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Rapid Inhibition of Cancer Cell Growth Induced by Lentiviral Delivery and Expression of Mutant-Template Telomerase RNA and Anti-telomerase Short-Interfering RNA

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ABSTRACT

In human cancers, telomeres are commonly maintained by elevated levels of the ribonucleoprotein enzyme telomerase, which contains an intrinsic templating RNA moiety (human telomerase RNA; hTER) and the core protein (human telomerase reverse transcriptase). We developed a lentiviral system for efficient overexpression of mutant-template human telomerase RNA (MT-hTer) to add mutant DNA to telomeres in cancer cells. We show that such MT-hTer overexpression rapidly inhibits cell growth and induces apoptosis in telomerase-positive precancerous or cancer cells but not in telomerase-negative cells. These rapid effects occurred independent of wild-type p53 and telomere length. Tumor growth and progression were significantly decreased in xenografts of human tumor cells overexpressing MT-hTers. Expression of a hairpin short-interfering RNA that specifically targeted the endogenous wild-type hTER template region, but spared the MT-hTers, also caused p53-independent cell growth inhibition and apoptosis, and when coexpressed with MT-hTer, synergistically killed cancer cells. Hence, anti-wild-type-hTER short-interfering RNA and MT-hTers may act through distinct pathways and, particularly in combination, represent a promising approach to anticancer therapies.

INTRODUCTION

Telomeres consist of terminal DNA repeats bound by telomere-specific DNA binding proteins. These, together with associated factors, preserve the integrity of the chromosome tip and the stability of the genome (1). In most eukaryotes, the telomeric repeat tracts are maintained by telomerase, a specialized ribonucleoprotein complex. The integral RNA component of telomerase, called human telomerase RNA (hTER) or hTR in humans, contains a short templating domain sequence that directs the synthesis of telomeric DNA repeats at chromosome tips (2, 3). In yeast, lack of telomerase eventually results in shortening of the bulk of the telomeres with triggering of cellular responses, including DNA damage responses (4–6). In mammals, genetic inactivation or lowering of telomerase disrupts telomere maintenance and causes age- and generation-dependent telomere shortening (7–11). The core essential components necessary for the reconstitution of human telomerase activity *in vitro* are the catalytic protein subunit, human telomerase reverse transcriptase (hTert), and the RNA subunit, hTER (12, 13). Although hTER is widely expressed, hTert and, consequently, telomerase activity are diminished in many adult somatic cells, although it is detectable in certain proliferating cells, stem cells, and germ cells (12–16). In contrast, telomerase activity is highly elevated in 85–90% of human cancers and >70% of immortalized human cell lines (14, 17). This is consistent with up-regulated telomerase conferring a strong selective advantage for continued growth of malignant cells (18). Although cells without telomerase can be tumorigenic in mouse xenografts, they cannot be immortalized (19).

A strategy to rapidly and specifically disrupt telomere maintenance in cancer cells has been explored previously; expressing hTER with mutations in the template region (mutant-template human telomerase RNA; MT-hTer) predicted to direct synthesis of mutated DNA that disrupts the binding of telomeric proteins (20–22). We reasoned that such MT-hTer expression would compromise the integrity of telomere structures in telomerase-positive tumor cells and, thereby, inhibit cancer cell growth, independent of telomere length. This strategy was based on the previous demonstration that, in *Tetrahymena* and yeasts, incorporation of even a few terminal mutant telomere repeats via expression of mutant-template telomerase RNAs can be rapidly deleterious, causing loss of cell viability without the necessity for prior telomere shortening (3, 23–26). Wild-type telomere repeats contain critical DNA binding sites for telomeric DNA sequence-specific binding proteins such as Cdc13p and Rap1p in *Saccharomyces cerevisiae* (27–30) and POT1, TRF1, and TRF2 in humans (31, 32). In addition, the uncapping of human telomeres by mutating the telomeric protein TRF2 can induce a p53-dependent growth arrest and cellular apoptosis in mammalian cells (33, 34).

As reported previously, introduction of MT-hTers into telomerase-positive human cancer cells produced observable cellular phenotypes and loss of tumor growth in xenografts (21, 22). However, in these previous attempts, the MT-hTers could only be stably expressed at low levels, and cell killing in the stably transfected clonal cell populations examined was not efficient. The relatively modest cellular phenotypes reported in those previous experiments, using such stable long-term expression of MT-hTer, could have been attributable to selection against any cells that had high MT-hTer expression. Furthermore, the abundance and stability of the wild-type endogenous telomerase RNA in most cancer cells (half-life of 4.4–32 days; Ref. 35) may prevent the rapid incorporation of ectopically expressed MT-hTer into a functional telomerase ribonucleoprotein complex. Therefore, to analyze short-term responses of cancer cells, improved delivery systems and strategies were necessary for the introduction and efficient overexpression of MT-hTer.

Here, we show that a lentiviral system can be used for an efficient delivery and overexpression of MT-hTer in human cancer cell lines. Overexpression of MT-hTer, achieved using an expression cassette from the IU1 small nuclear RNA gene, caused rapid cell growth inhibition and apoptosis both *in vitro* and in a mouse xenograft tumor model system. We report also that a non-mRNA, the wild-type human telomerase RNA (WT-hTER), can be efficiently and specifically targeted by a hairpin short-interfering RNA (siRNA; Refs. 36, 37) expressed from a lentivector to cause rapid cell growth inhibition and apoptosis. Coexpression of this siRNA with MT-hTer additionally

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increased the ratio of MT-hTer to WT-hTER and synergistically augmented cancer cell death. These effects were independent of wild-type p53 and telomere length and occurred without bulk telomere shortening. Thus, MT-hTer and anti-WT-hTER-siRNA expression represent a promising novel cancer therapeutic strategy.

