase-independent mechanism, as described in certain human cancers (28)? Would the time lag until a therapeutic effect of telomere shortening is obtained be prohibitive in vivo? Will organ systems dependent on telomerase activity, such as hematopoietic progenitor and germ cells, be adversely affected?

Many of these issues can best be explored in an animal model. Telomeres and telomerase have been studied extensively in the mouse. The telomeres in most murine strains are significantly longer than those in humans, causing some investigators to question the role that telomere shortening may have in murine cell life span and to be skeptical of the mouse’s utility as a model for humans (29, 30). Whereas the human and murine telomere sequences are identical, the mTR has only 65% nucleotide homology with hTR (31). Similarly, hTERT and mTERT share 64% amino acid homology (14). Low telomerase levels are evident in a great number of murine tissues, such as liver, kidney, and spleen, which may contribute to the increase in spontaneous immortalization of murine cells (13, 14, 32, 33). As do human tumors, most murine tumors have high telomerase levels (33, 34). mTERT knockout mice undergo progressive telomere shortening over multiple generations, leading to defects in spermatogenesis and the proliferative capacity of hematopoietic cells in late generations.
evidence supporting an important role for telomerase in self-renewing cells (35).

Described here is a novel cross-species approach to telomerase inhibition: retroviral transfer of the hTERT gene into murine cells. Ectopically expressed hTERT did not reconstitute telomerase activity in conjunction with native mTR or other mouse telomeric complex components but inhibited endogenous activity. Telomerase inhibition in immortal murine fibroblasts resulted in changes associated with critical telomere shortening followed by crisis with apoptotic cell death. This study provides an innovative dominant negative approach to telomerase inhibition as an anticancer therapy are discussed.

**MATERIALS AND METHODS**

**Cell Culture.** Murine bone marrow was harvested from femurs of 3-month-old BALB/c mice and then cultured in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc., Rockville, MD) with 10% FBS (Hyclone, Logan, UT), 100 units/ml penicillin, and 100μg/ml streptomycin (Life Technologies, Inc.). Stromal cells were isolated by serial depopulation of suspension hematopoietic cells on days 1, 3, and 5. Retroviral transduction of isolated MBMS was begun on day 7 after harvest.

MRC-5 cells, human fetal lung fibroblasts, were purchased from American Type Culture Collection (Manassas, VA) and cultured in minimal essential medium (Life Technologies, Inc.) supplemented with Earle’s balanced salt solution, 2 mM glutamine, 1.5 g/liter sodium bicarbonate, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

E86 retroviral producer cells and NIH 3T3 cells were cultured in DMEM-high glucose (Life Technologies, Inc.) with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. NIH 3T3 cells and derived clones were trypsinized, counted using 0.4% trypan blue (Life Technologies, Inc.) exclusion, and replated weekly.

All cells were incubated at 37°C in 5% CO₂.

All digital cell photographs were captured using a SPOT 2 camera with SPOT Advanced v2.2.2 software (Diagnostic Instruments, Sterling Heights, MI).

**Retroviral Vectors, Producer Cells, and Transductions.** As described previously (18), the retroviral vector GFP was constructed by inserting the coding sequence for EGFP into MFG (36), whereas TIG was generated by incorporating the hTERT cDNA linked to an internal ribosomal entry site element followed by EGFP. Retroviral plasmids were transiently transfected into 293GP cells (37) by a standard calcium phosphate transfection procedure, generating recombinant retroviral particles that were then used to stably infect the ecotropic retroviral producer cell line E86 (38). E86 cells transduced with TIG or GFP were subcloned by limiting dilution, and then individual clones were evaluated for viral titer on NIH 3T3 cells. The highest titer clones were identified and used in subsequent experiments. A marker rescue assay was performed on the two highest titer clones of each retrovirus, E86GFP 4 (titer 10⁷) and E86TIG 13 (titer 10⁷), using NIH 3T3 cells, demonstrating the absence of replication-competent retrovirus.

Target cells were transduced by incubation with viral supernatant and 8 μg/ml Polybrene (Sigma Chemical Co., St. Louis, MO). MBMS underwent six rounds of 8-h to overnight incubations. MRC-5 cells underwent two rounds of retroviral transduction 24 h after a 4-h infection with an adenovirus containing the ecotropic receptor (AdEcoR) at a multiplicity of infection of 100 plaque-forming units/cell. NIH 3T3 cells were transduced with one to two rounds of 8-h to overnight incubation. Transduction efficiency was quantitated by flow cytometric analysis (FACScan; Becton Dickinson, San Jose, CA) for GFP expression 3 days after transduction completion.

**Single-cell Cloning.** NIH 3T3 cells transduced with TIG or GFP were single-cell cloned in 96-well plates. The day of plating, 4 days after transduction completion and 1 day after FACS analysis, was designated day 0. A single cell proliferated to confluence in the 96-well plate, the entire clonal population was transferred into a T-25 flask. The number of days from single cell, designated PD 0, to confluence in the T-25 was designated as the time to initial confluence (21–23 PDs). Pellets from the day of initial confluence were used for initial analysis of telomerase activity.

