Chromosomal end fusion resulting from telomere erosion increases susceptibility to radiation via multinucleation: Effect of p53

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Abstract. Loss of p53 tumor suppressor facilitates acquisition of telomerase activity. In fact, both p53 inactivation and telomerase activation are frequently found in human cancers. p53 inactivation, however, eliminates or attenuates the biological responses to telomerase inhibition and the eventual telomere erosion. We show that telomere erosion can increase the susceptibility to radiation, irrespective of p53 status. Both telomerase inhibition and critically shortened telomere with significant change of chromosomal end-to-end fusion were essential for the enhancement of radiosensitivity. The enhancement was correlated with greater formation of multinucleated cells. p53 inactivation did not eliminate the observed generation of chromosomal fusion and multinucleation, and the resulting increased susceptibility to radiation, as opposed to the previously proved role of p53 in mediating cellular responses to telomere dysfunction. The present findings suggest the importance of chromosomal end fusion in modulating radiosensitivity rather than p53 DNA damage signaling. Thus, the suggested anticancer radiotherapeutic strategy combined with telomerase inhibition could clinically be applicable to cancers, irrespective of p53 status.

Introduction

One goal in modern cancer radiotherapies is to design a sensitizing agent to enhance the radiotherapeutic efficacy for cancer treatment with minimum cytotoxic effect on the normal cells. In this respect, telomerase is an attractive molecular target, since its activity has been found in >85% of human cancers, however, it is absent in most normal somatic tissues (1,2).

Due to the lack of telomerase activity, most normal somatic cells lose telomeric repeats after each cell division, and their telomeres eventually reach a level that activates p53 tumor suppressor to cease cell proliferation, termed replicative senescence (3,4). Deletion of p53 allows continued cellular proliferation beyond this Hayflick limit (5), thereby resulting in further severe telomere shortening and eventual erosion of one or more chromosomes (6,7). Interestingly, p53 involvement in biological response to telomere dysfunction has been found where p53 inactivation alleviates reproductive organ apoptosis against shortened telomeres (8) and abrogates cellular apoptosis to deprotected telomeres due to the loss of TRF2 telomere binding protein (9). In addition, p53 inactivation alters biological response type to telomere erosion (10,11). These findings underline that p53 is a key mediator of cellular responses to telomere dysfunction and plays an essential role in the maintenance of genomic stability related to telomere structure. Besides mediating telomere dysfunction, p53 also participates in repressing telomerase expression (12). Actually, p53 inactivation and telomerase activation are widespread alterations in human cancers (13,14) and these two factors can affect double-strand DNA repair pathways which maintain genomic stability (15,16). Therefore, a therapeutic approach which utilizes telomerase inhibition should consider the effect of p53 loss.

Late generation telomerase null mouse (mTERC$^{-/-}$) and primary cultured normal cells have increased radiosensitivity and an impaired double-strand DNA repair response upon exposure to ionizing radiation (15). To exploit this telomerase inhibition strategy for clinical application, it is necessary to consider that transformed tumor cells often exhibit highly differential responses against radiation when compared to

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untransformed primary cultured cells. A systematic analysis of radiosensitivity using genetically defined p53 null cells revealed that, although no significant differential radiosensitivity exists between untransformed $p53^{-/-}$ and $p53^{+/+}$ MEFs, the transformation makes $p53^{+/+}$ MEFs extremely sensitive against radiation, unlike $p53^{-/-}$ MEFs (17). Therefore, it is important to evaluate the relative contribution of telomerase inactivation in telomerase-positive tumor cells, compared to normal somatic cells. Furthermore, it is provocative to investigate whether telomerase inhibition can evoke radiosensitization of transformed tumor cells lacking p53 function, since p53 loss is thought to abrogate cellular response to telomere dysfunction (8,9,11,15,16). In the present study, employing Myc/Ras-transformed MEFs derived from both $mTERC^{-/-}INK4a^{-/-}$ and $mTERC^{-/-}p53^{-/-}$ mice (8,18), we investigated the functional relation of p53 and telomere dysfunction in modulating radiation response, by comparing their radiosensitivity profiles with the genomic instabilities via structural changes of telomere repeat. Contrary to earlier evidence that p53 is an extremely important factor in exhibiting cellular responses to telomere dysfunction, we found that p53 was not linked to enhancement of radiosensitivity resulting from chromosomal end-to-end fusions due to critically short telomere.

Materials and methods

Generation of transformed $mTERC^{-/-}$ MEFs. The Myc/Ras-transformed MEFs derived from mice carrying homozygous deletions of mTERC, and either the INK4a or p53 locus were obtained as described previously (8,18,19). The $INK4a^{-/-}$ $mTERC^{-/-}$ and $mTERC^{-/-}p53^{-/-}$ MEFs, grown in DMEM containing 10% FBS, were co-transfected with 2 μg each of expression constructs for Myc and $H\text{-}RAS^{G12}$, and either mTERC (20) or an empty Bluescript KS(+) (Stratagene) vector. The resulting single clones were picked, expanded 14 days after the transfection, and passaged until 220 population doubling levels (PDL). These passaged single clones were mixed in the same ratio at the indicated passage numbers according to each genotype. Serial passages of individual clones obtained from late generation were performed at confluence with a split ratio of 1:16 (corresponding to four population doublings).

Determination of cell survival and viability. The survival of transformed MEFs was determined by measuring the colony-forming ability of single cells exposed to radiation. Cells were seeded in triplicate at a density of 2,400 cells per 10-cm culture dish, and were exposed to γ-ray using a ^{137}Cs source, and the resulting colonies formed 8 days after the radiation were stained with 0.1% crystal violet for 30 min. The relative cell survivals were calculated as percentages of colonies counted in unirradiated cohorts.

For the determination of relative radiation sensitivity between transformed and untransformed MEFs with critically short telomeres, Myc/Ras-transformed G5 $mTERC^{-/-}INK4a^{-/-}$ and G6 $mTERC^{-/-}p53^{-/-}$ MEFs were transfected with retroviral pMFG-GFP (21) and selected with puromycin (3.0 μg/ml). These GFP-labeled transformed MEFs were co-incubated with untransformed normal G6 $mTERC^{-/-}$ MEFs at early passages within PDL 20, irradiated with 0, 3, 6, and 8 Gy, and the survived cells were then observed under fluorescence microscope (x100) 5 days after the radiation.