MATERIALS AND METHODS

Plasmid Construction. The three-plasmid-based lentivector system was generously provided by Dr. Didier Trono (University of Geneva, Geneva, Switzerland) (38). The pIU1-T7 and pTZ-U6 plasmids were gifts from Drs. Edouard Bertrand (Université Montpellier, Montpellier, France) and John J. Rossi (Beckman Research Institute of the City of Hope, Duarte, CA). Wild-type hTER was PCR cloned from human genomic DNA and subcloned into *Bgl*II/*Sal*I site in pIU1-T7 vector (39). MT-hTer constructs were generated by site-directed mutagenesis. The PCR fragments containing both IU1 promoter and WT-hTER or MT-hTer were subcloned into pHRCMVGFPWSin18 vector (38) to generate pHRIU1hTer-CMVGFPWSin18. The siRNA expression vectors were engineered by PCR U6 promoter using the following primers: 5′-AAAACTGCAGAAAAATTGTCTAACCCTAACTGAGAATCTCTTGAATTCTCAGTTAGGGTTAGACGGTGTTTCGTCCTTTCCACAAG-3′; and 5′-AAAAACTAGTAAGGTCGGGCAGGAAGAGGGC-3′. The PCR product was digested with *Spe*I and *Pst*I and subcloned into pHRCMVGFPWSin18 or pHRIU1hTer-CMVGFPWSin18 vectors. The hTert expression vector pBabe-puro-hTert was engineered as described previously (21).

Virus Production and Cell Culture. Lentivirus was generated as described previously (38). Briefly, 5 μg of pMD.G plasmid, 10 μg of pCMVDR8.91, and 15 μg of lentivector were cotransfected into 293T cells using the calcium phosphate coprecipitation method. Conditioned medium was harvested at 48 h and 72 h after transfection and filtered through 0.45-μm filters. Virus titers were calculated at 72 h after virus infection in all of the cell lines by counting the number of green fluorescent proteins expressing foci divided by the dilution factor. For virus infection, culture cells were incubated with culture medium-diluted virus supernatant supplemented with polybrene (8 μg/ml) for 8 h. To achieve >95% infection efficiency, virus titers of 20–40 transduction unit/cells were used. For cell growth measurement, 2×10^4 cells were reseeded in six-well plates at 48 h after virus infection, and the cell numbers were counted every day or every other day. Establishment of VA13(hTert) cells were described previously (21).

Telomerase Assays, Telomere Analysis, and RNA Analysis. Telomerase activity from cell extracts was analyzed using a PCR-based telomeric repeat amplification protocol assay. Telomere length was measured by hybridization of a ^{32}P-labeled $(CCCTAA)_4$ probe to genomic DNA digested with *Hin*fI and *Rsa*I. RNA was extracted using TRIzol reagent (Invitrogen) and separated on 1.5% agarose gel. RNA blots were hybridized with ^{32}P-labeled cDNA probes prepared by random primer labeling (Amersham Biosciences). For detection of endogenous WT-hTER and MT-hTer expression, RNase protection assays were performed with ^{32}P-labeled hTER and glyceraldehyde-3-phosphate dehydrogenase riboprobe as described previously (Ambion Inc., Austin, TX).

Cell Cycle and Apoptosis Analysis. Both floating and attached cells were collected from each sample. DNA content was analyzed by flow cytometry with propidium iodide staining, and the fraction of cells containing sub-G_1 DNA content was quantified.

Tumor Xenografts. UM-UC-3 cells were infected with control, WT, 47A, or AU5 virus, and viable cells were counted using trypan blue exclusion and a hemacytometer. Cell grafts were made by pelleting 1×10^6 UM-UC-3 cells and resuspending them in 50 μl of neutralized rat tail collagen prepared as described previously (40). The grafts were placed in the incubator at 37°C for 15 min to set. Ten grafts were made for each treatment group. The cells were grafted beneath the kidney capsule of adult male athymic "nude" mouse host (41). After 2 weeks, the hosts were euthanized, and the grafts were photographed, dissected from the kidney, and weighed. The tissue was fixed in 10% paraformaldehyde embedded in paraffin; 6-μm sections were cut and stained with H&E. Statistical comparisons among groups were made using an ANOVA followed by Fisher's project least significant difference test (Statview; Abacus Concepts, Cary, NC). Values were expressed as the mean ± 95% confidence interval.

RESULTS

Growth Inhibition by Lentiviral-Expressed MT-hTer Depends on hTert. For efficient delivery of ectopically expressed telomerase RNA into cultured human cells, we used a safety-engineered, HIV-1-based lentiviral system (38). To achieve high expression of the MT-hTers, we optimized the expression cassette. The IU1 small nuclear RNA promoter produced the best expression of functional telomerase RNA in this system (Supplementary Data, Fig. S1). Two mutants of the 11-nucleotide templating domain sequence of telomerase RNA, MT-hTer-AU5 and MT-hTer-47A, were constructed and analyzed (Fig. 1*A*). Telomerase enzymatic activity (telomeric repeat amplification protocol) is detectable when either of these MT-hTers is synthesized *in vitro* and telomerase activity reconstituted with hTert translated in rabbit reticulocyte lysates.[3] Hence, both mutant-template RNAs are capable of forming an enzymatically functional telomerase ribonucleoprotein complex and synthesizing mutant telomeric repeat DNA *in vitro*.

We tested the short-term response of telomerase-positive cells to the expression of MT-hTer. First, we tested whether MT-hTer effects require active telomerase. The recombinant lentiviruses with the expression cassette carrying either the MT-hTer or the wild-type control hTER were used to infect VA13 cells. This telomerase-negative cell line maintains its telomeres by a telomerase-independent mechanism, alternate lengthening of telomeres (ALT), and expresses neither hTert nor hTER (42). We infected either these hTert-negative VA13 parental cells or VA13 cells previously stably transfected with a hTERT gene expression vector, referred to here as VA13(hTert) cells. Expression of WT-hTER and MT-hTers was efficient in both VA13 and VA13(hTert) cell lines (Fig. 1*B*). The expression and stability of both MT-hTers was comparable with that of WT-hTER. Coexpression of hTert in the VA13(hTert) cells did not affect the level of the ectopically expressed telomerase RNAs.