**Telomerase Activity.** Cellular protein extracts were assayed for telomerase activity by using the PCR-based TRAP as described previously (18, 39) or by using the TRAPEZE telomerase detection kit (Intergen, Purchase, NY). After subculture extension by telomerase, the products were amplified by PCR in the presence of a [γ-32P]ATP-end-labeled TS primer, resolved on a 12.5% polyacrylamide gel, exposed to a Fujifilm Imaging Plate, scanned on a Fujifilm BAS-2500 Bio-imaging Analyzer, and then quantitated using MacBas v2.5 (Fujii, Stamford, CT). Background activity was subtracted, and the ratio of sample intensity to that of a 36-hp internal control was calculated. All values were then expressed as a percentage of telomerase activity in a control NB cell line.

**PCR Analysis.** Cells were lysed in buffer containing 5 mM Tris-HCl (pH 8.0), 0.45% Tween 20, and 100 μg/ml proteinase K (1.5 h at 50°C followed by 10 min at 95°C). PCR amplification was performed on lysates from 4 × 10⁵ cells/reaction using three sets of primers: (a) EGFP1 (5'-GGCACAAGTT-TACGGGTGTC-3') and EGFP2 (5'-AGCTCAGTCGGTCCAGC-3'); (b) β-actin upper (5'-GTGGGGGCGCCCAGCAACA-C-3') and β-actin lower (5'-CTCCTAATGTGACAGTTC-3'); and (c) hTERT R6 (5'-GGATGTGCTTTGAAGTCTGAG-3') and hTERT F1 (5'-AGCCGAC-GGATGGTCTTGAAGTCTGAG-3').

**Fig. 1.** Telomerase activity in hTERT-transduced human fibroblasts. TRAP assay was performed on 2 μg of cellular lysates of MRC-5 cells transduced with an adenovirus expressing the murine ecotropic retroviral receptor followed by a viral supernant from E86 packaging cell clones containing the TIG vector (AdE/TIG 2, 7, 9, 10, 13, 18, or 24) or GFP control vector (AdEGFP 4). R8, telomeric DNA; NB, NB cellular lysate; IC, internal control.

**Fig. 2.** Inhibition of endogenous telomerase activity in NIH 3T3 cells expressing hTERT. Viral supernant from E86 packaging cell clones containing either the TIG vector (E86TIG 2, 7, 9, 10, 13, 18, or 24) or a range of titers or the GFP control vector (E86GFP 4) was used to transduce NIH 3T3 cells at a range of transduction efficiencies. TRAP assay was performed on 2 μg of cellular lysates from transduced or control mock-infected cells, and then telomerase activity was quantitated by phosphorimaging, normalized against an internal control, and expressed as a percentage of telomerase activity in NB. TIG transduction efficiency was measured by FACS analysis for GFP expression that was plotted against telomerase activity. GFP-transduced control cells (data not shown) was 34.1% of NB control. r, correlation coefficient.
TACTCCAGCTAT-3'). A total of 30–35 PCR cycles (30 s at 94°C, 1 min at 60°C, and 1–2 min at 72°C) was performed, followed by 2% agarose gel analysis and imaging using Gel Doc with Quantity One v4.1 quantitation software (Bio-Rad, Hercules, CA).

Telomere Length Measurement. Telomeres were measured by modifying the previously described slot blot technique (40) for murine cells. Cell lysates were prepared as described above. Lysate DNA from 2–5 × 10⁴ cells/slot was denatured, blotted onto a Nytran nylon transfer membrane (Schleicher & Schuell, Keene, NH) using a Minifold Slot-Blot System apparatus (Schleicher & Schuell), and neutralized, all as described previously (40). The blotted DNA was then UV cross-linked. The membrane was prehybridized in 6× SSPE, 0.5% SDS, 5 × Denhardt’s solution, and 20 μg/ml tRNA (Sigma Chemical Co.) for 3 h at 55°C and then hybridized in 6× SSPE and 1% SDS with a [γ-32P]ATP-end-labeled oligonucleotide probe at 55°C overnight. Each blot was hybridized separately with two probes, a telomeric probe [(TTAGGG)₄] and a centromeric probe binding the 234-bp major satellite repeat [5'-GAAATGTCCACTGTAGGACGTGGAATATGGCAAGAAA-3'] (41–43). Between hybridizations with each probe, the membrane was dehybridized by washing three times for 15 min each in boiling 0.1× SSPE and 0.5% SDS. After hybridization, the membrane was washed three times in 6× SSPE and 0.1% SDS at room temperature and once at 55°C for 60 s. The membrane was rinsed in 5× SSPE and then exposed to a Fuji Imaging Plate for 24 h, scanned as described above, and analyzed using MacBas v2.5. Telomere and centromere signals were quantitated, and a T:C ratio was calculated for each slot. Each sample was blotted in triplicate. Means ± SEs for each sample were calculated, and relative telomere lengths were compared with the initial NIH 3T3 standard.

FISH with FITC-labeled peptide nucleic acid (CCCTAA) telomere probe (Oncor) was performed on methanol-acetic acid-fixed metaphase spreads according to the protocol provided by the manufacturer. Metaphases were analyzed using a Nikon Eclipse E800 microscope equipped with the Vysis path vision system (Vysis, Downers Grove, IL).

Cytogenetic Analysis. Cultures were harvested after overnight treatment with 0.005 μg/ml Colcemid. After hypotonic treatment with 0.075 M KCl for 20 min at 37°C, cells were fixed with methanol:acetic acid (3:1). Metaphase spreads were G-banded by the standard protocol and, when necessary, counterstained with 4',6-diamidino-2-phenylindole (Vysis) to identify the centromeres. Twenty metaphases were analyzed for each clone using a Nikon Eclipse E800 microscope equipped with the Vysis path vision system.

