

Research Article

Reprogramming Murine Telomerase Rapidly Inhibits the Growth of Mouse Cancer Cells *In vitro* and *In vivo*Tong Xu¹, Yucheng Xu¹, Chun-Peng Liao², Roy Lau¹, and Amir Goldkorn¹

Abstract

Telomerase plays a critical role in cancer, prompting the pursuit of various telomerase-based therapeutic strategies. One such strategy, telomerase interference, exploits the high telomerase activity in cancer cells and reprograms telomerase to encode “toxic” telomeres. To date, telomerase interference has been tested in human cancer cells xenografted into mice, an approach that does not recapitulate spontaneous malignancy and offers few insights about host toxicities, because human telomerase is targeted in a mouse host. To address these limitations, we designed and validated two new gene constructs specifically targeting mouse telomerase: mutant template mouse telomerase RNA (MT-mTer) and small interfering RNA against wild-type mouse telomerase RNA (α -mTer-siRNA). Using lentiviral delivery in mouse prostate cancer cells, we achieved α -mTer-siRNA-mediated knockdown of wild-type mTer (80% depletion) and concurrent overexpression of MT-mTer (50-fold). We showed that the two constructs effectively synergize to reprogram murine telomerase to add mutant instead of wild-type telomeric repeats, resulting in rapid telomeric uncapping (5-fold increase in DNA damage foci). This, in turn, led to rapid and significant apoptosis (>90% of cells) and growth inhibition *in vitro* (90% reduction in viable cell mass) and *in vivo* (75% reduction in tumor allograft wet weight). In summary, we have shown that mouse cancer cells are vulnerable to direct telomerase interference using novel murine telomerase-targeting constructs; this approach can now be used to study the true therapeutic potential of telomerase interference in mouse spontaneous cancer models. *Mol Cancer Ther*; 9(2); 438–49. ©2010 AACR.

Introduction

The enzyme telomerase preserves the proliferative capacity of the cells by lengthening and protectively “capping” telomeres, the tandem repetitive DNA sequences at the ends of human chromosomes (1). It is a ribonucleoprotein consisting of two core components: a reverse transcriptase protein (TERT) and telomerase RNA (Ter). Ter contains a short template sequence used by TERT to synthesize telomeric DNA (2). Whereas benign, terminally differentiated tissues have extremely low telomerase levels (3), malignant cells from a variety of cancers have significant telomerase expression and activity levels that correlate directly with malignant/metastatic potential (4–6). Attenuation of human telomerase function in

cancer cells has produced apoptosis and growth inhibition (7–9), underscoring the great clinical promise of this therapeutic approach.

Previously, we and others have explored various telomerase-based strategies against human cancer cells (7, 10–13) and tested them *in vivo* by targeting human telomerase in cancer cells xenografted into mice (7, 10, 11, 13). Telomerase depletion also has been studied in knockout models that lack telomerase entirely (14, 15). Such xenograft and knockout models necessitate a leap of faith: that the observed sequelae of telomerase manipulation will accurately predict efficacy and toxicity in an actual host with spontaneous malignancy and normal telomerase function. Such an assumption is particularly tenuous in the field of telomerase targeting because telomerase is known to play important roles both in tumors and in normal progenitor tissue compartments (5, 16–18).

In this study, we set out to engineer and validate two new gene constructs that can effectively reprogram mouse telomerase: (a) small interfering RNA against wild-type mouse telomerase RNA (α -mTer-siRNA) and (b) mutant template mouse telomerase RNA (MT-mTer) that encodes incorrect mouse telomeric repeats. When co-expressed in a mouse prostate cancer cell line derived from the *cPten*^{-/-} mouse (19–21), α -mTer-siRNA and MT-mTer coopt the activity of mouse telomerase and reprogram it to encode an altered telomeric sequence, eliciting a rapid cascade of telomeric uncapping, cellular apoptosis, and growth inhibition *in vitro* and *in vivo*.

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These experiments validate the mechanism of action and biological efficacy of α -mTer-siRNA and MT-mTer in mouse cancer cells, and these new constructs can now be used to study the systemic efficacy and toxicities of telomerase targeting in mouse models of spontaneous malignancy.

Materials and Methods

Plasmid Construction

The siRNA lentiviral vector was generated by PCR with U6 promoter as the template using the 5' primer 5'-GGACTAGTAAGGTTCGGGCAGGAAGAGGGC-3' and the 3' primer 5'-AAAACCTGCAGAAAAATTACCTAACCCCTGATTTTCATTCTCTTCAAATGAAAATCAGGGTTAGGTGGTGTTCGTCCTTCCACAAG-3' and inserted into *SpeI/PstI* sites of pHR'CMVPuroWsin18 (11, 22). The mutant mouse Ter expression construct (pIU1-MT-mTer) was PCR cloned using the 5' primer 5'-GGATCCACCAAACCCAGATTTTCAT-TAGCT-3' (mutated sites in bold) and the 3' primer 5'-CTCGAGGGTTGTGAGAACCGAGTTCCG-3' and subcloned into *BglII/SalI* sites in pIU1-T7 vector and then into pHR'CMVPuroWsin18 vector to generate Lenti-MT-mTer. To generate MT-mTer/siRNA vector, ϕ 1 origin from pBluescript (Promega) was PCR amplified using the 5' primer 5'-AGATCTTTGTTCCAGTTTGGAAACAAGAGT-3' and the 3' primer 5'-GAATTCGCATTAAGCGCGGCG-3' and inserted into *BamHI/EcoRI* sites of pIU1-MT-mTer. The DNA fragment containing ϕ 1 origin and MT-mTer was subcloned into α -mTer-siRNA.

Cell Culture

The mouse prostate cancer cell line E4 was generously provided by Dr. Pradip Roy-Burman (University of Southern California, Los Angeles, CA). Cells were cultured at 37°C and 5% CO₂ in DMEM (Cellgro) with 10% fetal bovine serum (Omega Scientific), 5 μ g/mL insulin (Sigma), 25 μ g/mL bovine pituitary extract, 6 ng/mL recombinant epidermal growth factor, 100 units/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen).

Virus Production and Infection

Lentivirus was generated as previously described (23). Virus-containing supernatant from several different preps was combined and concentrated by centrifugation at 28,000 rpm (SW32T rotor, Beckman) for 2 h and resuspended in culture medium and used to infect E4 cells as described previously (10).

RNA Extraction, Reverse Transcription, and PCR

Total RNA from infected cells was extracted using RNA-Bee reagent (Tel-Test). First-strand cDNA was synthesized using the RETROscript reverse transcription kit (Applied Biosystems, Inc.). To differentiate MT-mTer expression from wild-type mTer (WT-mTer), PCR was designed using one primer against the mTer coding

region and one against a sequence that is downstream of the mTer coding region in the construct. This extra sequence resulted from pIU1-T7 vector and was located before the transcription termination signal; thus, it was transcribed in MT-mTer but was not included in endogenous WT-mTer (see Supplementary Table S1 for PCR primer sequences).