Cytogenetic analysis. For metaphase preparation, the cells were incubated with 0.1 μg/ml colcemid for 1 h, with hypotonic 0.075 M KCl for 20 min, and then fixed with a mixture of methanol and acetic acid (3:1 vol/vol). The fixed cells dropped onto slides were then baked at 60˚C for 2 h and stained with Giemsa. To perform the fluorescence *in situ* hybridization (FISH) of metaphase chromosome, air-dried slides were fixed with 4% formamide/PBS and then hybridized to Cy3-labeled telomere-specific $(CCCTAA)_3$ peptide nucleic acid probe, and counterstained with 4',6'-diamidino-2-phenylindole (DAPI) as described previously (18). The frequency of chromosomal end-to-end fusions was determined by counting 50 metaphases per sample.

Measurement of telomere length. Telomere length determination was performed on early and long-term passaged cells as described previously (20). Briefly, cells embedded in agarose plugs at a concentration of $1x10^6$ cells per plug were treated with proteinase K. The extracted DNA embedded in plugs was digested with *Hin*fI and *Rsa*I and then electrophoresed through 1% agarose gel in 0.5X TBE buffer at 14˚C, using a ramped pulse speed of 1-6 sec at 6 V/cm for 17 h with CHEF MAPPER DR III pulsed-field gel electrophoresis apparatus (Bio-Rad). The gel was blotted and hybridized with telomeric oligonucleotide probe $(CCCTAA)_3$ and subsequently with mouse major satellite repeat probe (GGACCTGGAATA TGGCGAGAAA) (22). Telomere length was presented as mean telomeric restriction fragment length (TRF), which was calculated as previously defined (23).

Measurement of mitotic death. Mitotic cell death after radiation was determined by counting the cells with multinuclei as described previously (24). Cells growing at low density on slide glass were exposed to radiation doses indicated on the text, and the irradiated cells were fixed after 5 days in 70% methanol for 1 h and stained with either hematoxylin/eosin or DAPI. The percentages of cells with multinuclei or micronuclei (≥3) were determined by counting at least 200 cells for each sample under light and fluorescence microscopes.

Results

Both telomerase inhibition and short telomeres are required for the enhancement of radiosensitivity, which can occur irrespective of p53 status. The serial intercrosses of $mTERC^{-/-}$ mice lacking tumor suppressor INK4a or p53 produce mice with progressively shortened telomere (8,18). However, these two compound mutant mice exhibit different phenotypes with the emergence of telomere dysfunction. These differences result mainly from their p53 status principally due to DNA damage, since $INK4a^{-/-}$ null mice retain functional p53 against DNA damage, including anticancer agents and radiation (25,26, and data not shown). Thus, the responses against telomere dysfunction according to p53 status, especially resulting from DNA damage, could systemically be elucidated by comparing cells derived from these two compound mutant

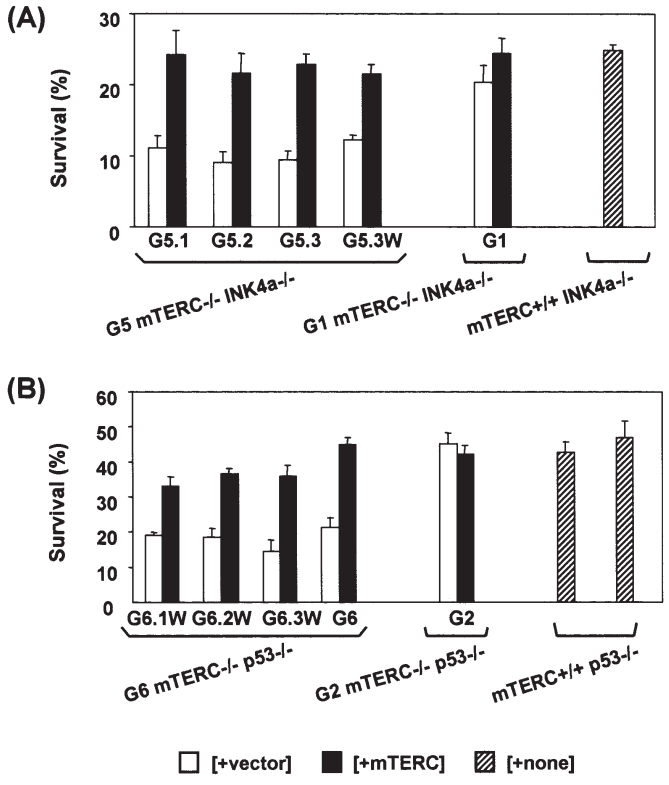

Figure 1. Enhanced radiosensitivity of Myc/Ras-transformed late generation telomerase-deficient MEFs, irrespective of p53 status. Survivals of Myc/Ras-transformed pooled populations of telomerase-deficient MEF cultures with (closed bars) and without mTERC reconstitution (open bars), and telomerase-proficient MEF cultures (hatched bar) were measured 8 days after exposure to 3 Gy of radiation. (A) Pooled populations of Myc/Ras-transformed G5 and G1 mTERC$^{-/-}$INK4a$^{-/-}$ MEFs were prepared by mixing individually isolated clones derived from G5 and G1 MEFs (G5.1, 13 and 10 clones; G5.2, 10 and 8 clones; G5.3, 8 and 6 clones; G1, 10 and 10 clones with and without mTERC-reconstitution, respectively) and by mixing whole clones formed in one culture dish, which was derived from G5.3 MEFs (G5.3W). Pool of Myc/Ras-transformed mTERC$^{+/+}$INK4a$^{-/-}$ MEF culture was composed of 10 clones isolated individually. (B) Pooled populations of Myc/Ras-transformed G6 and G2 mTERC$^{-/-}$p53$^{-/-}$ MEFs were prepared by mixing whole clones formed in one 10-cm culture dish, which was derived from three G6 MEFs and one G2 MEF (G6.1W, G6.2W, G6.3W, and G2W), and in one set of individually isolated clones derived from G6.1 MEFs (5 and 10 clones with and without mTERC reconstitution, respectively). Pools of Myc/Ras-transformed mTERC$^{+/+}$p53$^{-/-}$ MEF culture were composed of independently derived whole clones.