Cell Cycle Analysis and Apoptosis Assays. Both adherent and floating cells were collected for each sample. Cell cycle analysis after EtBr staining for DNA content of extracted nuclei (44, 45) was evaluated by FACScan. PE-labeled annexin V staining was performed following the manufacturer’s instructions (PharMingen, San Diego, CA; Ref. 46). PI was subsequently added to evaluate for cell necrosis. Three-color analysis (GFP, PE, and PI) was performed on a FACScan.

RESULTS

Transduction of MBMS with hTERT. To determine whether expression of retrovirally transduced hTERT would reconstitute functional telomerase activity in murine cells, murine bone marrow was harvested from femurs of 3-month-old BALB/c mice. The stromal
cells (MBMS) were isolated and then retrovirally transduced with ecotropic retrovirus generated by an E86GFP clone as well as several different E86TIG clones. FACS analysis for GFP expression revealed transduction efficiencies in a range of 7.1–24.5% for TIG and 40.8% for GFP. TRAP analysis detected a very low level of endogenous telomerase activity in the mock-transduced stromal cells, but no endogenous telomerase activity was detected in the TIG-transduced cells (data not shown). This is consistent with previously described results in which telomerase activity could not be reconstituted in extracellular extracts containing hTERT and mTR.

**Transduction of Human MRC-5 Cells with hTERT after Infection with Ecotropic Receptor-containing Adenovirus.** To confirm an interspecies incompatibility rather than a recombinant event or defective vector expression in the transduced MBMS, supernatant from the same viral producer cells was used to transduce MRC-5 cells, human fetal lung fibroblasts that express hTERT, with a highly significant negative correlation between transduction efficiency and telomerase activity ($r = -0.921$; Fig. 2). Analyzed from another perspective, all 3T3 cell populations with >45% TIG transduction efficiency had a reduction in telomerase activity to <45% of the GFP-transduced controls. Because telomerase activity in the mock-transduced cells was 34.1% of NB control (data not shown), transduction with GFP alone did not inhibit telomerase activity but actually appeared to increase it. Thus, hTERT did appear to act as a specific inhibitor of telomerase activity in the mouse cell.

Because E86 packaging cells are derived from NIH 3T3 cells (38), it is notable that the highest viral titers attainable from E86TIG and E86GFP clones were $10^3$ and $10^7$, respectively (see “Materials and Methods”). The significantly lower titer for E86TIG producers may be explained by a negative growth selection for those clones producing high levels of hTERT-containing virus. Harmful hTERT activity in the cells may also explain the abnormal morphological characteristics of E86TIG clones as compared with normal-appearing E86GFP clones (data not shown).

**Inhibition of Endogenous Murine Telomerase Activity after hTERT Transduction.** Based on the above-mentioned results, we investigated whether hTERT not only fails to generate functional telomerase in murine cells but actually inhibits endogenous murine telomerase activity in a dominant negative fashion. NIH 3T3 cells, immortalized murine fibroblasts with high endogenous telomerase activity, were transduced with virus from an E86GFP control clone and several E86TIG clones to achieve a range of transduction efficiencies. Telomerase activity decreased significantly in cells expressing hTERT, with a highly significant negative correlation between transduction efficiency and telomerase activity ($r = -0.921$; Fig. 2). Analyzed from another perspective, all 3T3 cell populations with >45% TIG transduction efficiency had a reduction in telomerase activity to <45% of the GFP-transduced controls. Because telomerase activity in the mock-transduced cells was 34.1% of NB control (data not shown), transduction with GFP alone did not inhibit telomerase activity but actually appeared to increase it. Thus, hTERT did appear to act as a specific inhibitor of telomerase activity in the mouse cell.

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**Fig. 4.** TIG and GFP vector presence in clones and negative correlation between TIG vector expression and cell proliferation. A, PCR for GFP (days 152–156) was performed to evaluate for the presence of GFP or TIG vector. A sample PCR is shown. PCR for β-actin was performed as a control for DNA content. B, late-passage cells (days 223–226) were evaluated by FACS analysis for GFP expression. Mean GFP fluorescence of the entire cell population was plotted against the proliferation rate over the previous week. Clones marked with x eventually progressed to widespread cell death. Clones with normal proliferation patterns had no detectable GFP expression. $r$, correlation coefficient.

**Fig. 5.** Telomere length analysis of 3T3TIG and 3T3GFP clones. A, slot blot hybridized with telomere probe is shown for 3T3 Mock cells, 3T3GFP clones, and 3T3TIG clones of varying growth characteristics. Each sample was blotted in triplicate. Calculations are presented in Table 1. B–D, representative FISH images of metaphase spreads with fluorescent spots on telomeres. Metaphase spreads of (B) 3T3 Mock (day 195) and (C) 3T3GFP B5 (day 226) showing bright telomere signals of visually equal intensity on all chromosomal ends. D, partial metaphase of 3T3TIG G9 (day 230) showing marked heterogeneity in the telomere signal intensities. Many telomeres do not show signals. Bright or weak telomere staining can be seen on some chromosomal ends.
Table 1 Telomere length analysis by slot blot of 3T3TIG and 3T3GFP clones