Next, the cellular effects of MT-hTer overexpression in both VA13 and VA13(hTert) cells were evaluated. Because of the high infection efficiency of the lentivirus (>95%), we could evaluate the cellular effects of expression of the anti-hTER siRNA in the pooled cell population without any selection for a drug resistance gene, eliminating potential variation generated by analysis of clonal cell cultures. These data also showed that the cellular toxicity induced by lentivirus infection, *per se*, is negligible. Overexpression of WT-hTER and MT-hTers did not affect the growth of the hTert-negative VA13 cells during the 16 days after lentivirus infection (Fig. 1*C*, *left*). In contrast, MT-hTers expression in VA13(hTert) cells caused rapid growth inhibition (Fig. 1*C*, *right*) and cell death (data not shown). Similar but quantitatively much weaker results for the 47A mutant expressed from a plasmid were reported previously for VA13 cells (20). The lack of any cellular effect in hTert-negative VA13 cells *versus* the rapid cellular response in VA13(hTert) cells indicates that the effect of MT-hTer is dependent on hTert, which is up-regulated in the majority of human cancers.

Interestingly, coexpressing the WT-hTER in VA13(hTert) caused a slight but consistent increase in cell growth rate. Hence, reconstitution of telomerase function might promote cell growth compared with the VA13 cells that use only the ALT mechanism for telomere maintenance. Promotion of cell growth by excess telomerase has also been observed in K5- mTert mice, which overexpress mTert under the K5 promoter (43).

To test cancer cell responses to MT-hTer expression, several telomerase-positive cancer cell lines were infected with recombinant

[3] M. A. Rivera and E. H. Blackburn. Processive utilization of the human telomerase template: lack of a requirement for template switching, submitted for publication.