Real-time PCR

Real-time PCR (RT-PCR) for mTer quantitation was done using B-R SYBR Green Supermix for iQ (Quanta BioSciences; see Supplementary Table S1 for real-time primer sequences) on a MyiQ single-color RT-PCR detection system (Bio-Rad) for 40 cycles at 95°C for 10 s and 55°C for 45 s. The iQ5 optical system software version 2.0 was used to analyze results as normalized to β -actin internal controls.

Telomeric Repeat Amplification Protocol Assay

Telomerase activity from cell extracts was analyzed using RT-PCR-based telomeric repeat amplification protocol (TRAP) on a Bio-Rad MyiQ system as described (24). For the second (amplification) step, two reverse primers were used: ACX reverse primer specific for wild-type TTAGGG encoded by endogenous WT-mTer and 4A10A reverse primer specific for mutant TTTGGG encoded by MT-mTer (see Supplementary Table S2 for oligo sequences). The reactions were run for 40 cycles at 95°C for 0 s, 50°C for 5 s, and 72°C for 10 s to amplify WT-mTer product (TTAGGG) or MT-mTer product (TTTGGG), respectively. The iQ5 optical system software version 2.0 was used to analyze the results.

Telomere Length Assay

Genomic DNA was extracted from infected E4 cells using DNeasy Blood and Tissue kit (Qiagen), and relative telomere lengths were analyzed in triplicate by RT-PCR (Bio-Rad MyiQ) as described previously using T and S primers (see Supplementary Table S1 for T and S primer sequences; ref. 25).

Cell Viability and Apoptosis Assays

Assays were done on E4 cells 3 and 4 d after infection using MTS ((Promega), *In Situ* Cell Death Detection kit [terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)], and Fluorescein and Homogeneous Caspases Assay kit (Roche Applied Science) per the manufacturers' protocols. For TUNEL assays, fluorescence-activated cell sorting (FACS) was done on BD LSR-II (BD Biosciences). For MTS and caspase assays, the absorbance was measured at 490 nm by Plate Chameleon Multi-technology plate reader (Hidex).

Immunofluorescence Microscopy

E4 cells (10⁴) were plated on coverslips in 12-well plates and infected with lentivirus overnight in the presence of 8 μ g/mL polybrene. On day 4, cells were stained as described previously (10) using primary antibodies

against p53BP1 (Bethyl Laboratories) and TRF2 (BD Biosciences), secondary antibodies conjugated to Alexa Fluor 647 (Invitrogen) and Alexa Fluor 568 (Invitrogen), and DAPI for DNA visualization. p53BP1 foci were enumerated using a Zeiss Imager.Z1 microscope with Axiovision software at $\times 63$ magnification. Confocal colocalization was analyzed using Zeiss LSM 510 with Zeiss LSM 510 software at $\times 63$ magnification.

Subcutaneous Tumor Allografts

Six- to 8-wk-old male nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice were purchased from NIH. E4 cells were infected overnight with control or MT-mTer/siRNA lentiviruses and cultured at 37°C and 5% CO_2 for 1 d after changing medium. Before inoculation into mice, total RNA and genomic DNA from E4 cells were isolated as described above to check the expression of mTer. For each mouse, 10^6 cells were resuspended in medium, mixed with 50 μL ice-cold Matrigel (BD Biosciences), and placed on ice until inoculation. Insulin syringe (1 mL) was used for subcutaneous inoculation onto the flank of each mouse (five mice per

treatment group). All experiments were approved and done following the rules of the Institutional Animal Care and Use Committees at University of Southern California. Thirty days after inoculation, mice were sacrificed, and tumors were resected and weighed. A portion of tumor tissues was fixed and used to make paraffin-embedded slides for H&E staining. Another portion of tumors from three mice were also digested with 1 mg/mL collagenase (Sigma), 1 $\mu\text{g}/\text{mL}$ DNase I (Invitrogen), and 1 mg/mL hyaluronidase (Sigma) at 37°C for 1 h to obtain a single-cell suspension. These cells were then stained with biotinylated antibodies against CD13, CD45, and Ter119 (BD Biosciences) and streptavidin-phycoerythrin/Cy5 secondary antibody against biotinylated antibodies, Sca-1–phycoerythrin, and CD49f–Alexa Fluor 647 (BioLegend). $\text{Lin}^{-}\text{Sca-1}^{+}\text{CD49f}^{+}$ E4 cells were sorted by BD FACSAria (BD Biosciences). Genomic DNAs from FACS-purified E4 cells were isolated, and levels of genomically integrated MT-mTer were measured by PCR using the single-copy gene *36B4* to control for DNA loading (see Supplementary Table S1 for PCR primer sequences).