Relative telomere lengths were quantitated by phosphorimaging of the blot after hybridization with both telomere and centromere probes. All samples were blotted in triplicate, and a T:C ratio was determined for each slot. Means ± SE for each sample were calculated and normalized to the NIH 3T3 initial T:C ratio.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Day</th>
<th>PD</th>
<th>Telomere length (% NIH 3T3 initial)</th>
<th>Telomere shortening (% telomere loss/PD)</th>
</tr>
</thead>
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<tr>
<td>NIH 3T3 Initial</td>
<td>12</td>
<td>17</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>NIH 3T3 Mock</td>
<td>230</td>
<td>321</td>
<td>100.3 ± 0.5</td>
<td>-0.001 ± 0.002</td>
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<tr>
<td>3T3GFP B5</td>
<td>226</td>
<td>307</td>
<td>85.2 ± 2.0</td>
<td>0.048 ± 0.006</td>
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<tr>
<td>3T3GFP C3</td>
<td>226</td>
<td>277</td>
<td>75.5 ± 3.8</td>
<td>0.088 ± 0.014</td>
</tr>
<tr>
<td>3T3GFP D1</td>
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<td>286</td>
<td>90.9 ± 1.2</td>
<td>0.032 ± 0.004</td>
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<tr>
<td>3T3TIG B4</td>
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<td>292</td>
<td>92.9 ± 7.0</td>
<td>0.024 ± 0.024</td>
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<tr>
<td>3T3TIG H7</td>
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<td>295</td>
<td>81.8 ± 2.1</td>
<td>0.062 ± 0.007</td>
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<td>228</td>
<td>67.2 ± 0.8</td>
<td>0.144 ± 0.003</td>
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<td>0.120 ± 0.018</td>
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<td>39.7 ± 2.1</td>
<td>0.286 ± 0.014</td>
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<td>38.1 ± 2.1</td>
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<td>208</td>
<td>61.5 ± 8.5</td>
<td>0.186 ± 0.041</td>
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<tr>
<td>3T3TIG D10</td>
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<td>59.4 ± 1.7</td>
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<td>3T3TIG G8</td>
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<td>212</td>
<td>29.0 ± 0.5</td>
<td>0.335 ± 0.002</td>
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<td>3T3TIG H2</td>
<td>238</td>
<td>232</td>
<td>45.6 ± 0.9</td>
<td>0.234 ± 0.003</td>
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*a* Clone with normal growth.

*b* Clone with intermediate, steady growth.

*c* Clone with slowed growth followed by widespread cell death.

Fig. 6. Morphological changes of late PD hTERT-expressing NIH 3T3 clones with slowing growth. Phase-contrast micrographs demonstrate increased size, flattening, multinucleation, and cytoplasmic extensions in day 226 3T3TIG clones.

Proliferation of hTERT-transduced NIH 3T3 Clones. To further evaluate the effects of ectopic hTERT activity on murine cells and to assess the impact of targeted telomerase inhibition on previously immortal murine cells, additional experiments were conducted on NIH 3T3 cells. NIH 3T3 cells were transduced with either TIG or GFP or were mock-infected (incubated with media and Polybrene only). Transduction efficiencies, measured by FACS analysis, were 99.9% for GFP and 97.7% for TIG. Whereas mock- and GFP-transduced mass cultures maintained high telomerase activity levels (74.7% and 71.8% of NB control, respectively), the TIG-transduced mass culture demonstrated significant telomerase activity inhibition (9.2% of NB control; Fig. 3).

To analyze long-term effects of telomerase inhibition without the proliferative selection for untransduced cells seen over time in the mass cultures (data not shown), single-cell clones of the 3T3TIG and 3T3GFP mass cultures were generated. Twenty-five 3T3TIG clones and 12 TIGGFP clones, along with the mock-transduced mass culture (3T3 Mock), were followed. All 3T3TIG clones showed initial telomerase activity inhibition by TRAP with a mean ± SD telomerase activity of 3.9 ± 4.1% of the activity in mock-transduced mass cultures (representing >95% telomerase activity inhibition). In contrast, the 3T3GFP clones demonstrated mean telomerase activity levels of 64.2 ± 40.3% of activity in mock-transduced mass cultures.

A sample TRAP for one set of clones is shown in Fig. 3B. Each clone was counted weekly for over 300 days (Fig. 3C). After 120–200 PDs, 16 of 25 3T3TIG clones developed marked proliferative slowing, followed by cessation of proliferation and widespread cell death. This is consistent with the timing expected for an effect of critical telomere shortening. In contrast, all 12 3T3GFP clones and the 3T3 Mock cells continued to grow at a constant linear rate.

To evaluate whether differing proliferative characteristics among 3T3TIG clones were secondary to cells remaining untransduced or losing the TIG vector, PCR for GFP was performed on all 3T3TIG and 3T3GFP clones. As illustrated in Fig. 4A, all clones were vector positive.

FACS analysis of late-passage clones (days 223–226) was then performed to determine whether 3T3TIG clone growth characteristics reflected variable vector expression (Fig. 4B). Indeed, a negative correlation was seen between mean GFP fluorescence and cell proliferative rate (r = -0.701), with two distinct groups based on presence or absence of progression to widespread cell death of the clonal population. Two clones, 3T3TIG A2 and 3T3TIG C6, were not included in this analysis because their entire populations were already dead by days 223–226. The two most rapidly proliferating clones, 3T3TIG B4 and 3T3TIG H7, had no detectable GFP fluorescence.