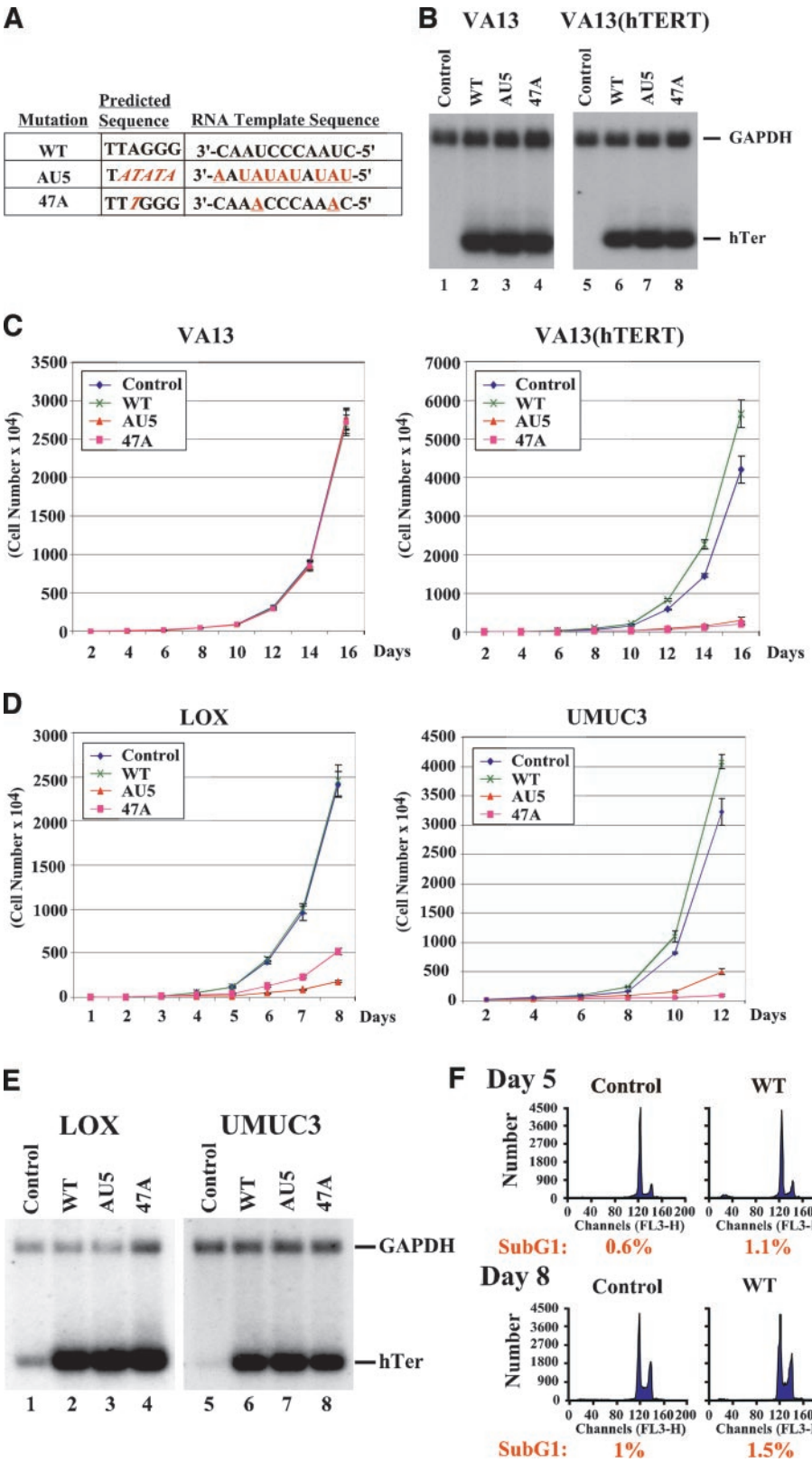

Fig. 1. Cell growth inhibition induced by lentiviral-expressed mutant-template human telomerase RNA (MT-hTer). *A*, the 11-nucleotide WT, AU5, and 47A telomerase RNAs template sequences (*right*) and their predicted copied DNA sequences (*left*). *B*, Northern blotting analysis of wild-type human telomerase RNA (WT-hTER) and MT-hTer expression in VA13 or VA13(hTert) cells infected with lentivirus expressing WT, AU5, or 47A telomerase RNA; total RNA was isolated 4 days after viral infection. The expression level of glyceraldehyde-3-phosphate dehydrogenase is used as the RNA loading control. *C*, cell growth inhibition is induced by MT-hTer expression in VA13(hTert) but not in VA13 cells. Lentivirus infection was >95% efficient as indicated by green fluorescent protein positivity. Thus, no drug marker selection step was used, and cell numbers were counted for the entire unselected cell population every 2 days after infection. *D*, cell growth effects induced in LOX and UM-UC-3 cancer cells mock-infected or infected with lentivirus expressing WT-hTER or MT-hTer at efficiencies close to 100%. Total unselected cell population numbers were counted every day (*LOX*) or every 2 days (*UM-UC-3*). *E*, Northern blotting analysis of endogenous (*Lanes 1* and *5*) and ectopically expressed telomerase RNA in LOX and UM-UC-3 cells. Ectopically expressed telomerase RNA comigrates with endogenous telomerase RNA. *F*, DNA content of LOX cells mock-infected or infected with WT-hTER or MT-hTer lentivirus as analyzed by fluorescence-activated cell sorter at day 5 or day 8 after virus infection. Percentages of cells with sub-G_1 DNA content are indicated in *red*. *Bars*, ±SD.

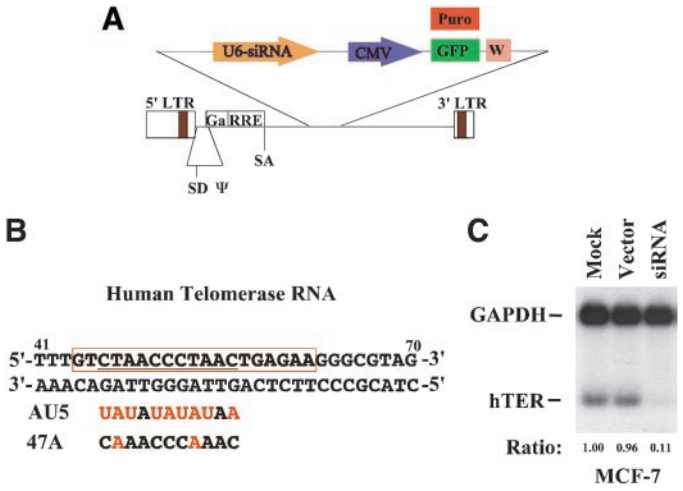

Fig. 2. Hairpin short-interfering RNA (siRNA) construct targeting endogenous wild-type telomerase RNA. *A*, schematic of lentivector expressing siRNA from the U6 promoter. Two alternative markers, green fluorescent protein or puromycin resistance marker cassette, were tested (see "Materials and Methods"). *B*, the siRNA sequence showing the targeted wild-type hTER sequence (*red rectangle*) containing the 11-nucleotide templating domain (*underlined*): *bottom*, AU5 and 47A MT-hTers template sequences; and *red*, mutated bases. *C*, efficient reduction of wild-type hTER, as shown by Northern hybridization, by siRNA using the green fluorescent protein containing lentiviral construct. MCF-7 cells were mock-infected or infected with lentivirus expressing siRNA or control lentivirus, and RNA was prepared and analyzed at day 4 after virus infection.

lentiviruses expressing MT-hTers or the control WT-hTER. Again, the high-infection efficiency of the lentivirus allowed measurement of the cellular effects in the pooled population without the necessity for prior selection of drug-resistant cells (Fig. 1*D*). Northern blotting showed that overexpression of ectopically expressed WT-hTER or MT-hTer was achieved in the LOX (human melanoma) and UM-UC-3 (human transitional epithelial carcinoma bladder) cells compared with the endogenous WT-hTER levels (Fig. 1*E*). Hence, expression of telomerase RNA from the IU1 promoter may bypass the regulation of total hTER accumulation that was inferred in previous studies in other cancer cell lines (21, 22).

LOX and UM-UC-3 cells were highly sensitive to the lentiviral-expressed AU5 or 47A MT-hTers; rapid cell growth inhibition was evident by day 5 after lentivirus infection in LOX cells and by day 8 in UM-UC-3 cells (Fig. 1*D*). Later, day 8 for LOX cells and day 12 for UMUC-3 cells, small populations of cells were recovered (Fig. 1*D*). Fluorescence microscopy showed that they exhibited either no or low green fluorescent protein expression, indicating that only those cells that did not receive the lentivirus or had low or no expression of MT-hTers were able to grow out (data not shown). The failure of these cells to express the MT-hTer may be due either to the inability of the lentivirus to infect every cancer cell with the virus titers used here or to silencing or loss of provirus during cancer cell growth. When these surviving cells were reinfected with lentivirus-expressing MT-hTers, the kinetics and extent of the initial rapid growth inhibition and cellular apoptosis were recapitulated, and the same was seen after a third cycle of infection (data not shown). Hence, as was also reported previously with human breast and prostate cancer cells expressing very low levels of MT-hTers over several months (21), no cell subpopulation resistant to, or adapted to, MT-hTer has been detected. Fluorescence-activated cell sorter analysis of cellular DNA contents of LOX cells mock-infected or infected with recombinant lentiviruses showed an increased sub-G_1 population, indicative of apoptosis, in cells overexpressing AU5 or 47A MT-hTer, especially the AU5 mutant (Fig. 1*F*). Apoptosis was also confirmed by Annexin V and terminal deoxynucleotidyl transferase-mediated nick end labeling staining (data not shown). The onset of apoptosis was rapid, being evident in LOX cells by day 5 after infection, reaching a plateau at day 8 (Fig. 1*F*) and at day 9 in UM-UC-3 cells (Supplementary Data, Fig. S2). Fluorescence-activated cell sorter analysis of both LOX and UM-UC-3 cells also showed a G_2-M arrest and an increased fraction of polyploid nuclei in the cells expressing 47A or AU5 MT-hTers (Fig. 1*F*; data not shown).