mice. In this study, we initially assessed the radiosensitivity of Myc/Ras-transformed MEFs which were derived from mTERC$^{-/-}$INK4a$^{-/-}$ mice. These transformed MEFs grew rapidly in culture and formed the tumors on nude (data not shown) and SCID mice (18). Myc/Ras-transformed late generation G5 mTERC$^{-/-}$INK4a$^{-/-}$ MEFs have chromosomal end-to-end fusions, whereas these same cells that have been reconstituted with mouse telomerase RNA component (mTERC) have minimal chromosomal aberrations (18). Therefore, these comparable sets of G5 transformed MEFs with or without mTERC-reconstitution are useful to examine the contribution of telomerase inactivation of telomerase-positive tumor cells, maintaining critically short telomeres, in evaluating cellular response to radiation. Myc/Ras transformation leads to gross genetic changes that are distinct from parental cells and differ among the individual clones; therefore, these resulting clones would be expected to differ in their sensitivity to radiation. To exclude such bias as clonal variation due to Myc/Ras transformation, we examined radiation sensitivity in two differently prepared pooled transformed populations: one was a mixture of individually isolated clones expanded in culture, designated as clonal pool or clonal pooled population, and the other was a mixture of whole clones formed in one MEF culture pooled together, designated as whole pool or whole pooled population. Reconstitution of mTERC affected both growth and survival rates of G5 mTERC$^{-/-}$NK4a$^{-/-}$ cultures. The growth rates of the mTERC-reconstituted pools were higher than those of the corresponding pools without mTERC-reconstitution, irrespective of pooling methods (data not shown). In a survival analysis, the mTERC-reconstitution exerted a profound effect on radiation resistance of G5 mTERC$^{-/-}$INK4a$^{-/-}$ cultures (Fig. 1A), indicating that telomerase inactivation can elicit an enhancement of radiation sensitivity in telomerase-positive transformed cells. Since rapidly growing cancer cells are generally considered to be more sensitive to radiation than slowly growing cancer cells, we could reasonably exclude the possibility that the faster growth by mTERC-reconstitution resulted in the radiation resistance in our survival analysis. Specifically, the mTERC$^{-/-}$ clonal pools showed an average 2.32-fold lower survival rate to 3 Gy of radiation than the corresponding mTERC-reconstituted pools, which was evidently found in all three clonal pooled populations (Fig. 1A, 11.1% vs 24.3% for G5.1; 9.1% vs 21.6% for G5.2; and 9.5% vs 22.9% for G5.3). This observation was further supported by comparison of radiosensitivities in pools composed of whole clones. The mTERC$^{-/-}$ whole pool, designated as G5.3W in Fig. 1A, also exhibited a lower survival rate than that of the mTERC-reconstituted whole pool. In all experiments using Myc/Ras-transformed pooled populations, the telomerase-reconstituted G5 mTERC$^{-/-}$INK4a$^{-/-}$ MEF cultures exhibited a survival rate comparable to that of mTERC$^{+/+}$INK4a$^{-/-}$ MEF culture (Fig. 1A, hatched bar).

Next, to address whether the influence of p53 loss on the observed radiosensitivity profile was dependent on telomerase status, we investigated the radiotherapeutic efficacy of telomerase inhibition using Myc/Ras-transformed mTERC$^{-/-}$p53$^{-/-}$ MEFs. Similar to those seen in G5 mTERC$^{-/-}$INK4a$^{-/-}$ MEF cultures, the whole pools mixed with all the clones formed in one G6 mTERC$^{-/-}$p53$^{-/-}$ MEF culture were more radiosensitive than those of the corresponding mTERC-rescued pools, as shown in all three independently isolated whole pools (Fig. 1B; G6.1W, G6.2W, and G6.3W). Further support of this enhanced radiosensitivity came from the comparison of radiosensitivities between the clonally isolated pool of G6 mTERC$^{-/-}$p53$^{-/-}$ clones (n=10) and that of the mTERC-rescued clones (n=5) (Fig. 1B, G6). These clonally isolated pools also exhibited a radiosensitivity profile similar to that seen in all three whole pools. The present findings illustrate that mTERC-reconstitution exerts a profound effect on the responses of G6 mTERC$^{-/-}$p53$^{-/-}$ cultures to radiation, similar to G5 mTERC$^{-/-}$INK4a$^{-/-}$ cultures. However, the mTERC-reconstitution did not affect the survival rates of early generation G1 mTERC$^{-/-}$INK4a$^{-/-}$ or G2 mTERC$^{-/-}$p53$^{-/-}$ cultures which retain intact telomere function (8,18) (Fig. 1), suggesting that critically short telomere as well as telomerase inhibition are required to elicit an enhanced radiosensitivity of telomerase-positive