Telomere Shortening in hTERT-transduced NIH 3T3 Clones. The standard measurement of telomere length in human cells has been by TRF measurements. However, the excessive length of murine telomeres, with TRFs of 20–150 kb compared with 5–15 kb in humans, makes them difficult to measure accurately using conventional gel electrophoresis. Murine chromosomes have long subtelo-

Table 2 Chromosomal aberrations of hTERT-expressing NIH 3T3 cells in crisis

<table>
<thead>
<tr>
<th>Clone</th>
<th>Day</th>
<th>PD</th>
<th>54-64</th>
<th>65-125</th>
<th>Total no. of aberrations/cell</th>
<th>Ploidy (%)</th>
<th>Media (SD)</th>
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<tr>
<td>Mock</td>
<td>195</td>
<td>270</td>
<td>100</td>
<td></td>
<td>0.05 (0.22)</td>
<td>—</td>
<td>—</td>
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<tr>
<td>GFP B5</td>
<td>198</td>
<td>268</td>
<td>80</td>
<td>20</td>
<td>—</td>
<td>0.10 (0.31)</td>
<td>—</td>
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<tr>
<td>TIG B4</td>
<td>197</td>
<td>250</td>
<td>90</td>
<td>10</td>
<td>0.85 (1.04)</td>
<td>—</td>
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<tr>
<td>TIG A3</td>
<td>197</td>
<td>193</td>
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<td>15</td>
<td>1.65 (1.39)</td>
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<td>TIG B9</td>
<td>193</td>
<td>201</td>
<td>85</td>
<td>15</td>
<td>2.95 (0.94)</td>
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<tr>
<td>TIG G5</td>
<td>197</td>
<td>193</td>
<td>85</td>
<td>15</td>
<td>5.75 (2.10)</td>
<td>—</td>
<td>—</td>
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<tr>
<td>TIG C5</td>
<td>197</td>
<td>206</td>
<td>70</td>
<td>30</td>
<td>3.85 (2.68)</td>
<td>2.35 (0.88)</td>
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<td>TIG F10</td>
<td>197</td>
<td>190</td>
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<td>20</td>
<td>7.90 (2.88)</td>
<td>3.60 (1.95)</td>
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<td>201</td>
<td>60</td>
<td>40</td>
<td>9.70 (3.96)</td>
<td>0.70 (0.95)</td>
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<td>210</td>
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<td>75</td>
<td>3.75 (2.84)</td>
<td>1.50 (0.73)</td>
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<td>TIG G5</td>
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<td>3.05 (1.73)</td>
<td>0.50 (0.22)</td>
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<td>TIG G9</td>
<td>225</td>
<td>220</td>
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<td>45</td>
<td>5.65 (2.21)</td>
<td>0.70 (0.22)</td>
<td>—</td>
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<tr>
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<td>215</td>
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<td>50</td>
<td>4.20 (3.74)</td>
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<td>—</td>
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<tr>
<td>TIG H2</td>
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<td>65</td>
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<td>—</td>
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<tr>
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<td>233</td>
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<td>65</td>
<td>3.50 (1.76)</td>
<td>0.70 (0.22)</td>
<td>—</td>
</tr>
</tbody>
</table>

*a* A total of 20 metaphases were analyzed for each clone except for TIG G1 day 202 (12) and day 232 (4); TIG H2 day 200 (10).

*b* Mean (SD).

*c* Includes p-arm fusions, q-arm fusions, p- and q-arm fusions, rings, acentric fragments, triradials, tetraradials, and chromosomal breaks.

*d* Cells not in crisis.

*e* Vector lost over time.

*f* Ploidy 47–51.

*g* Ploidy 8n ±.

*h* Mitotic index very low; poor morphology; highly aberrant karyotype.
meric regions lacking restriction sites so that the TRF measurement also overestimates the true telomere length (30). Consequently, a recently developed flow cytometric method using FISH with a labeled probe for the telomeric sequence (flow-FISH) has been used to measure murine telomere lengths (49). Because this technique measures total cell telomere content, the presence of cytogenetic abnormalities, such as aneuploidy, may generate inaccurate telomere length measurements. A third technique described for telomere length measurement is the slot blot (40). Similar in principle to the flow-FISH, it is a DNA blot technique in which a radiolabeled telomeric sequence probe is used to measure total telomere content in the cell. In addition, the blot is hybridized with a centromeric probe. A telomeric to centromeric DNA ratio proportional to telomere length is obtained. This corrects for chromosomal number in the cell and generates results independent of cell ploidy. The technique has been modified here for murine cells.

Cells from the 3T3 Mock culture, several 3T3GFP control clones, and 3T3TIG clones with variable growth characteristics were evaluated by slot blot (Fig. 5A). Telomere lengths at days 226–245 were compared with the initial NIH 3T3 length on day 12. Relative telomere lengths and the rate of telomere shortening (percentage of telomere loss/PD) are shown in Table 1. The 3T3 Mock cells showed no evidence of telomere shortening, whereas the 3T3GFP clones and normally growing, non-vector-expressing 3T3TIG B4 and 3T3TIG H7 had minimal shortening. Those 3T3TIG clones with slowed proliferation followed by widespread cell death demonstrated significantly increased telomere shortening at a rate expected for telomerase-inhibited cells. It is noteworthy that 3T3TIG clones with intermediate proliferative rates also had intermediate telomere shortening rates. These intermediate clones were part of the group of clones that did not progress to widespread cell death during the experimental time period. However, we hypothesize that given their slower rate of telomere shortening, they would have eventually progressed to widespread cell death if the experiment had been carried on for a long enough period.