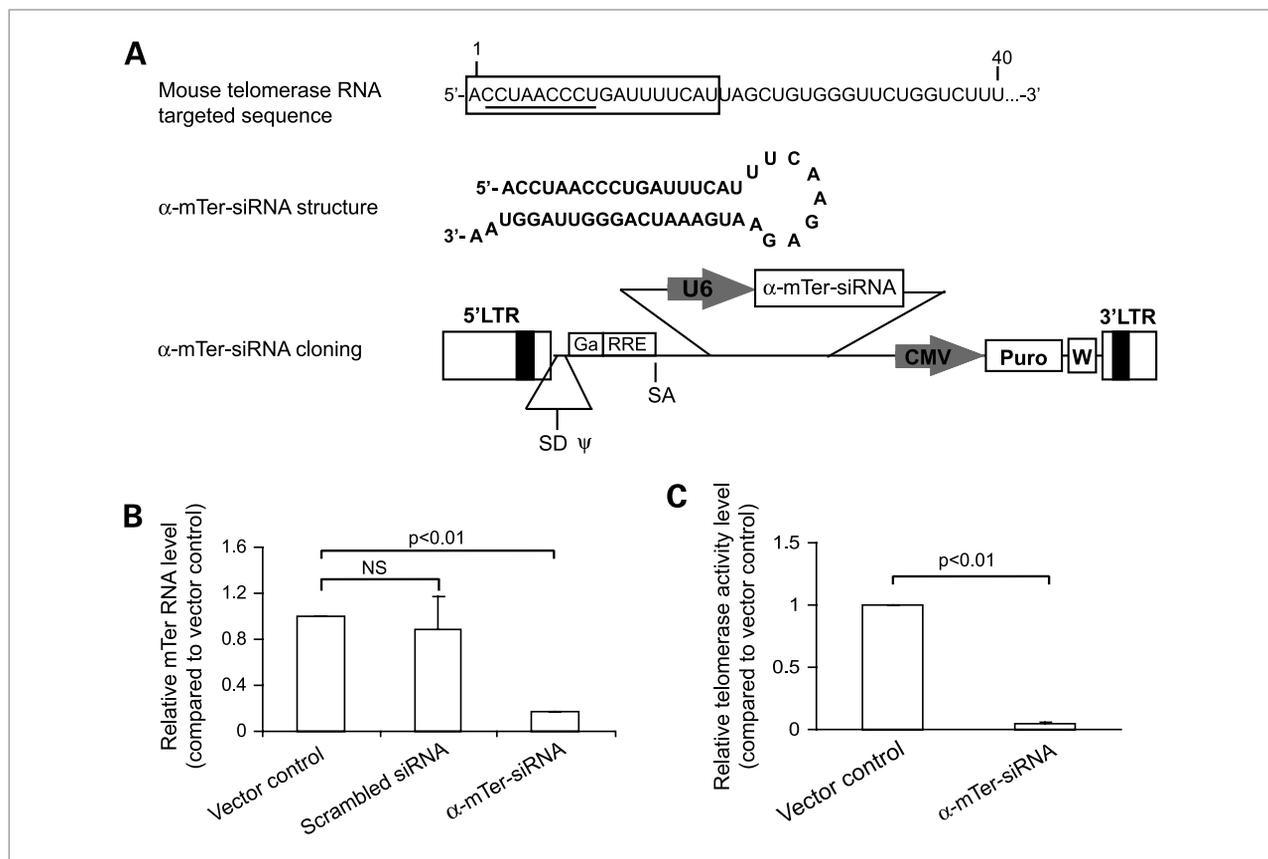


Figure 1. α -mTer-siRNA knocks down WT-mTer and inhibits telomerase activity in E4 mouse prostate cancer cells. **A**, α -mTer-siRNA structure and cloning into lentiviral vector system (target sequence is boxed, template region is underlined). **B**, α -mTer-siRNA knocks down WT-mTer by 80% 3 d after expression in E4 cells, as quantified by RT-PCR. **C**, α -mTer-siRNA inhibits telomerase activity by 95% 3 d after expression in E4 cells, as quantified by RT-PCR TRAP.

Results

α -mTer-siRNA Knocks Down WT-mTer and Inhibits Telomerase Activity in E4 Mouse Prostate Cancer Cells

We synthesized a short hairpin α -mTer-siRNA specifically targeting the template region of WT-mTer component and cloned it into a three-plasmid-based lentiviral system under the U6 promoter (Fig. 1A). Lentivirus bearing the α -mTer-siRNA was introduced into E4 cells, a mouse prostate cancer cell line derived from prostate tumors arising in the transgenic *cPten*^{-/-} mouse prostate cancer model (generously provided by the laboratory of Dr. Pradip Roy-Burman). The E4 cells bear a Lin⁻Sca-1⁺CD49f⁺ surface marker signature, stain positive for androgen receptor, strongly express telomerase, and are tumorigenic based on data presented in this report, which are consistent with the detailed phenotypic and biological characteristics of this novel cell line determined by the Roy-Burman group (26).

To confirm lentiviral infection and expression efficiency, we conducted an initial control experiment in which lentivirus containing only a puromycin selection marker ("vector control") was used to infect E4 cells. After 4 days of puromycin selection, >95% of these cells remained viable versus <5% viable cells in mock-infected E4 cells. Having confirmed >95% lentiviral infection and expression efficiency in this manner, we dispensed with the 4-day puromycin selection period in subsequent experiments and thus were able to measure the immediate short-term effects of telomerase interfering constructs relative to vector control.

Using RT-PCR, we found that cells infected with α -mTer-siRNA had an 80% depletion of endogenous WT-mTer 3 days after infection compared with cells infected with vector control or with a nonspecific scrambled siRNA (Fig. 1B). RT-PCR-based TRAP assays done on cell protein extracts 3 days after infection showed a 95% reduction of telomerase activity in α -mTer-siRNA-infected cells compared with cells infected with vector control (Fig. 1C).

MT-mTer Incorporates into Mouse Telomerase and Reprograms It to Add TTTGGG Mutant Telomeric Repeats

Using PCR-based mutagenesis, we introduced two mutations (T→A) at the 4 and 10 positions of the mouse telomerase RNA gene. This new MT-mTer construct was cloned into lentiviral vector under the IU1 small nRNA promoter and introduced into E4 target cells, where it was transcribed to generate mouse telomerase RNA with two mutations (U→A) in the template region (Fig. 2A). The MT-mTer construct also was cloned into a lentiviral vector already containing α -mTer-siRNA for coexpression, so-called MT-mTer/siRNA (Fig. 2A).

Lentiviral introduction of the MT-mTer and MT-mTer/siRNA constructs into E4 cells achieved 50-fold overex-

pression of MT-mTer by RT-PCR relative to vector control or a scrambled constructs after 3 days (Fig. 2B). The RT-PCR primers detected both MT-mTer and WT-mTer; therefore, we further confirmed the specific expression of MT-mTer by regular PCR using primers designed specifically for MT-mTer. This approach showed that the α -mTer-siRNA component of MT-mTer/siRNA had no effect on the expression of MT-mTer and specifically knocked down only endogenous WT-mTer (Fig. 2C).

We investigated whether overexpressed MT-mTer RNA could successfully incorporate into an active telomerase enzyme and serve as a template for the addition of TTTGGG repeats in place of TTAGGG. For this purpose, we used a modified RT-PCR TRAP assay using the ACX and 4A10A reverse primers, which were specific for the telomeric repeats generated by endogenous WT-mTer or ectopically expressed MT-mTer, respectively (experiment scheme depicted in Fig. 2D and results summarized in Table 1). In TRAP assays for the detection of wild-type TTAGGG repeats (Table 1, *left half*), expression of MT-mTer alone had no effect on telomerase activity detected with the ACX wild-type-specific primer, whereas expression of MT-mTer/siRNA reduced wild-type telomerase activity to 5% of vector control as expected from α -Ter-siRNA knockdown of WT-mTer. In TRAP assays for the detection of mutated TTTGGG repeats (Table 1, *right half*), expression of either MT-mTer or MT-mTer/siRNA increased telomerase activity detected with the 4A10A mutant-specific primer by 2.7- and 3.6-fold, respectively, indicating that MT-mTer RNAs incorporated with mouse telomerase reverse transcriptase to form active telomerase enzyme capable of adding mutant TTTGGG tandem repeats. This effect was potentiated by concurrent siRNA depletion of competing WT-mTer (hence, 3.5-fold increase in mutant telomerase activity with MT-mTer/ α -Ter-siRNA and only 2.7-fold with MT-mTer alone).

We confirmed these findings by doing additional TRAP reactions using only dTTP and dGTP instead of all four deoxynucleotide triphosphates (dNTP), thus taking advantage of the sequence difference between the wild-type (TTAGGG) and mutant template (TTTGGG). This was accomplished by providing only dTTP and dGTP in the extension (first) step of the TRAP reaction and then supplementing dATP and dCTP for the amplification (second) step (illustrated in Fig. 2D). As predicted, addition of dTTP and dGTP alone produced no detectable telomerase activity when the ACX (wild-type-specific) reverse primer was used. In contrast, telomerase activity was readily detectable from cells expressing MT-mTer when the 4A10A (mutant-specific) reverse primer was used in the presence of dTTP and dGTP alone (Table 1).