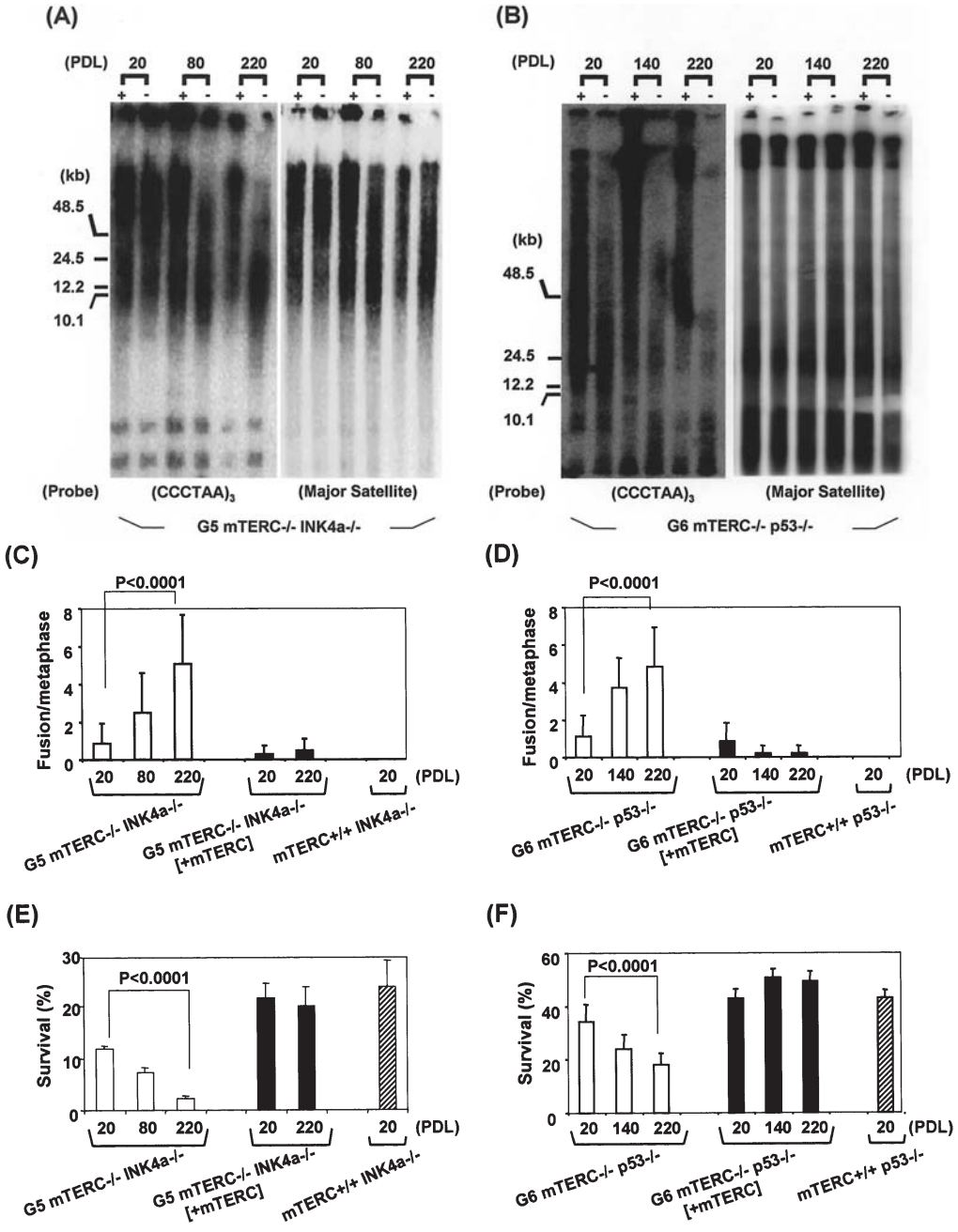

Figure 2. Correlation between radiosensitivity, chromosomal end-to-end fusion and telomere length as a function of cell division. The individual clones of Myc/Ras-transformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ (n=8) and G5 mTERC$^{-/-}$INK4a$^{-/-}$[+mTERC] MEFs (n=10), and the individual clones of Myc/Ras-transformed G6 mTERC$^{-/-}$p53$^{-/-}$ (n=10) and G6 mTERC$^{-/-}$p53$^{-/-}$[+mTERC] MEFs (n=5) were passaged until PDL 220, and the respective genotype-matched clones were mixed to prepare pooled clonal populations at three steps during the passages as indicated in the panels. (A and B) Telomere lengths analyzed by Southern blot analysis of terminal restriction fragment (TRF). The blots of digested DNA were probed with telomeric repeat $(CCCTAA)_3$ or major satellite DNA described in Materials and methods. +, the telomere lengths of cells with mTERC-reconstitution; -, the telomere lengths of cells without mTERC-reconstitution. (C and D) Chromosomal end-to-end fusions measured by counting 50 metaphases per clone. (E and F) Survivals measured after exposure to 3 Gy of radiation. In C-F closed bars indicate addition of mTERC, open bars the addition of empty vector, and hatched bars no addition.

tumor cells. In addition, the lower survival rates found in both G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ cultures than their corresponding mTERC-reconstituted cultures clearly indicate that telomerase inhibition can sensitize telomerase-positive cells with critically short telomeres toward radiation, and that this sensitization could occur regardless of p53 status, due to DNA damage response.

Correlation of radiosensitization level with the degrees of genomic instability represented by chromosomal end fusion. To further evaluate the observed enhancement of radiosensitivity by telomerase inhibition, Myc/Ras-transformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ clones (10 clones with and 8 clones without mTERC-reconstitution) and Myc/Ras-transformed G6 mTERC$^{-/-}$p53$^{-/-}$ clones (5 clones with and 10 clones without mTERC-reconstitution) were passaged until PDL 220. These four sets of the individual clones with and without telomerase reconstitution were mixed by their genotypes at early, middle, and late passages (PDL 20, 80 or 140, and 220, respectively) in the same ratio to make genotype-matched clonal pools,

and then the correlations between radiosensitivity and either telomere length or chromosomal end-to-end fusions were investigated. As shown in Fig. 2A and B, increasing passages of G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ MEF cultures during the period of PDL 20 to 220 led to a progressive shortening of telomere length (Δ119 bp and Δ74.5 bp per PD, respectively). As the telomere shortened during the passages, both cultures exhibited concomitant increase of chromosomal end-to-end fusions (Δ0.021 and Δ0.019 fusions per PD, respectively) (Fig. 2C and D), where the telomere signals were not detectable at the fusion points by fluorescence *in situ* hybridization with telomeric repeat probe (Supplementary Fig. 1). On the contrary to telomerase-deficient cultures, however, the mTERC-reconstituted cultures did not show reduction of telomere lengths (Fig. 2A and B) and showed negligible changes in the number of chromosomal end-to-end fusions (Fig. 2C and D) during the passages. The remarkable increase of telomere length in the mTERC-reconstituted G6 mTERC$^{-/-}$p53$^{-/-}$ pool was found to be due to the increase of only one out of five clones, however, long-term passages of this clone did not affect the survival rate (data not shown), thus eliminating possible bias in the evaluation of the mTERC-reconstituted pool. The significantly reduced telomere length and increased chromosomal end-to-end fusions, shown in both mTERC$^{-/-}$INK4a$^{-/-}$ and mTERC$^{-/-}$p53$^{-/-}$ cultures, but not in the mTERC-reconstituted cultures, illustrate that telomerase-deficient cultures are accompanied with increasing genomic instability, represented by chromosomal end-to-end fusions during long-term passages, whereas telomerase-reconstituted cultures maintain the genomic stability with nearly the same amounts of initial fusions.