To corroborate the slot blot data and evaluate telomere shortening on individual chromosomal ends, metaphase spreads of several 3T3TIG clones, whose telomeres were measured by slot blot as discussed above, were also evaluated by FISH for telomere signal. As illustrated in Fig. 5, B and C, FISH revealed bright telomere signals of visually equal intensity on all chromosomal ends of the 3T3 Mock (day 195) and 3T3GFP B5 (day 226) control metaphases. Normally growing, non-vector-expressing 3T3TIG B4 (day 225) also showed a widespread bright signal, similar in intensity to the controls (data not shown). In contrast, the other five 3T3TIG clones evaluated, which were characterized by slowing growth followed by cell death, dem-
Clones. As proliferation decreased in clones expressing TIG at high levels, the number of aberrations/cell increased and the mitotic index decreased over time on serial analysis. The intermediate time point (data not shown) had intermediate results between the first and last time points. In all, p-arm fusions were the most common, followed by the presence of both p- and q-arm fusions or q-arm fusions only. The greater number of p-arm fusions is consistent with telomeres being shorter in the p-arm than the q-arm in mouse chromosomes (30, 50). Over time, most clones showed karyotypic instability, reflected by the presence or increase in triradials, tetradials, chromosomal breaks and fragmentation, rings, and unusual chromatid interactions. Examples of observed aberrations are shown (Fig. 7). These results illustrate the evolution of cytogenetic instability at a time of slowed proliferation, critical telomere shortening, and eventual cell death and are consistent with the cytogenetic abnormalities observed in cells cultured from late-generation mTR knockout mice (50–52).

**Morphological Characteristics of hTERT-transduced NIH 3T3 Clones.** As proliferation decreased in clones expressing TIG at high levels, the cells underwent morphological changes characterized by very large size, formation of cytoplasmic extensions, flattening, and multinucleation (Fig. 6). In contrast, 3T3GFP clones and 3T3 Mock cells maintained normal appearance.

**Cytogenetic Analysis of hTERT-transduced NIH 3T3 Clones.** To ascertain the relationship between telomere shortening in our telomerase-inhibited cells and telomere dysfunction leading to the cytogenetic instability characterizing cells in crisis, ten 3T3TIG clones showing characteristics of crisis were evaluated along with the normal-growing 3T3TIG B4, 3T3GFP B5, and 3T3 Mock control populations. Metaphase spreads were scored for ploidy and for individual chromosomal aberrations [chromosomal fusions (p-arm, q-arm, and mixed p- and q-arm), ring chromosomes, triradials, tetradradials, and chromosomal fragmentation and breaks] at three time points, separated by 14-day intervals. The results of the first and last time points are summarized in Table 2.

Both the 3T3 Mock and 3T3GFP B5 cells were near triploid, with an occasional cell showing chromosomal fusion. 3T3TIG B4 was primarily near triploid, and the mean number of aberrations/cell decreased from 0.85 (day 197) to 0.05 (day 225). In all other 3T3TIG clones, the ploidy and mean number of aberrations/cell increased and the mitotic index decreased over time on serial analysis. The intermediate time point (data not shown) had intermediate results between the first and last time points. In all, p-arm fusions were the most common, followed by the presence of both p- and q-arm fusions or q-arm fusions only. The greater number of p-arm fusions is consistent with telomeres being shorter in the p-arm than the q-arm in mouse chromosomes (30, 50). Over time, most clones showed karyotypic instability, reflected by the presence or increase in triradials, tetradradials, chromosomal breaks and fragmentation, rings, and unusual chromatid interactions. Examples of observed aberrations are shown (Fig. 7). These results illustrate the evolution of cytogenetic instability at a time of slowed proliferation, critical telomere shortening, and eventual cell death and are consistent with the cytogenetic abnormalities observed in cells cultured from late-generation mTR knockout mice (50–52).

**Cell Cycle Analysis and Apoptosis of hTERT-transduced NIH 3T3 Clones.** Cell cycle analysis was performed by EtBr staining of extracted nuclei for DNA content, thereby controlling for cell multinucleation. Cultures of 3T3 Mock, 4 3T3GFP clones, and 18 3T3TIG clones (with a range of growth characteristics) were evaluated at one or more of three time points (days 148, 204, and 224; data not shown). The 3T3 Mock and 3T3GFP clones remained at their baseline near triploid, with normal cell cycles at all time points. In contrast, 15 of the 18 (83%) 3T3TIG clones evaluated showed the development of hypertriploidy, which increased over the crisis time points.
mirror those targeting the hTERT for inhibition in human cancer cells (26, 27).

Proliferative Recovery after Crisis in Two hTERT-transduced NIH 3T3 Clones. Two 3T3TIG clones, 3T3TIG G3 and 3T3TIG G5, having undergone crisis including widespread apoptotic death, subsequently exhibited recovery in culture. As shown in Fig. 10A, after reinitiating proliferation, the clones continued to proliferate at a linear, albeit slower, overall rate. PCR for both GFP and hTERT confirmed that both genes of the bicistronic TIG vector were still present on day 407 (Fig. 10B). Interestingly, the PCR demonstrated loss of the vector in 3T3TIG B4, a result consistent with the clone’s normal phenotype. FACS analysis for GFP on day 448 showed that the vector was not only present in 3T3TIG G3 and 3T3TIG G5 but was being expressed (Fig. 10C). Again, the lack of expression in 3T3TIG B4 was consistent with vector loss. Positive selection after the TIG vector loss can also explain the loss of chromosomal aberration of 3T3TIG B4 over time.