Knock-Down of WT-hTER by siRNA. Among the cancer cell lines we analyzed, LOX and UM-UC-3 responded rapidly to MT-hTer overexpression. However, responses of other cell lines, such as the colon cancer line HCT116, the breast cancer line MCF-7, and the prostate cancer cell line LNCaP, were slower (Fig. 3*E*; data not shown). The relative abundance of endogenous WT-hTER is higher in these cancer cells than in UM-UC-3 or LOX cells (data not shown). Although these cell lines also differ in other respects, we reasoned that by selectively reducing the amount of endogenous WT-hTER by RNA interference and, hence, increasing the ratio of MT-hTer to endogenous WT-hTER, the incorporation of MT-hTer into functional ribonucleoprotein might be augmented relative to the hTER, because the siRNA would reduce the competition of endogenous WT-TER with the MT-hTer for assembly into telomerase.

We designed a hairpin siRNA to target specifically the region encompassing the 11-nucleotide template sequence of the WT-hTER. On testing various siRNA expression cassettes and sequences in the lentiviral system, optimal effects were found with the siRNA shown in Fig. 2, *A* and *B*, placed under the control of the U6 promoter. First, the effectiveness of the siRNA was confirmed by mock infecting or infecting MCF-7 cells with lentivirus expressing siRNA or control lentivirus. Expression of this siRNA efficiently knocked down the level of endogenous WT-hTER (Fig. 2*C*).

Coexpression of siRNA Sensitizes Cancer Cells to MT-hTer. To avoid the need for sequential infections, a single lentivector was engineered to express the siRNA together with MT-hTer or the control WT-hTER. This lentivector was also constructed to carry a green fluorescent protein reporter for monitoring virus infection efficiency or a puromycin resistance gene cassette for cell selection (Fig. 3*A*). Despite the efficient reduction of WT-hTER by the siRNA construct (Fig. 2*C* and Fig. 3*C*), the virus titer for lentivirus coexpressing siRNA and WT-hTER was only ~2-fold lower than lentivirus expressing WT-hTER alone or lentivirus coexpressing siRNA plus the nontargeted MT-hTer (Fig. 3*B*). The siRNA expressed from the same lentivector efficiently reduced both endogenous and ectopically expressed WT-hTER transcripts (Fig. 3*C*, *Lanes 5* and *12*). Hence, we surmise that the rapid assembly of virus RNA transcript into lentiviral particles may shield the access of this siRNA to its target, largely preventing the degradation of virus RNA. Northern blotting analyses showed efficient expression of ectopically expressed MT-hTers from lentiviruses coexpressing siRNA and MT-hTer (Fig. 3*C*). Because the endogenous WT-hTER and ectopically expressed MT-hTers comigrated at the same position, RNase protection analysis was done to distinguish the differential effect of the siRNA on the expression level of WT-hTER *versus* MT-hTers. This analysis showed that coexpression of siRNA in HCT116 cells decreased the total WT-hTER levels (Fig. 3*D*, *Lanes 2* and *6*) without affecting the expression of ectopically expressed MT-hTers (Fig. 3*D*, *Lanes 3*, *4*, *7*, and *8*). Because the coexpression of the siRNA with each MT-hTer specifically depleted the endogenous hTER, it increased the ratio of MT-hTer to WT-hTER (Fig. 3*D*, *Lanes 3*, *4*, *7*, and *8*).

Expression of anti-hTER siRNA alone or MT-hTer alone each inhibited HCT116 cell growth to comparable extents (Fig. 3*E*). However, when the siRNA and MT-hTer were coexpressed, the cellular responses were dramatically sensitized (Fig. 3*E*). Notably, under each condition in these isogenic HCT116 cell lines with or without p53, the effects were similar. Hence, the rapid cell growth inhibition and apoptosis do not require p53 function (Fig. 3, *E* and *F*).