Next, using these pools with differential genomic instability according to passage numbers, we analyzed survival rates to find out whether there was any correlation between radiosensitivity and either telomere length or chromosomal end-to-end fusions. Increasing passages of both mTERC$^{-/-}$INK4a$^{-/-}$ and mTERC$^{-/-}$p53$^{-/-}$ pools led to decreased survivals after the exposure to radiation (Fig. 2E and F). Specifically, the initial 11.8% and 34.5% survivals of G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ MEFs at PDL 20 decreased gradually to 2.3% and 18.2% at PDL 220, respectively. Comparing dose-response curves of mTERC$^{-/-}$ and mTERC-reconstituted cells at PDL 220, we found marked difference in survival fractions between the G5 mTERC$^{-/-}$INK4a$^{-/-}$ or G6 mTERC$^{-/-}$p53$^{-/-}$ MEF culture and the corresponding mTERC-rescued culture (Supplementary Fig. 2). The gradual reduction of survivals according to the degree of chromosomal end-to-end fusions and telomere shortening indicates that the genomic instability generated by this chromosomal end fusion which resulted from telomere shortening might be the basis of sensitization in telomerase-deficient cells to radiation.

Abundant mitotic death in telomere dysfunctional cells. In response to radiation, severely damaged cancer cells undergo mitotic cell death which is characterized by the formation of micronuclei and/or multinuclei (24,27). Critically short telomere during mitosis often leads to bridge-breakage-fusion cycle of telomeric fusion and impairment of G2/M checkpoint control, which may be one mechanistic basis of the generation of aneuploidy and polyploidy population (28,29). In order to evaluate whether the reduced survival rate of telomere dysfunctional cells after exposure to radiation might have been due to mitotic cell death, we examined the formation of micronuclei and multinuclei.

Myc/Ras-transformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ cells and their corresponding mTERC-rescued cells showed a dose-dependent increase of multinuclei (n≥3) upon exposure to radiation, however, micronuclei were rarely generated even in high-dose radiation (data not shown). In our transformed MEF cultures, the most prominent feature was an appearance of enlarged cells containing multiple nuclei within a cell, determined by both hematoxylin/eosin and DAPI stainings (Fig. 3A), which could optimally be assessed 5 days after the exposure to radiation. In addition, the changing pattern of multinucleation after the exposure to radiation was a gradual increase of number of multinuclei in both G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ cultures, accompanied by a reduction of diploid population (Fig. 3B and C). Under a steady growing condition, G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ cells and their mTERC-rescued cells maintained very low levels of <0.1% multinuclei (Fig. 3D and E). Upon the exposure to radiation, however, both G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ cultures produced significantly more multinuclei at PDL 20 than their corresponding mTERC-rescued cultures (Fig. 3D and E). Specifically, G5 mTERC$^{-/-}$INK4a$^{-/-}$ cells and the mTERC-rescued cells produced 19.7% and 11.0% of multinuclei (p=0.0026) at 6 Gy, respectively (Fig. 3D). The significantly enhanced formation of radiation-induced multinuclei was also evident in G6 mTERC$^{-/-}$p53$^{-/-}$ cells at PDL 20, compared to the mTERC-rescued cells [21% vs 14% at 6 Gy, respectively (p=0.0015)] (Fig. 3E). These findings indicate that abundant formation of multinuclei in the telomere dysfunctional cells can occur irrespective of p53 status. This view was further strengthened by the observation that increasing passages up to PDL 220 led to an increasing formation of multinuclei in both G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ cells, but not in the corresponding mTERC-rescued cells. Similar to unchanged survival rates of early generation cultures despite the mTERC-reconstitution, shown in Fig. 1, there were no discernible differences in radiation-induced multinuclei production between Myc/Ras-transformed G1 mTERC$^{-/-}$INK4a$^{-/-}$ or G2 mTERC$^{-/-}$p53$^{-/-}$ cultures with maintaining robust telomere function, and their corresponding mTERC-rescued cultures. The above findings clearly show that the reduction of survival rates and resulting enhancement of radiosensitivity were due to more abundant mitotic death of cells with genomic instability represented by chromosomal end fusions, which is part of telomere dysfunction, than cells with robust telomere function.

Relative contribution of telomerase deficiency in transformed and untransformed cells with critically shortened telomere. For clinical application of telomerase inhibition strategy, it is important to consider that telomere shortening, as occuring normally with age, would be expected to place normal tissues of elderly patients at increased risk of enhanced radiosensitization. Therefore, we can not exclude the possibility that normal cells of older patients would be more radiosensitive than their telomerase-inhibited tumor cells, thus losing any advantage of telomerase inhibition with increasing age. To clarify this point,