Telomerase activity was measured at three time points: (a) at first confluence after single-cell cloning (days 20–29); (b) shortly after the time of crisis escape (days 293–301); and (c) during later stable proliferation (day 490; Fig. 10D). As expected, the initial time point demonstrated significant telomerase inhibition in 3T3TIG B4, 3T3TIG G3, and 3T3TIG G5 in comparison with 3T3 Mock and 3T3 GFP B5. By the intermediate time point, 3T3TIG B4, which was not expressing the TIG vector by that point (Fig. 4B) and was subsequently found to have lost the TIG vector, recovered telomerase activity to a level comparable with that of the 3T3 Mock and 3T3GFP B5 controls. This restoration of telomerase activity with vector loss further supported a direct, reversible role for hTERT in telomerase activity inhibition. Interestingly, a drop in telomerase activity was seen for 3T3 Mock and 3T3GFP B5 control cells between the first and second time points, although levels remained stable by the third point. This may explain the slow reduction in telomere length seen over time in the controls (Fig. 10E), perhaps because telomerase levels were no longer high enough to fully maintain telomere integrity. Both 3T3TIG G3 and 3T3TIG G5, having resumed linear rates of proliferation by the second time point, maintained markedly inhibited telomerase activity levels as compared with 3T3 Mock, 3T3GFP B5, and 3T3TIG B4. The levels of telomerase activity at the second time point were even lower in the escaping clones than the initial inhibited levels. Thus, the mechanism of proliferative escape for these two clones was not related to directly overcoming the telomerase inhibition.

By day 490, the 3T3 Mock, 3T3GFP B5, and 3T3TIG B4 continued to maintain similar, stable telomerase activity. As expected, the telomerase activity level in the 3T3TIG G3 clone was extremely low. Surprisingly, there was a rise in telomerase activity seen in the 3T3TIG G5 clone at this late point, although levels were still lower than in the control cells and within the SE of the initial inhibited level. No phenotypic changes were seen as a result of this rise, and telomeres continued to shorten steadily (see below).

Consistent with persistent telomerase activity inhibition in 3T3TIG G3 and 3T3TIG G5, telomeres continued to progressively shorten more rapidly in the 3T3TIG G3 and 3T3TIG G5 clones than in the controls (Fig. 10E). This continued telomere shortening was reflected in the increasing development of chromosomal aberrations. From the time of early crisis (Table 2), 3T3TIG G5 developed an increase in chromosome number and aberrations. In contrast, 3T3TIG G3 eliminated its aberrations over the 4-week early crisis time period, indicating a selection occurring for cytogenetically normal cells. However, cytogenetic analysis of both 3T3TIG G3 and 3T3TIG G5 over 200 days later, after crisis escape and resumption of linear proliferation, revealed the development of abnormal ploidy in almost all cells as well as a significant increase in the number of total aberrations/cell

![Diagram](https://example.com/diagram.png)

Fig. 9. Apoptosis of hTERT-expressing NIH 3T3 clones. A. sub-G1 percentage was determined by cell cycle analysis, performing FACS for DNA content on EtBr extracted nuclei. An example is shown, illustrating the development of sub-G1 during crisis of 3T3TIG G9 as compared with 3T3 Mock and 3T3GFP B5 controls. B, annexin V-PE binding to the same 3T3TIG G9 clone in crisis is shown in comparison with 3T3 Mock and 3T3GFP B5 controls. Necrotic cells were gated out using PI counterstaining. A quantitative analysis is presented in Table 3.

interval. All three 3T3TIG clones that remained near triploid, including 3T3TIG B4, maintained normal proliferative and morphological characteristics. Those clones developing hypertriploidy also showed a decrease in S phase and an increase in G1 phase over time, particularly within the hypertriploid subpopulation. An example of 3T3TIG G1 at day 224 is shown (Fig. 8).

To evaluate whether cell death was secondary to apoptosis, appearance of a sub-G1 peak corresponding to the time of crisis was examined with each cell cycle analysis (Table 3; Fig. 9A). In contrast to controls, those 3T3TIG clonal populations in crisis developed an increasing sub-G1 peak over time, a characteristic of apoptosis. Similar results were obtained by evaluating selected clones undergoing widespread cell death for binding of PE-labeled annexin V, another marker of apoptosis (Ref. 46; Table 3; Fig. 9B).

The development of slowed proliferation, morphological changes, and cytogenetic instability followed by apoptotic cell death is consistent with a crisis associated with critically shortened telomeres in telomerase-inhibited, previously immortal murine cells. These results, using hTERT as a dominant negative type inhibitor in murine cells,
Thus, the telomeric dysfunction associated with telomerase inhibition was still present in these escape clones, suggesting that the mechanism of escape may be related to loss of some part of the DNA damage signal pathway responsible for stimulating apoptosis.

**DISCUSSION**

We have used a novel cross-species approach to telomerase inhibition, demonstrating that expression of hTERT in murine cells resulted in a dominant negative type inhibition of endogenous telomerase activity. We speculate that this represents a form of competitive inhibition, with the hTERT having enough homology to combine with other components of the murine telomerase complex but being unable to generate functional telomerase activity. Alternatively, the hTERT may bind the telomeres themselves, thereby directly preventing mTERT from binding.