MT-mTer and α -mTer-siRNA Inhibit E4 Mouse Prostate Cancer Proliferation *In vitro* and *In vivo*

We introduced MT-mTer and α -mTer-siRNA by lentiviral infection into E4 cells and measured cell numbers

Table 1. MT-mTer reprograms telomerase enzyme to add mutated telomeric repeats

	Telomerase activity (as % of vector control)*			
	Amplification: ACX reverse primer (specific to wild-type TTAGGG)†		Amplification: 4A10A reverse primer (specific to mutant TTTGGG)†	
	Extension: 4 dNTPs†	Extension: dGTP and dTTP†	Extension: 4 dNTPs†	Extension: dGTP and dTTP†
Vector control	100	0	0	5
MT-mTer	100	0	270	270
MT-mTer/siRNA	5	0	350	350

NOTE: When MT-mTer and MT-mTer/siRNA are overexpressed, significantly more telomeric products (270% and 350%, respectively) are detected using a TTTGGG-specific ("4A10A") reverse primer in the second (amplification) step of RT-PCR TRAP, an effect that is preserved even when just dGTP and dTTP are added in the first (extension) step of the reaction. Activity values are % of vector control and are means of triplicates; all values differ from vector control with statistical significance ($P < 0.01$).

*All activity values are means of triplicates.

†Extension and amplification steps illustrated in Fig. 2D.

inoculation, we sacrificed the mice and excised and recorded the wet weight of tumors, as this was a more accurate readout than the caliper measurements. Mice inoculated with E4 cells expressing MT-mTer/siRNA formed smaller tumors at all time points, and their excised final tumor weights were 50% smaller than those of the control groups (mean wet weight, 0.12 g versus 0.25 g; $P < 0.01$; Fig. 4A).

Although E4 cells expressing MT-mTer/siRNA formed significantly smaller tumors, we were curious as to how they were able to form tumors at all. To gain insight into this question, we first isolated the E4 cells from mouse tumors using FACS sorting for Lin⁻Sca-1⁺CD49f⁺ cells, the known unique surface marker signature of the *cPten*^{-/-} mouse prostate tumor cells (Fig. 4B, left; ref. 20). Using RT-PCR, we found no significant difference in MT-mTer RNA levels between MT-mTer/siRNA tumor cells

and vector control tumor cells at day 30 (Fig. 4B). At the same time, MT-mTer genomic DNA levels were significantly lower in MT-mTer/siRNA tumor cells at day 30 than in E4 cells at day 0, which is immediately before inoculation into the mice (Fig. 4C). Taken together, these experiments suggested that the development of MT-mTer/siRNA tumors, albeit significantly smaller tumors, could be attributed to outgrowth of cells lacking genomic MT-mTer/siRNA (failed integration or subsequent loss) as well as to downregulation of MT-mTer/siRNA expression in cells that did possess the construct.

MT-mTer/siRNA Induces Rapid DNA Damage and Apoptosis without Significantly Altering Telomere Lengths

TUNEL assay done 4 days after expression of MT-mTer/siRNA showed that 90% of cells were TUNEL positive versus <5% in cells expressing vector control (Fig. 5A). Similarly, caspase activity after 4 days increased >4-fold in cells expressing MT-mTer/siRNA relative to cells expressing vector control (Fig. 5A). Together, these assays strongly suggested that ectopic overexpression of MT-mTer in combination with knockdown of WT-mTer inhibited proliferation by inducing rapid and marked apoptosis. To verify whether cellular apoptosis was generated by DNA damage and "uncapped telomeres" as shown in human systems (10, 11, 27, 28), we analyzed p53BP1 foci by immunofluorescent staining and found significantly more p53BP1 foci in MT-mTer/siRNA-infected cells than in vector control-infected cells (Fig. 5B). Moreover, the p53BP1 foci seemed to localize at telomeres by TRF2 colocalization, in contrast to p53BP1 foci generated by γ irradiation, which presumably induced DNA breaks indiscriminately throughout the genome (Fig. 5B), suggesting that the DNA damage induced by MT-mTer/siRNA indeed occurred preferentially at telomeres. Having observed rapid

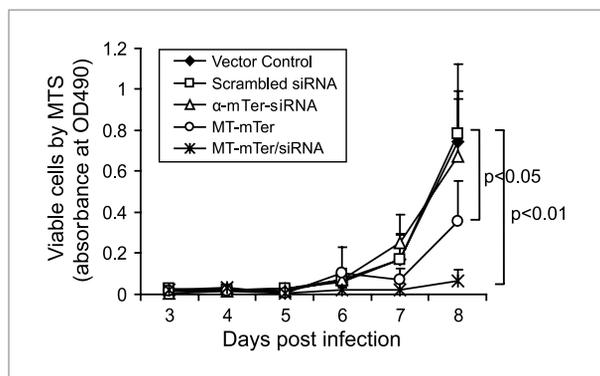


Figure 3. MT-mTer and MT-mTer/siRNA inhibit E4 mouse prostate cancer proliferation *in vitro*. Cell growth by MTS colorimetric cell viability assay is significantly inhibited by day 7 after expression of MT-mTer or MT-mTer/siRNA constructs (50% and 90% reduction, respectively).

onset of DNA damage, apoptosis, and growth inhibition within days, we suspected that minimal bulk telomere shortening had occurred during this short time period. Indeed, RT-PCR-based telomere length assay on E4 cells 3 days after MT-mTer/siRNA expression showed no significant changes in telomere lengths relative to control cells (Fig. 5C).

Discussion

The role of telomerase in cancer and progenitor cells has been studied intensely; however, current xenograft and knockout mouse models cannot recapitulate the effects of telomerase targeting in an actual host with spontaneous malignancy and normal telomerase

function. To address this limitation, we designed and validated two novel gene constructs, α -mTer-siRNA and MT-mTer, which specifically target murine telomerase and can be used to modulate telomerase activity in mouse models. The constructs were validated *in vitro* and *in vivo* and were shown to effectively reprogram mouse telomerase and induce telomeric uncapping, cellular apoptosis, and growth inhibition.