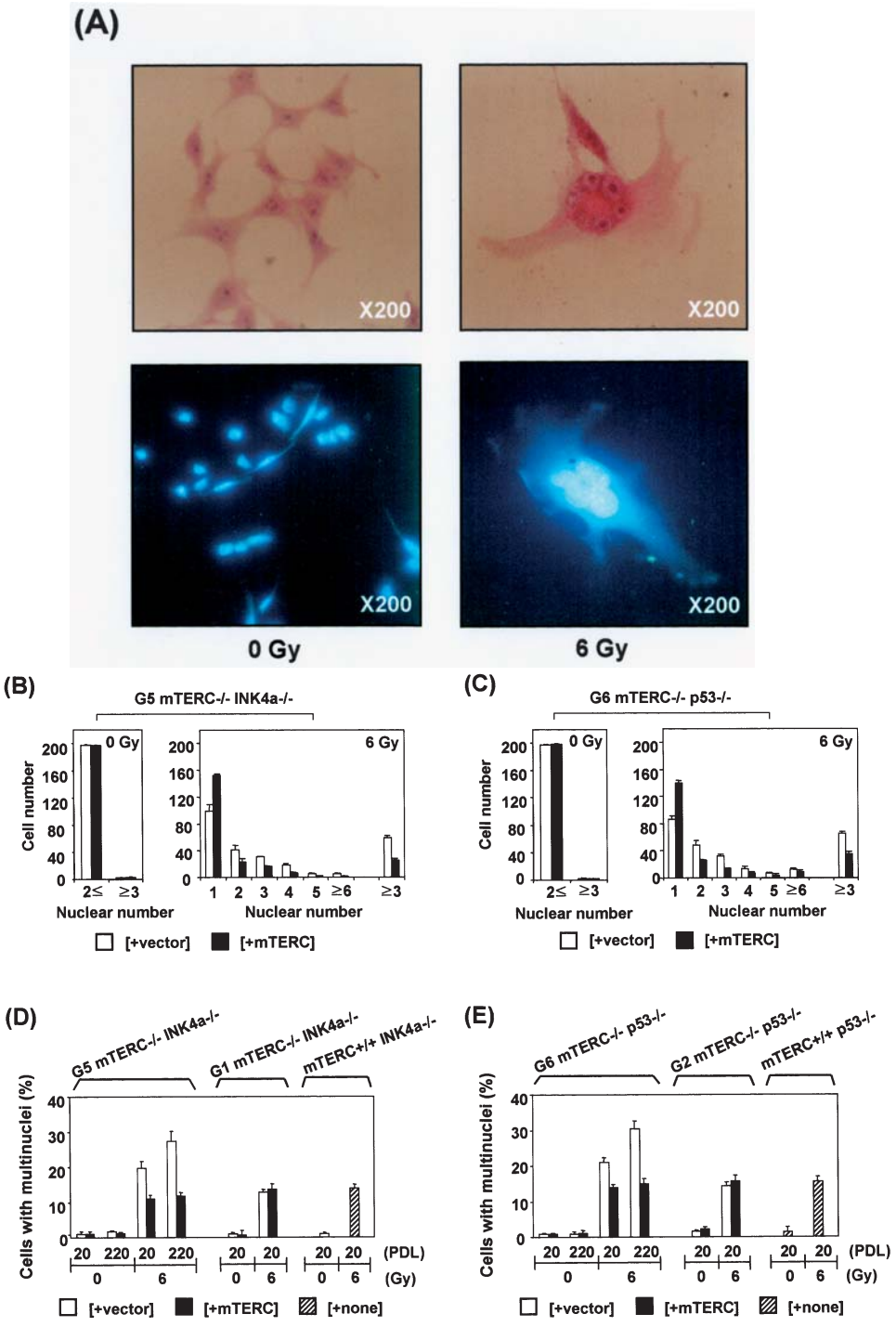

Figure 3. Increased multinuclei formation in telomere dysfunctional cells by radiation. The multinuclei formation of Myc/Ras-transformed mTERC$^{-/-}$INK4a$^{-/-}$, mTERC$^{-/-}$p53$^{-/-}$ cultures (open bars), and their corresponding mTERC-rescued cultures (closed bars) were counted 5 days after exposure to 6 Gy of radiation. (A) Representative photographs showing radiation-induced mitotic cell death, determined by either hematoxylin/eosin (upper) or DAPI staining (bottom) of G5 mTERC$^{-/-}$INK4a$^{-/-}$ cultures which were exposed to 0 and 6 Gy of radiation (magnification x200). Cells with multinuclei (≥3 per cell) were measured by hematoxylin/eosin staining and confirmed by DAPI staining under a light and fluorescence microscope, respectively, by counting 200 cells/sample. (B and C) Changing pattern of multinuclei number after exposure to 6 Gy of radiation. (D and E) Difference of multinucleated cell numbers between short-term (PDL 20) and long-term (PDL 220) passaged cells.

we investigated radiation sensitivity by culturing transformed late generation telomerase-deficient culture together with untransformed normal late generation telomerase-deficient culture, both of which retained a critically short telomere length with a significant amount of chromosomal end-to-end fusions. As shown in Fig. 4, transformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ cultures labeled with GFP were seen under fluorescence microscope before irradiation, however, after irradiation with more than 6 Gy, they completely disappeared, even in the presence of untransformed normal G6 mTERC$^{-/-}$ cells which do not form tumor mass on SCID or nude mouse skin (20). In survival analyses, both Myc/Ras-transformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ cultures also exhibited lower survival rates, compared to those

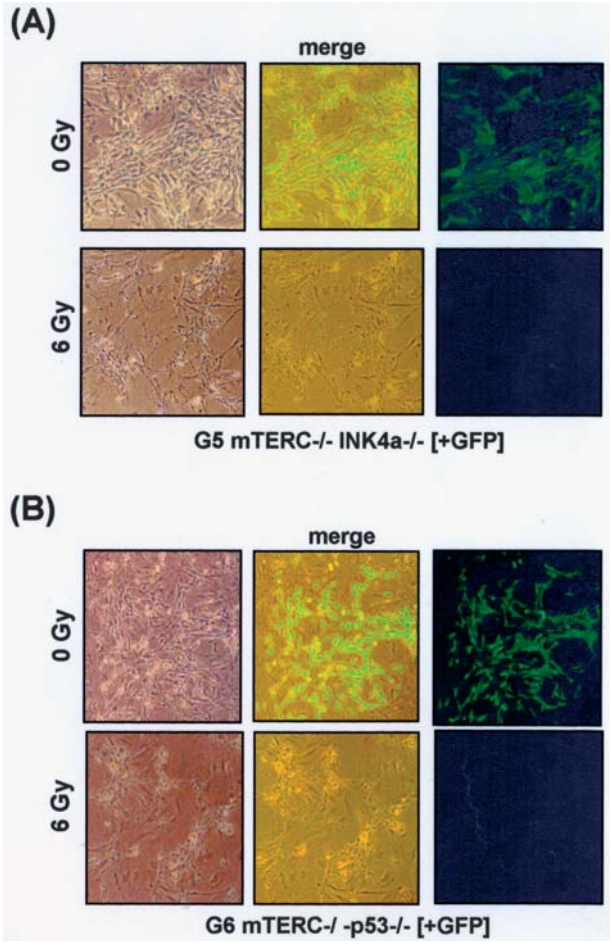

Figure 4. Higher radiation sensitivity of transformed late generation telomerase-deficient cells compared to untransformed late generation telomerase-deficient cells. Myc/Ras-transformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$p53$^{-/-}$ cells labeled with retroviral GFP vector were co-incubated with untransformed G6 mTERC$^{-/-}$ cells at an early passage within PDL 20, irradiated with 0, 6 and 8 Gy, and then inspected under fluorescence microscope (x100). Representative image pictures of co-incubated experiments which received 6 Gy irradiation and observed under light (left columns of A and B) or fluorescence filter (right columns) or both images of which merged together (middle columns).