Applying this strategy, we found that telomerase inhibition in immortal murine fibroblasts led to a crisis associated with critically shortened telomeres, manifested by slowing and subsequent cessation of proliferation, abnormal morphology, altered cell cycle, telomere dysfunction with cytogenetic instability, and eventual apoptotic cell death. These results provide further evidence of the importance of telomerase in cell immortalization and telomere maintenance and are similar to results obtained in human cancer cells after gene transfer of a dominant negative mutant of hTERT (26, 27). Thus, although telomeres of most mouse strains are longer than those in humans and telomerase is more ubiquitously expressed at low levels (13, 14, 32, Table 4). Thus, the telomeric dysfunction associated with telomerase inhibition was still present in these escape clones, suggesting that the mechanism of escape may be related to loss of some part of the DNA damage signal pathway responsible for stimulating apoptosis.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Day</th>
<th>PD</th>
<th>Ploidy (%)</th>
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<tr>
<td>Mock</td>
<td>441</td>
<td>613</td>
<td>54-64</td>
<td>0.30 (0.47)</td>
</tr>
<tr>
<td>GFP B5</td>
<td>441</td>
<td>588</td>
<td>95</td>
<td>4.95 (0.60)</td>
</tr>
<tr>
<td>TIG B4</td>
<td>441</td>
<td>580</td>
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<td>9.60 (2.39)</td>
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<td>TIG G3</td>
<td>441</td>
<td>433</td>
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<td>95</td>
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<td>441</td>
<td>322</td>
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<td>3T3 Mock</td>
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</table>

* A total of 20 metaphases were analyzed for each clone.
* Mean (standard deviation).
* Includes p-arm fusions, q-arm fusions, p- and q-arm fusions, rings, acentric fragments, triradials, tetraradials, and chromosomal breaks.
* Clone status after vector loss.
* Clones status after crisis escape.
33), telomeres do play an important role in murine cells, and telomerase appears crucial for telomere maintenance. Because most murine tumors have high telomerase levels (33, 34), the approach for directed telomerase inhibition described here can be used to target specific cell types, allowing study of telomerase inhibition as an anticancer treatment in the murine model both in vitro and in vivo.

We have already begun using this dominant negative strategy to target murine leukemias for telomerase inhibition. MOPC 315, a BALB/c-derived IgA-secreting myeloma, has shown marked telomerase inhibition after transduction with hTERT, demonstrating that the dominant negative inhibitory effect of hTERT in murine cells is not unique to immortal fibroblasts and can be applied to other cells, including malignant ones.

The telomere dysfunction associated with cytogenetic instability seen in our study parallels that seen in cells derived from mTR knockout and combined mTR and p53 knockout mice. In addition, telomere dysfunction in those mice is associated with increased tumor formation despite telomerase deficiency (51, 52). This potential cancer predisposition secondary to telomere dysfunction has important implications, including implications for the use of telomerase inhibition as an anticancer therapy. Long-term side effects, particularly in those organ systems dependent on telomerase for self-renewal, such as the hematopoietic system, may include both decreased proliferative capacity, as reported previously in mTR knockout mice (35), and an increased potential for de novo malignant transformation. This needs to be investigated further in an in vivo model of telomerase inhibition.

One limitation of the mouse model is the long telomere length found in most strains, increasing the time necessary for telomeres in telomerase-inhibited cells to shorten critically enough to induce apoptosis. Thus, the time lag until therapeutic effect that is a concern in humans is even more pronounced in mice. Possible approaches to this problem in the mouse model include injecting late-passage telomerase-inhibited tumor cells with already shortened telomeres, proportional tumor debulking to allow time for therapeutic effect, serial passage of tumor cells between mice, or the use of mouse strains, such as mus spretus, with telomere lengths comparable with those in humans (32, 53).

Another crucial aspect of the time lag required for telomerase inhibition to be therapeutic is the possibility for escape. Unlike most chemotherapies, where the effect is immediate, the mechanism of telomerase inhibition requires the inhibitor to remain active for a prolonged period. This gives each cell time to overcome that inhibition. One study in telomerase-inhibited human cancer cells shows escape by reactivation of telomerase activity, although the mechanism remains unknown (27). We described two clones that progressed to crisis, including widespread apoptosis, until a subpopulation of each culture showed proliferative recovery. Telomerase still remained inhibited in these escape clones, and their telomeres continued to shorten at the same rate, leading to telomere dysfunction characterized by increasing cytogenetic instability. This suggests that the cells lost part of the DNA damage signal pathway that stimulates apoptosis. As our knowledge of the telomeric complex increases, other mechanisms of escape will undoubtedly become apparent. Telomerase-independent mechanisms of telomere maintenance in both human and murine cells, evidenced by the existence of telomerase-negative cancers (28) and the transformation of fibroblasts derived from mTR knockout mice (54), present another route for bypassing telomerase inhibition.

Whether these escape mechanisms will be clinically relevant remains to be seen. The study described here shows that telomerase inhibition in immortal murine cells leads to apoptotic death, echoing results reported in human cancer cells (25–27). The cross-species inhibitory approach applied here can be used for future investigations into telomerase inhibition in murine cancers, including evaluation of escape mechanisms. In addition, this study shows the mouse to be an appropriate model for telomerase inhibition in humans, providing an in vivo standard to evaluate telomerase inhibition as a clinical anticancer treatment.

ACKNOWLEDGMENTS

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