We did our studies in E4 mouse prostate cancer cells, which are derived from prostate tumors arising in the transgenic *cPten*^{-/-} mouse, regarded as perhaps the best mouse model of spontaneous prostate cancer currently available (19–21, 29). The *cPten*^{-/-} mouse accurately recapitulates human disease, progressing from prostatic hyperplasia, to prostate intraepithelial neoplasia, to

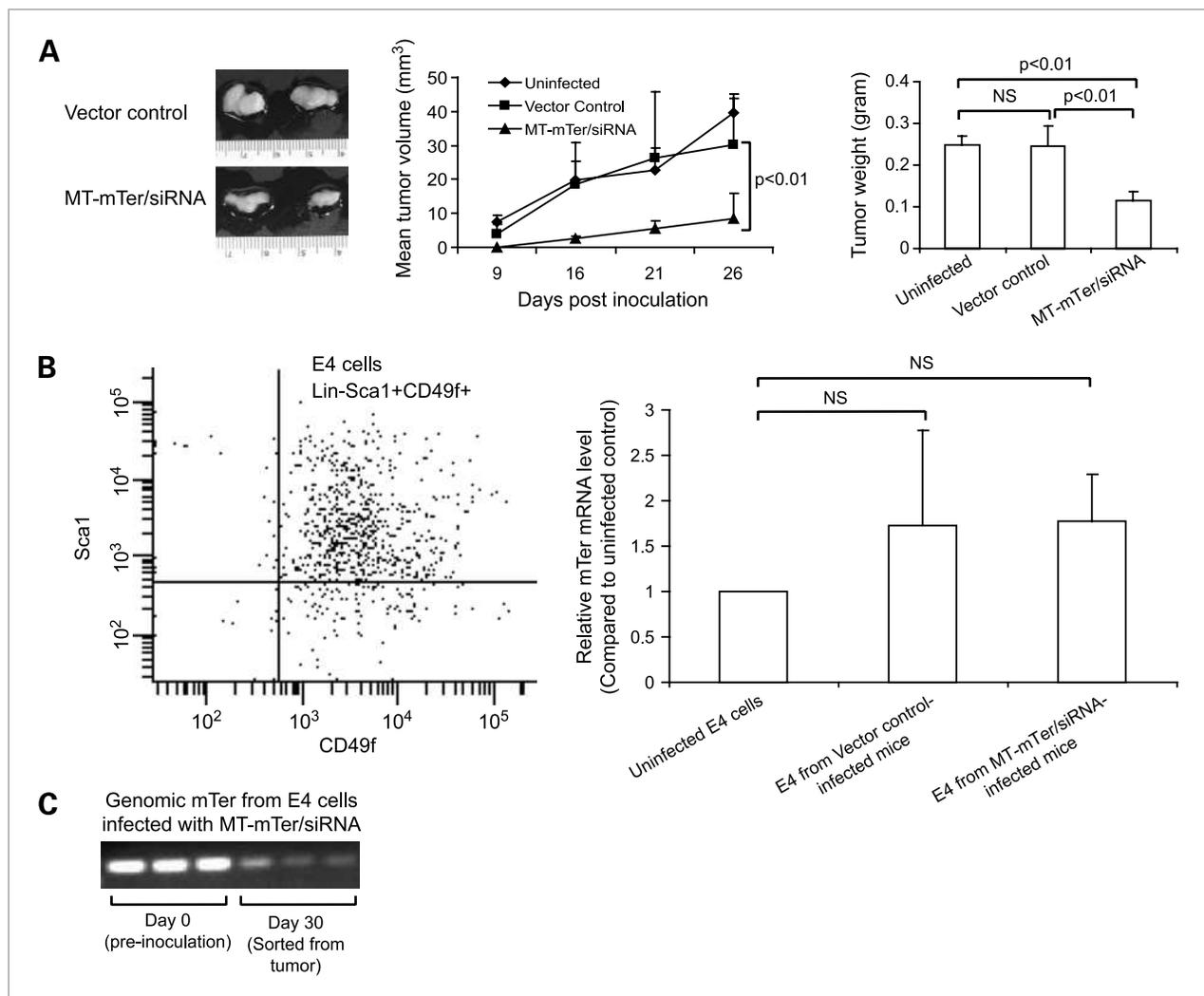


Figure 4. MT-mTer/siRNA inhibits E4 tumor growth. **A**, left, representative samples of MT-mTer/siRNA or vector control tumors. E4 cells were infected *in vitro* with lentivirus expressing active or control constructs and then allografted subcutaneously into NOD-SCID mice. Middle, growth of tumors was inhibited by E4 cells infected by MT-mTer/siRNA lentivirus; right, wet weights of tumors resected 4 wk after inoculation. **B**, left, E4 cells (Lin⁻Sca1⁺CD49f⁺) were sorted by FACSaria from freshly resected, disaggregated, and digested tumors; right, MT-mTer RNA levels from sorted E4 cells were quantified by RT-PCR. **C**, bottom, genomic mTer DNA levels from sorted E4 cells were quantified by PCR using the single-copy gene *36B4* to control for DNA loading. Representative bands from three different samples are shown.