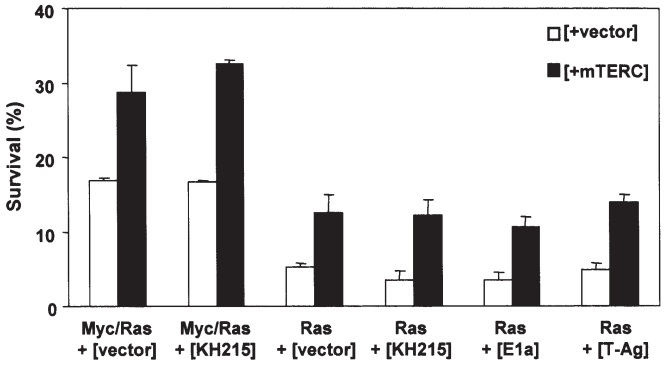

Figure 5. Radiosensitization by telomerase-deficiency, irrespective of p53 inactivation. Survivals of transformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ MEF cultures with and without mTERC reconstitution, whose p53 was inactivated by dominant negative p53 (KH215) or viral oncogene (E1a or SV40 Large T antigen), were measured 8 days after exposure to 3 Gy of radiation.

of their corresponding untransformed cultures (Supplementary Fig. 3). These late generation transformed telomerase-deficient cultures exhibited levels of telomere lengths and chromosomal end-to-end fusions comparable to their corresponding untransformed normal cultures (Supplementary Fig. 4 and data not shown). The present findings obtained from mixed cultures of transformed and untransformed cultures, both of which maintained critically shortened telomere length with significant chromosomal end-to-end fusions, suggest that, although normal tissues of older individuals would possibly be expected to have increased radiation sensitivity due to telomere shortening over cellular replication time, the radiosensitivity combined with telomerase inhibition would result in significant therapeutic gain.

p53 inactivation did not alter radiosensitization mediated by telomerase inhibition. To further confirm the present finding that the observed enhancement of radiosensitivity occurred, irrespective of p53 status, we generated telomerase-deficient and -rescued transformed foci of G5 mTERC$^{-/-}$INK4a$^{-/-}$ cultures whose p53 could be inactivated by cotransfecting dominant negative p53 with either Myc/Ras or Ras. The co-transfection of Ras and KH215, a dominant negative mouse p53, leads to more abundant formation of transformed foci through p53 inactivation than that of Ras alone (30), which we also confirmed in our present transformed foci analysis using G5 mTERC$^{-/-}$INK4a$^{-/-}$ MEFs (data not shown). As shown in Myc/Ras-transformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ cells, Ras-transformed telomerase-deficient cells were also more radiosensitive than those with mTERC-reconstitution (Fig. 5). The introduction of KH215 did not affect the observed differential radiosensitivity profile between them, which was also found in Myc/Ras-transformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ cells with and without mTERC-reconstitution. We then examined whether viral oncoproteins interacting with and inactivating p53 might influence the observed radiosensitivity profile, and found that G5 mTERC$^{-/-}$INK4a$^{-/-}$ cells cotransfected with Ras and either Ela (31) or SV40 Large T antigen (32) were more radiosensitive than their corresponding transformed cells with mTERC-reconstitution. The chromosomal end-to-end fusions were also found in these telomerase-deficient cells transformed with viral oncogene or dominant negative p53, but negligibly in their corresponding mTERC-reconstituted cells (data not shown). These findings obtained through the inactivation of p53 by dominant negative p53 or viral oncogenes strongly support the notion that telomerase inhibition and resulting telomere dysfunction can radiosensitize telomerase-positive tumor cells irrespective of p53 status.

Discussion

Based on the recent observation that telomerase is activated in human cancers but inactivated in normal somatic tissues, attempts have been made to develop a telomerase inhibitor as an anticancer therapy (33,34). The present study demonstrated the possibility that the combination radiotherapy with telomerase inhibitor could clinically be applicable for the enhancement of radiotherapeutic efficacy. Although the loss of p53 has been shown to attenuate the adverse cellular effects of telomere dysfunction (8,9,11), our results described herein

showed that dysfunctional telomeres could sensitize p53-deficient cells as well as p53-proficient cells toward radiation.

The mechanistic basis of the enhanced radiosensitivity was most likely due to the genomic instability, represented by chromosomal end-to-end fusions following critical telomere shortening. This conclusion was derived from our findings described herein as well as other earlier observations. After co-transfection of Myc/Ras with or without mTERC reconstitution, the difference of telomere length was hardly detectable initially between mTERC$^{-/-}$ and the corresponding mTERC-rescued cells (present finding), whereas the chromosomal end-to-end fusions were abundantly found initially in the mTERC$^{-/-}$ cells, but were negligible in the mTERC-rescued cells (8,18,19, and the present study), indicating the genomic instability in mTERC$^{-/-}$ cells. In cells passaged for 200 PDLs, markedly increased fusions were the prominent feature of mTERC$^{-/-}$ cells, but not in mTERC-rescued cells, suggesting that the loss of chromosomal end capping capacity by telomerase inhibition is important to elicit enhanced radiosensitivity. Before clinically applying this combination radiotherapy, however, more careful considerations are needed, since cancers in most cases are recognized as diseases of the elderly and the normal cells of elderly patients would be at an increased risk of radiosensitization due to telomere shortening with age. Indeed, shortened telomere causes severe impairment of gastrointestinal tract exposed to radiation (15). Nevertheless, the previous findings that human cancers have relatively shorter telomere than their matched somatic or peripheral blood cells (35,36), together with our present results of complete regression of transformed late generation telomerase-deficient tumor cells, even in the presence of untransformed late generation telomerase-deficient normal cells upon exposure to 8 Gy irradiation, would give significant therapeutic gain to clinical application of our present combination radiotherapy with telomerase inhibition.