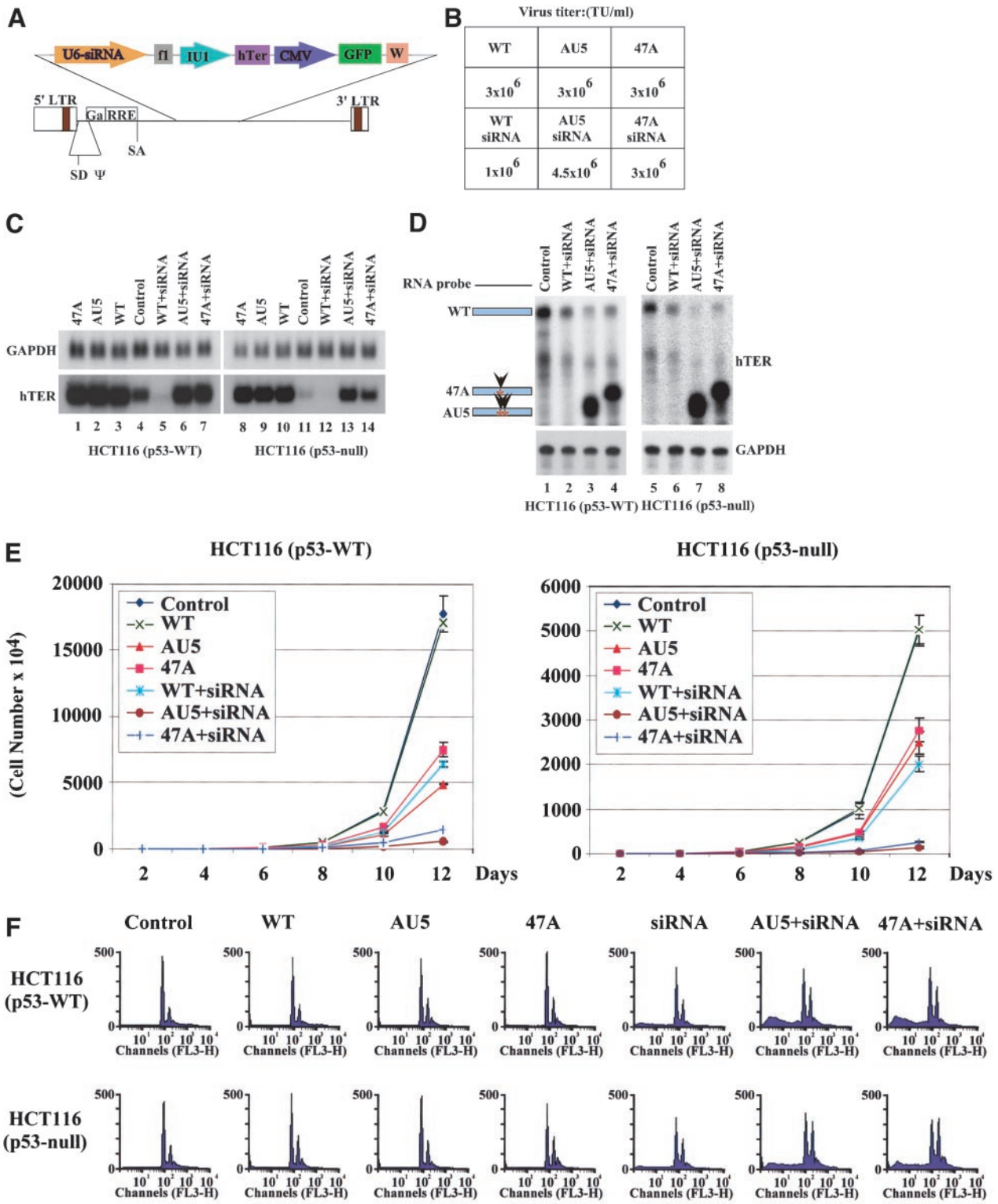

Fig. 3. Coexpression of short-interfering RNA (siRNA) sensitizes cellular responses to mutant-template human telomerase RNA (MT-hTer). *A*, schematic of lentivector coexpressing siRNA, telomerase RNA, and green fluorescent protein reporter gene. *f1*, phage f1 replication origin. *B*, titers of various lentivirus constructs measured directly from 293T packaging cells shown before adjustment to equal titers for infections. *C*, Northern blot analysis of endogenous and ectopically expressed telomerase RNA transcripts in HCT116 cells with or without functional p53. Total RNA was extracted 4 days after infection with various lentiviruses as indicated above the lanes. Ectopically expressed telomerase RNAs comigrate with endogenous telomerase RNA. *D*, RNase protection assays, using a 120-nucleotide RNA probe complementary to the 5′ portion of human telomerase RNA (*hTER*), showing differential effects of the siRNA on levels of wild-type RNAs and MT-hTers. Protected RNA fragments are indicated schematically for WT, AU5, or 47A telomerase RNA; ∗, mutations in MT-hTers; *arrows*, predicted cutting sites. RNA loading controls below are glyceraldehyde-3-phosphate dehydrogenase mRNA. *E*, rapid cell growth inhibition is induced by MT-hTer independent of cellular p53 status. HCT116 cells with (p53-WT) or without (p53-null) functional p53 were mock-infected or infected with lentivirus expressing wild-type human telomerase RNA (WT-hTER) or MT-hTer, with or without coexpression of siRNA as indicated, at efficiencies close to 100%. Total unselected cell numbers were counted every 2 days. *F*, DNA content of HCT116 cells mock-infected or infected with lentivirus expressing WT-hTER or MT-hTer with or without coexpression of siRNA, as indicated, was analyzed by fluorescence-activated cell sorter at day 10 after virus infection. Increased sub-G_1 population was observed in cells expressing anti-hTER siRNA alone or coexpressing MT-hTer and siRNA. *GFP*, green fluorescent protein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *bars*, ±SD.