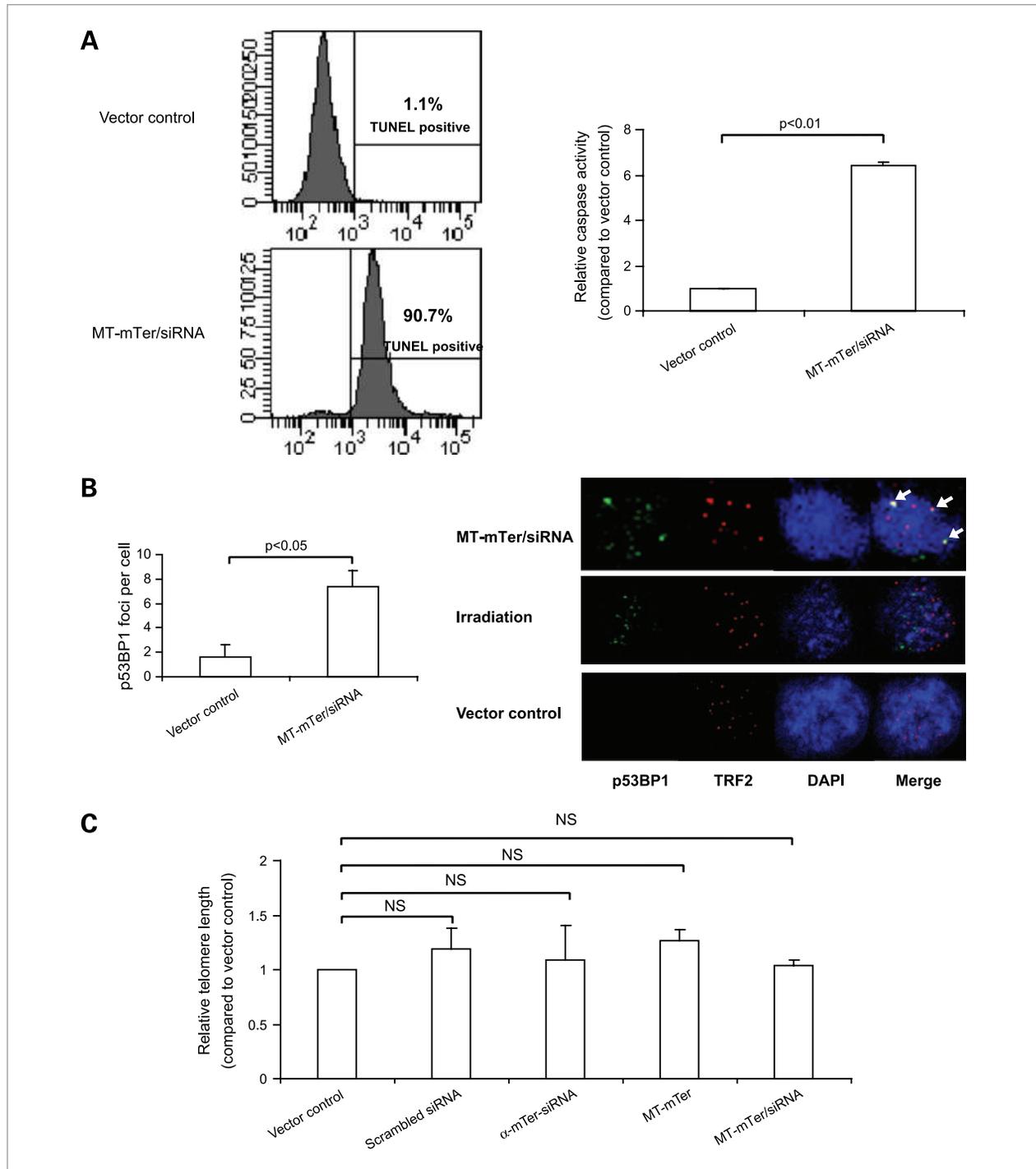


Figure 5. MT-mTer/siRNA induces rapid apoptosis and DNA damage in E4 cells without altering bulk telomere length. **A**, left, TUNEL assay done 4 d after lentiviral expression of MT-mTer/siRNA shows brisk apoptotic cell death, 90.7% versus 1.1% with vector control by FACS analysis. Shown are representative plots of three different assays with statistical significance ($P < 0.01$). Right, caspase assay done at the same day 4 time point reveals significantly increased caspase activity consistent with apoptosis in cells expressing MT-mTer/siRNA versus control. **B**, left, quantitation of p53BP1 DNA damage foci in MT-mTer/siRNA-infected or vector control-infected cells. Cells were grown on glass coverslips, fixed, and stained on day 4 after lentiviral infection, and foci were counted under $\times 63$ magnification using a Zeiss Imager.Z1. Right, representative fluorescence micrographs depicting p53BP1 foci, TRF2, DAPI, and merge. Cells were grown, infected, fixed, and stained as before and then photographed at $\times 63$ magnification using a Zeiss LSM 510 confocal scope. MT-mTer/siRNA-infected cells seem to have more p53BP1 DNA damage foci than vector control-infected cells and more colocalization of p53BP1 DNA damage foci with telomeres (TRF2) than irradiated cells (colocalization indicated by arrows, right). **C**, cells infected with lentivirus expressing the various constructs were harvested 3 d after infection and assayed for bulk telomere lengths using RT-PCR (mean of triplicate experiments).

locally advanced prostate adenocarcinoma, to eventual micrometastatic and hormone-refractory disease. We were confident that the *Pten*^{-/-} phenotype of the E4 cells would not unduly influence their susceptibility to treatment, as telomerase interference had previously been found equally efficacious in human cancer cell lines possessing wild-type or null *Pten* status (11, 30). Hence, the E4 cells constituted not only a reliable *in vitro* model for construct validation but also a direct link to our future *in vivo* studies of systemic telomerase interference in the *cPten*^{-/-} spontaneous prostate cancer mouse model.

When ectopically expressed in the E4 cells, the first construct, α -mTer-siRNA, achieved significant knockdown of mouse telomerase RNA and telomerase activity. We engineered the α -mTer-siRNA construct using a hairpin loop structure targeting the 5' mTer region that encompasses the template sequence, as this strategy had previously proven effective for knockdown of human telomerase RNA (10, 11, 31). Notably, we observed that coexpression of α -mTer-siRNA with MT-mTer (so-called MT-mTer/siRNA) did not affect MT-mTer levels (Fig. 2B and C); that is, the α -mTer-siRNA specifically depleted only WT-mTer and thus potentiated the "substitution" of MT-mTer for WT-mTer.

The second gene construct, MT-mTer, contained a mutated template sequence intended to alter the telomeric repeat sequence in the target cells. This approach was first introduced in ciliates and yeast (32, 33) and subsequently accomplished in human cell lines (10, 11, 30, 34–36). In the human studies, U→A template mutations were used successfully, prompting us to engineer analogous mutations in the mTer template. Successful incorporation of MT-Ter into active telomerase enzyme had been assessed previously either by blotting genomic DNA with a probe specific for the predicted telomere sequence (32, 35, 36) or by traditional PCR TRAP assay with amplification primers specific to the predicted altered sequences added to a telomeric substrate (30, 34, 36, 37). In our current study, we adapted the latter approach to next-generation RT-PCR TRAP, which showed that MT-mTer successfully partnered with mTERT to form enzymatically active telomerase that added incorrect telomeric repeats (TTTGGG instead of TTAGGG) and that this effect was potentiated by coexpression of siRNA against WT-mTer.

Expression of MT-mTer alone induced a significant (50%) growth-inhibitory effect, consistent with previous reports of MT-mTer expression in other model systems such as ciliates, yeast, and human immortal or cancer cell lines (11, 30, 33, 35, 36, 38); these earlier studies used a spectrum of mutant template strategies and growth readouts, but all observed some degree of growth disruption. In contrast to the effects of MT-mTer, introduction of α -mTer-siRNA alone produced no significant short-term growth inhibition despite inhibition of telomerase activity. This was consistent with past reports in human cancer cell lines, where hTer depletion achieved either no significant growth inhibition or inhibition only with long-term

culture over a period of weeks, attributed to a necessary "lag" period of telomere shortening in the absence of telomerase activity (10, 11, 31, 39). In the current study, coexpression of α -mTer-siRNA and MT-mTer induced marked (90%) growth inhibition, suggesting once again that α -mTer-siRNA potentiated the effects of MT-mTer by removing competing WT-mTer.

We tested the effects of MT-mTer/siRNA on tumor growth in NOD-SCID mice. This experiment potentially could have been conducted in immune-competent syngeneic mice, but such an approach would have risked lower engraftment rates while still not addressing the ultimate question of efficacy in spontaneously arising tumors. Therefore, we elected to use immune-deficient NOD-SCID mice to maximize engraftment, thereby enabling a more robust validation of tumor inhibition by telomerase interference relative to vector control. When allografted into mice, E4 cells expressing MT-mTer/siRNA formed tumors that were 50% smaller compared with tumors from mock- or vector-infected cells. We found a significant reduction in MT-mTer RNA and DNA levels in MT-mTer/siRNA tumor cells, suggesting that these small tumors likely formed from E4 cells that had downregulated MT-mTer expression or genomically lacked the construct altogether. From a therapeutic standpoint, such escape mechanisms are to be expected. Even highly efficacious therapies rarely, if ever, achieve total eradication of tumor growth with a single treatment; rather, systemic treatment (in mouse spontaneous malignancy models or in patients) will necessitate repeat dosing to ensure maximal delivery and cell kill with successive cycles.

We investigated whether the growth inhibition mediated by MT-mTer/siRNA was the result of "uncapped" telomeres, an inability to assume a protected configuration, presumably because the altered telomere sequence cannot appropriately interact with members of the protective shelterin complex (11, 27, 28, 30, 36). Indeed, we found that cells expressing MT-mTer/siRNA accumulated significantly higher numbers of p53BP1 DNA damage foci that seemed to localize preferentially at telomeres, which in turn led to marked increases in apoptosis (>90% of cells). Although quite promising from an anti-tumor perspective, such a high rate of apoptosis in telomerase⁺ cells raises the specter of toxicity in the *in vivo* setting. Two factors that mitigate this concern are the following. (a) We have shown previously that telomerase interference is dependent on the presence of active telomerase (TERT), which generally is expressed at much higher levels in tumor cells than in host tissues, thus conferring mechanistic specificity to this therapeutic approach. (b) As with all antineoplastic strategies, efficacy and toxicity will be titrated to an optimal ratio (therapeutic index) by modulating the dose intensity and frequency, first in animal models and ultimately in human clinical trials. Such optimization requires the ability to systemically deliver telomerase interference in a host

with cancer, precisely the rationale for designing these constructs.

We measured the effects of telomerase interference on the length of mouse telomeres, which are significantly longer than their human counterparts (40, 41). In the first report (in ciliates) of MT-mTer expression (32), Blackburn and colleagues found an increase in bulk telomere length, possibly due to “inability to bind some length-regulating factor.” A subsequent study done by that group in yeast found variable telomere length effects depending on the specific template mutations induced (33). In human immortalized cells or cancer cell lines, a preponderance of studies found no significant telomere length changes with short-term (days to weeks) expression of MT-hTer (10, 11, 30, 35, 36). Similarly, studies depleting human telomerase RNA with siRNA found no short-term telomere length changes (10, 31), and a study of long-term telomerase inhibition with an oligonucleotide that binds hTer noted telomere shortening only after a period of several weeks (39). A recent study was reported wherein two mouse immortal cell lines (telomerase⁺ or mTer⁻) were directly transfected with a MT-mTer (42); however, that study used direct transfection and long-term clone selection to introduce the MT-mTer, resulting in low levels of expression. Consequently, no mutant telomerase activity or biological effects were demonstrable in the telomerase⁺ line, and the effects noted in the mTer⁻ line were not consistent among clones (42).

In our study, MT-mTer/siRNA induced telomere dysfunction without bulk telomere shortening in mouse cancer cells. The length-independent rapid effects of these new constructs may prove particularly useful in mice, which typically possess very long telomeres (40, 41). Alternative telomerase strategies that directly inhibit the enzyme can induce cell growth inhibition only after a prolonged “lag phase” of cell division and progressive telomere shortening (39). Such approaches may be of limited utility in the setting of very long mouse telomeres, which must progressively shorten over numerous cell divisions and several generations to manifest a phenotype (14, 15, 43, 44). In contrast, reprogramming mouse telomerase with MT-mTer/siRNA can effectively elicit telomere dysfunction, apoptosis, and growth inhibition in a matter of days without the need for significant telomere shortening.

Given the recent enthusiasm and substantial efforts allocated to telomerase-based therapies, it is critical to develop more informative models that accurately reflect the potential efficacy and toxicity of these strategies. Current models may not accurately predict the efficacy of telomerase-based strategies. Although telomerase targeting may seem efficacious against homogeneous, rapidly dividing cancer cells xenografted into mice, it may be less effective against spontaneously arising tumors consisting of subpopulations of tumor cells with varying degrees of differentiation and telomerase activation. Indeed, such phenotypic heterogeneity within tumors has been observed in a large spectrum of common malignancies, ranging from leukemia, to breast cancer, to prostate cancer, to glioblastoma (16–18). At the same time, exist-

ing models provide minimal insight about the potential toxicity of telomerase manipulation. Most telomerase-targeting agents are engineered specifically against human telomerase; therefore, when tested in a mouse xenograft model, many of these human telomerase-targeting agents may be blind to the active mouse telomerase present in renewable host tissue compartments such as bone marrow, skin, and gut. Several studies have shown that telomerase activation within these compartments plays a critical role in cycling, proliferation, and differentiation of progenitor cells and that telomerase knockout in mice eventually leads to defects in these very same compartments (15, 44–49). Although telomerase knockout mice do illuminate some of these progenitor cell toxicities, they are not cancer models and do not recapitulate the scenario of acute telomerase targeting in a telomerase wild-type host with cancer.

MT-mTer/siRNA will help to surmount the limitations of current xenograft and knockout models, providing a ready set of new tools for directly studying the effects of telomerase disruption on tumors and progenitor compartments in mouse models of spontaneous malignancy. As with all mouse models, the implications of MT-mTer/siRNA for human therapy should be interpreted with a recognition of important differences between human and mouse telomere and telomerase biology, notably the greater length of mouse telomeres and the higher expression of telomerase in normal mouse tissues (43, 50). One speculates that both of these factors would endow telomerase interference with an even better efficacy/toxicity profile in humans than in mice, although such conclusions would have to await direct testing. Even with their acknowledged differences from human biology, mouse models inarguably have yielded many of our seminal insights about mammalian telomere and telomerase function, and they continue to provide a valuable and necessary preclinical setting for studying and manipulating telomeres and telomerase. Systemic delivery of MT-mTer/siRNA in mice will enable, for the first time, controlled depletion of telomerase activity (α -mTer-siRNA) and uncapping of telomeres (MT-mTer) in tumor cells and in progenitor compartments of an immune-competent mammalian host.