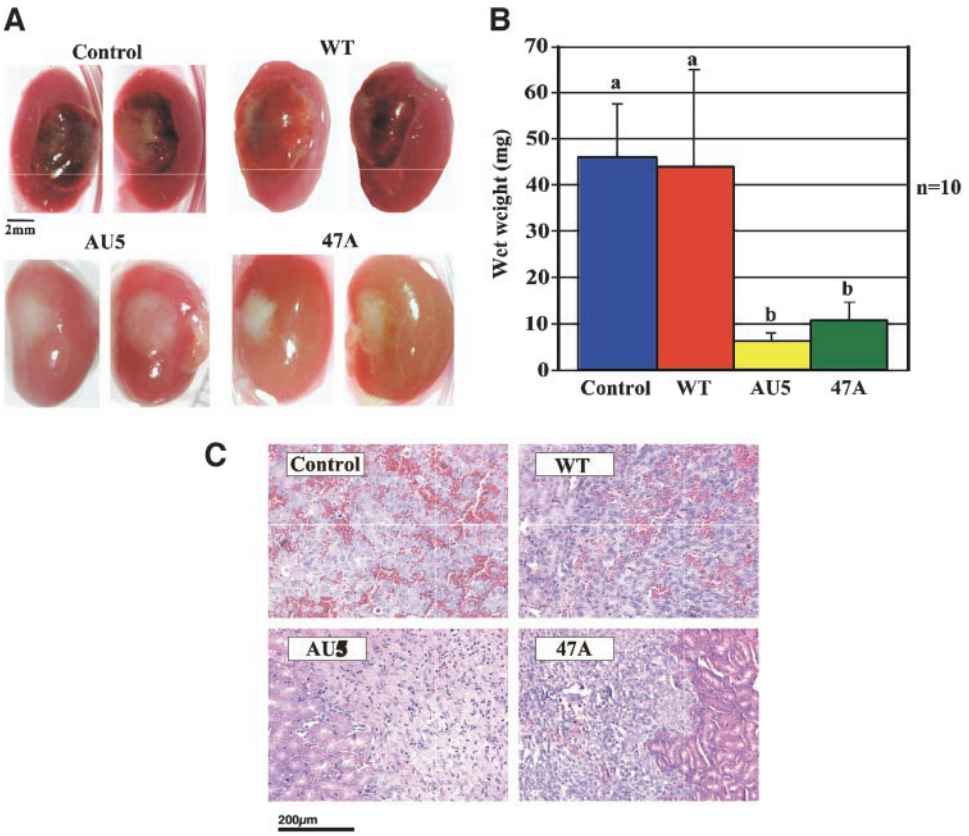

Fig. 4. UM-UC-3 cells expressing mutant-template human telomerase RNA form smaller and less vascularized tumors in mice than wild-type human telomerase RNA controls. *A*, representative tumors formed from UM-UC-3 xenografts in the subrenal capsule of nude mice. UM-UC-3 cells were mock-infected or infected with virus expressing wild-type or mutant telomerase RNA as indicated, then subcultured for 4 days *in vitro*, before being xenografted into the subrenal capsule of nude mice. The xenografted tumors were examined 2 weeks after implantation. *B*, statistical analysis of tumor wet weights. Statistical comparisons among groups were made using an ANOVA followed by a Fisher's project least significant difference test (Statview; Abacus Concepts). Values are expressed as the mean ± 95% confidence interval ($n = 10$/group). *Bars* with the same superscript are not significantly different. *Bars* with different superscripts are statistically different ($P < 0.001$). *C*, histology of xenografted tumors. Tumors formed by xenografts of UM-UC-3 cells mock-infected or infected with lentivirus expressing wild-type human telomerase RNA show rich angiogenesis compared with that of UM-UC-3 cells infected with mutant-template human telomerase RNA. *Bars*, ±SD.

MT-hTer-Expressing Cells Form Smaller, Less Vascular Tumors in Mice. To validate the effect of lentiviral-delivered MT-hTer on human cancer cells in an *in vivo* setting, UM-UC-3 bladder cancer cells were first mock-infected or infected with lentivirus expressing WT-hTER or MT-hTer. Cells were allowed to recover, and the populations expanded *in vitro* for 4 days before being xenografted into the subrenal capsule of nude mice. The rich vascularization of the subrenal capsule provides an ideal environment for tumor xenografts; high survival rates of xenografted tumors can be achieved (40, 41). Two weeks after tumor implantation, and 18 days after virus infection, mice were sacrificed, and the xenografted tumors were examined. As shown in Fig. 4*A*, large tumors were readily observable on the mouse kidneys in xenografts of UM-UC-3 cells that had been either mock-infected previously or infected with WT-hTER lentivirus. These UM-UC-3 xenograft tumors were very rich in blood vessels. In contrast, xenografts of the UM-UC-3 cells infected with the AU5 or 47A MT-hTer lentivirus showed much smaller tumor volumes (Fig. 4, *A* and *B*) and minimal tumor angiogenesis. Tumor histology confirmed the rich angiogenesis of UM-UC-3 xenografts that had been either mock infected or infected with WT-hTER lentivirus compared with UM-UC-3 xenografts infected with MT-hTer lentiviruses (Fig. 4*C*). In summary, lentiviral-expressed MT-hTer dramatically inhibited tumor growth and reduced angiogenesis *in vivo*.

MT-hTers Induce Cell Growth Inhibition Without Changing the Bulk Telomere Length. Although rapid growth inhibition and apoptosis were readily observed in cells overexpressing MT-hTers, no bulk telomere shortening was observed by Southern blotting analysis in the HCT116 cell lines with or without functional p53 (Fig. 5*A*). This lack of a requirement for bulk telomere shortening was supported additionally by the rapid growth inhibition and apoptosis, which occurred in LOX cells overexpressing MT-hTer, despite the lack of shortening of their very long telomeres, which are >40 kb in length (data not shown).

A prediction of the incorporation of mutant telomere repeats caused by MT-hTer telomerase action is that telomere uncapping would be induced. Therefore, we monitored activation of DNA damage response genes by Northern blotting analysis of the HCT116 cells with or without functional p53. Increased p21 and GADD45 gene expression was observed in both HCT116 (p53-WT) and HCT116 (p53-null) cells (Fig. 5*B*). Although the basal level of p21 transcript was reduced in the HCT116 (p53-null) cells, upon expression of MT-hTers, p21 and GADD45 transcripts were reproducibly induced. This is consistent with our finding that MT-hTers induce cellular phenotypes independent of endogenous p53. Additional analysis is required to address the nature of the response pathways activated in HCT116 (p53-WT) and HCT116 (p53-null) cells.