Having validated rapid telomerase interference in mouse cancer cells, we are currently packaging MT-mTer/siRNA for targeted systemic delivery by repeat dosing in the *cPten*^{-/-} spontaneous prostate cancer mouse. Notably, although our current studies use a prostate cancer model, we expect telomerase interference to achieve significant efficacy in a wide spectrum of malignancies, as >90% of human cancers upregulate telomerase activity by way of TERT expression, thus rendering them susceptible to targeting with MT-Ter/siRNA. It is our profound hope that introduction of these new constructs will open the door to systemic studies in mice, in larger mammals, and ultimately in human clinical trials to fully explore and develop this powerful and near-universal anticancer strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- De Lange T, Lundblad V, Blackburn EH. *Telomere*. 2nd ed New York: Cold Spring Harbor Laboratory Press; 2005.
- Greider CW, Blackburn EH. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature* 1989;337:331–7.
- Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW. Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet* 1996;18:173–9.
- Meecker AK. Telomeres and telomerase in prostatic intraepithelial neoplasia and prostate cancer biology. *Urol Oncol* 2006;24:122–30.
- Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer* 1997;33:87–791.
- Tomoda R, Seto M, Tsumuki H, et al. Telomerase activity and human telomerase reverse transcriptase mRNA expression are correlated with clinical aggressiveness in soft tissue tumors. *Cancer* 2002;95:1127–33.
- Dikmen ZG, Gellert GC, Jackson S, et al. *In vivo* inhibition of lung cancer by GRN163L: a novel human telomerase inhibitor. *Cancer Res* 2005;65:7866–73.
- Hahn WC, Stewart SA, Brooks MW, et al. Inhibition of telomerase limits the growth of human cancer cells. *Nat Med* 1999;5:1164–70.
- Zhang X, Mar V, Zhou W, Harrington L, Robinson MO. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev* 1999;13:2388–99.
- Goldkorn A, Blackburn EH. Assembly of mutant-template telomerase RNA into catalytically active telomerase ribonucleoprotein that can act on telomeres is required for apoptosis and cell cycle arrest in human cancer cells. *Cancer Res* 2006;66:5763–71.
- Li S, Rosenberg JE, Donjacour AA, et al. Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA. *Cancer Res* 2004;64:4833–40.
- Shay JW, Wright WE. Telomerase therapeutics for cancer: challenges and new directions. *Nat Rev Drug Discov* 2006;5:577–84.
- Gowan SM, Harrison JR, Patterson L, et al. A G-quadruplex-interactive potent small-molecule inhibitor of telomerase exhibiting *in vitro* and *in vivo* antitumor activity. *Mol Pharmacol* 2002;61:1154–62.
- Blasco MA, Lee HW, Hande MP, et al. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* 1997;91:25–34.
- Lee HW, Blasco MA, Gottlieb GJ, Horner JW II, Greider CW, DePinho RA. Essential role of mouse telomerase in highly proliferative organs. *Nature* 1998;392:569–74.
- Collins AT, Maitland NJ. Prostate cancer stem cells. *Eur J Cancer* 2008;42:1213–8.
- Pardal R, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer* 2003;3:895–902.
- Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001;414:105–11.
- Wang S, Gao J, Lei Q, Rozengurt N, et al. Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* 2003;4:209–21.
- Wang S, Garcia AJ, Wu M, Lawson DA, Witte ON, Wu H. Pten deletion leads to the expansion of a prostatic stem/progenitor cell subpopulation and tumor initiation. *Proc Natl Acad Sci U S A* 2006;103:1480–5.
- Liao CP, Zhong C, Saribekyan G, et al. Mouse models of prostate adenocarcinoma with the capacity to monitor spontaneous carcinogenesis by bioluminescence or fluorescence. *Cancer Res* 2007;67:7525–33.
- Naldini L, Blomer U, Gally P, et al. *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996;272:263–7.
- Ramezani A, Hawley RG. Generation of HIV-1-based lentiviral vector particles. *Curr Protoc Mol Biol* 2002. Chapter 16: Unit 16.22.
- Herbert BS, Hochreiter AE, Wright WE, Shay JW. Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. *Nat Protoc* 2006;1:1583–90.
- Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA. Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet* 2003;361:393–5.
- Liao C-P, Mengmeng Liang, Cohen MB, et al. Mouse prostate cancer cell lines established from primary and post-castration recurrent tumors. *Hormones & Cancer*. In press.
- de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 2005;19:2100–10.
- Xu L, Blackburn EH. Human cancer cells harbor T-stumps, a distinct class of extremely short telomeres. *Mol Cell* 2007;28:315–27.
- Ahmad I, Sansom OJ, Leung HY. Advances in mouse models of prostate cancer. *Expert Rev Mol Med* 2008;10:e16.
- Kim MM, Rivera MA, Botchkina IL, Shalaby R, Thor AD, Blackburn EH. A low threshold level of expression of mutant-template telomerase RNA inhibits human tumor cell proliferation. *Proc Natl Acad Sci U S A* 2001;98:7982–7.
- Li S, Crothers J, Haqq CM, Blackburn EH. Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. *J Biol Chem* 2005;280:23709–17.
- Yu GL, Bradley JD, Attardi LD, Blackburn EH. *In vivo* alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. *Nature* 1990;344:126–32.
- Lin J, Smith DL, Blackburn EH. Mutant telomere sequences lead to impaired chromosome separation and a unique checkpoint response. *Mol Biol Cell* 2004;15:1623–34.
- Feng J, Funk WD, Wang SS, et al. The RNA component of human telomerase. *Science* 1995;269:1236–41.
- Guiducci C, Cerone MA, Bacchetti S. Expression of mutant telomerase in immortal telomerase-negative human cells results in cell cycle deregulation, nuclear and chromosomal abnormalities and rapid loss of viability. *Oncogene* 2001;20:714–25.
- Marusic L, Anton M, Tidy A, Wang P, Villeponteau B, Bacchetti S. Reprogramming of telomerase by expression of mutant telomerase RNA template in human cells leads to altered telomeres that correlate with reduced cell viability. *Mol Cell Biol* 1997;17:6394–401.
- Cerone MA, Londono-Vallejo JA, Bacchetti S. Telomere maintenance by telomerase and by recombination can coexist in human cells. *Hum Mol Genet* 2001;10:1945–52.
- Blackburn EH. Cell biology: telomeres sans frontieres. *Nature* 1990;343:122.
- Herbert B, Pitts AE, Baker SI, et al. Inhibition of human telomerase in

- immortal human cells leads to progressive telomere shortening and cell death. *Proc Natl Acad Sci U S A* 1999;96:14276–81.
40. Zijlmans JM, Martens UM, Poon SS, et al. Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. *Proc Natl Acad Sci U S A* 1997;94:7423–8.
 41. Hemann MT, Greider CW. Wild-derived inbred mouse strains have short telomeres. *Nucleic Acids Res* 2000;28:4474–8.
 42. Marie-Egyptienne DT, Brault ME, Nimmo GA, Londono-Vallejo JA, Autexier C. Growth defects in mouse telomerase RNA-deficient cells expressing a template-mutated mouse telomerase RNA. *Cancer Lett* 2009;275:266–76.
 43. Prowse KR, Greider CW. Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc Natl Acad Sci U S A* 1995;92:4818–22.
 44. Samper E, Fernandez P, Eguia R, et al. Long-term repopulating ability of telomerase-deficient murine hematopoietic stem cells. *Blood* 2002;99:2767–75.
 45. Forsyth NR, Wright WE, Shay JW. Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again. *Differentiation* 2002;69:188–97.
 46. Allsopp RC, Morin GB, DePinho R, Harley CB, Weissman IL. Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation. *Blood* 2003;102:517–20.
 47. Ferron S, Mira H, Franco S, et al. Telomere shortening and chromosomal instability abrogates proliferation of adult but not embryonic neural stem cells. *Development* 2004;131:4059–70.
 48. Flores I, Cayuela ML, Blasco MA. Effects of telomerase and telomere length on epidermal stem cell behavior. *Science* 2005;309:1253–6.
 49. Gonzalez-Suarez E, Samper E, Ramirez A, et al. Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes. *EMBO J* 2001;20:2619–30.
 50. Davis T, Kipling D. Telomeres and telomerase biology in vertebrates: progress towards a non-human model for replicative senescence and ageing. *Biogerontology* 2005;6:371–85.

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