DISCUSSION

We have shown here that targeting a combination of two aspects of telomerase function, telomerase activity and telomeric repeat sequence specification, is highly effective in killing human melanoma, colon, and bladder cancer cell lines. Similar overall results were also obtained with telomerase-positive T24 bladder cancer cells, MCF-7 breast cancer cells, and LNCaP prostate cancer cells (data not shown). Tumorigenesis of MT-hTer-overexpressing human cancer cells in immunodeficient mice was also suppressed. The requirement for up-regulated telomerase (a characteristic of most human cancers), the rapidity of the killing, the failure to see any resistant cell subpopulation, and the lack of dependence on p53, initial telomere length, or progressive telomere shortening make the siRNA and MT-hTers very attractive anticancer therapeutic approaches.

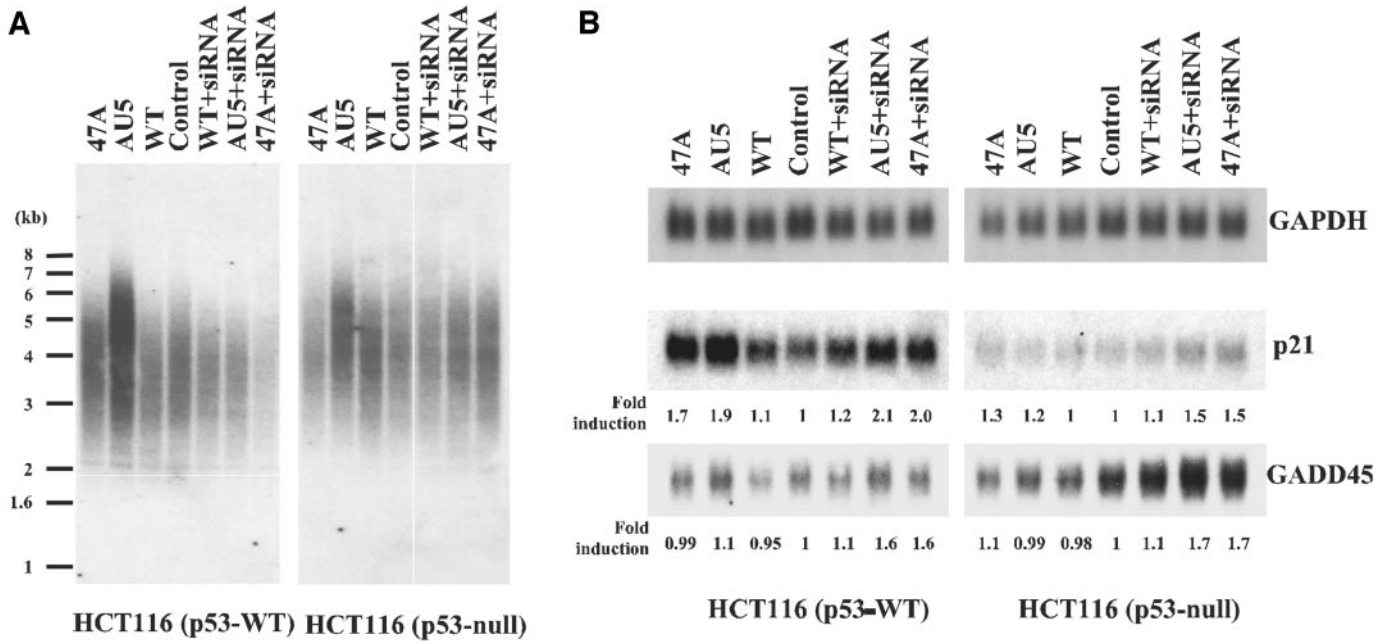

Fig. 5. Telomere length and DNA damage responses in HCT116 cells expressing wild-type human telomerase RNA (WT-hTer) or mutant-template human telomerase RNA (MT-hTer). *A*, Southern blotting analysis of telomere lengths (TRF). Genomic DNA was isolated 14 days after lentivirus infection. Cells were mock-infected (*Control*) or infected with lentivirus expressing WT-hTER alone (*WT*); MT-hTer-AU5 alone (*AU5*); MT-hTer-47A alone (*47A*); WT-hTER and short-interfering RNA (*WT+siRNA*); and MT-hTer-AU5 and siRNA (*AU5+siRNA*) or MT-hTer-47A and siRNA (*47A+siRNA*) as indicated. *B*, Northern blotting analysis of p21 and GADD45 mRNA induction in HCT116 cells with or without functional p53. RNA was prepared 4 days after virus infection. Note that the basal level of p21 transcript is lower in the p53-null HCT116 cells.

A notable feature of these rapid growth-inhibitory effects was that bulk telomere shortening was not required. Our analyses showed that MT-hTer expression induces a sustained DNA-damage response in telomerase-positive cancer cells. It is of interest that the rapid cell growth inhibition induced in HCT116 cells by MT-hTer alone, the antitelomerase RNA siRNA alone, or both together did not depend on the cellular status of p53, which is frequently inactivated in human cancer. Also, MT-hTer expression caused rapid cancer growth inhibition and apoptosis of VA13(hTert) fibroblasts immortalized by SV40 large T-antigen, which inactivates cellular p53 and retinoblastoma protein function. This lack of reliance on p53 function contrasts with previous studies using a different type of disruption of telomere function: expression of $TRF2^{\Delta B\Delta M}$, a dominant-negative form of the telomere-protective protein TRF2 (33, 34), which induced p53-dependent apoptosis and growth inhibition (33, 34). Therefore, we propose that different response pathways are involved in telomere uncapping by MT-hTer *versus* $TRF2^{\Delta B\Delta M}$.

This work has established the feasibility of suppressing human cancer cell and tumor growth by the lentiviral-mediated introduction of mutant-template telomerase RNA and anti-hTER siRNA. The unique properties of the lentivector allow the efficient delivery and coexpression of several different gene expression cassettes in a single virus construct, allowing the simultaneous expression of MT-hTer and depletion of endogenous hTER and causing rapid inhibition of cell growth and induction of apoptosis. Thus, MT-hTer and anti-hTER-siRNA expression represent promising novel anticancer strategies.

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