Since p53 mutation is frequently found in human cancers and abrogates opposing effects of telomere dysfunction (8,9), it is necessary to consider weather this combination radiotherapeutic strategy could be applicable to p53 mutant cancers. In fact, p53 is up-regulated during processing various biological responses to telomere dysfunction, such as apoptosis, senescence, and cardiac failure (37,38). Moreover, it has been suggested that mutation of p53 or inactivation of telomerase could generate secondary mutation through increasing genomic instability during cell proliferation (39,40). In the present study, however, the analysis of the long-term passaged telomerase-deficient cells revealed that progressive telomere shortening and concomitant chromosomal end-to-end fusions during cell division occurred in both p53 DNA damage response-deficient and -proficient cells. The comparison of radiosensitivity and the degree of chromosomal end-to-end fusions showed a strong correlation between radiosensitizing efficacy and genomic instability irrespective of p53 status due to DNA damage response. Furthermore, the fact that p53 inactivation did not alter the radiosensitization effect of telomerase inhibition strongly suggests that telomerase inhibition can sensitize telomerase-positive tumor cells irrespective of p53 status. This result also suggests that the genomic instability induced by chromosomal end-to-end fusion is important in evoking the sensitivity toward radiation rather than p53 signaling after irradiation. Therefore, this combination anticancer radiotherapy with telomerase inhibition could be applicable to cancers, regardless of p53 mutation.

Previously, the therapeutic enhancement of doxorubicin in telomere dysfunctional cells was proved to be due to generation of multichromosomal fusions in which the p-arm of one chromosome is connected to the q-arm of another (19). In contrast to doxorubicin exposure, however, post-radiation did not produce any multichromosomal fusion (Supplementary Fig. 5), suggesting the possibility that the mechanistic basis of radiosensitization by telomerase inhibition is different from that of doxorubicin. Nevertheless, the more massive chromosomal fragmentation of telomere dysfunctional cells than telomerase-reconstituted cells seemed to be a common occurrence upon exposure to radiation or doxorubicin.

In summary, the observations presented herein provide an important rationale for understanding the mechanism of response of telomere dysfunctional cells to radiation. To accomplish the clinical therapeutic efficacy of telomerase inhibitors, tumor cells should divide until the emergence of telomere dysfunction, as seen by increased chromosomal aberrations. The mass of solid tumor in some cases would limit sufficient divisions required for generating telomere-free chromosomes, which could be a problem for clinical application of telomerase inhibition strategy. However, recurrent tumors after radiotherapy or after surgical resection of tumor burdens would potentially be targeted to achieve the purpose of combination radiotherapy with telomerase inhibitor, since the recurrent tumors might allow sufficient division to generate telomere-free chromosomes. A more precise relationship between telomere dysfunction and responses to radiation in various types of human tumors should be explored in order to achieve a successful outcome of telomerase inhibitor-based radiation therapy.

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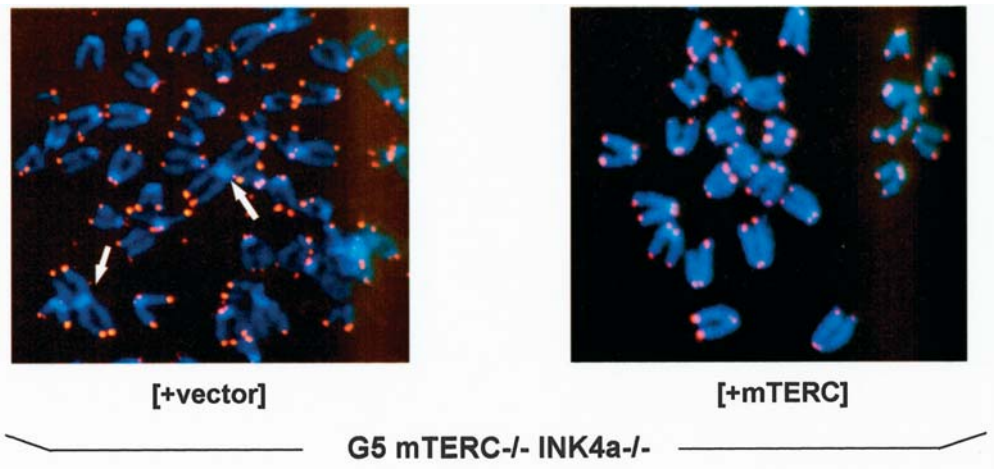
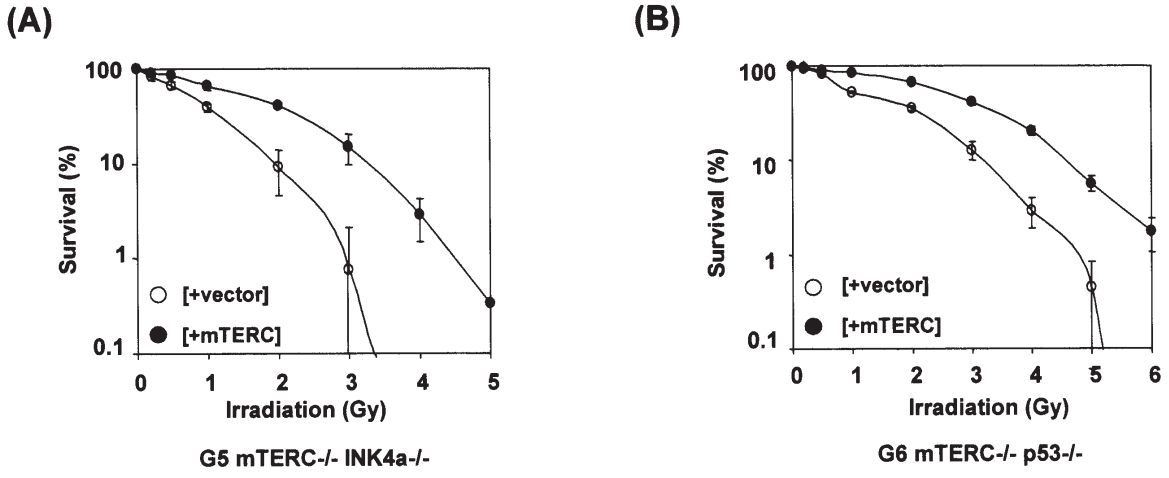
References

1. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PLC, Coviello GM, Wright WE, Weinrich SL and Shay JW: Specific association of human telomerase activity with immortal cells and cancer. Science 266: 2011-2015, 1994.
2. Shay JW and Bacchetti S: A survey of telomerase activity in human cancer. Eur J Cancer 33: 787-791, 1997.
3. Vaziri H, West MD, Allsopp RC, Davison TS, Wu YS, Arrowsmith CH, Poirier GG and Benchimol S: ATM-dependent telomere loss in aging human diploid fibroblasts and DNA damage lead to the post-translational activation of p53 protein involving poly(ADP-ribose) polymerase. EMBO J 16: 6018-6033, 1997.
4. Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P and Campisi J: Reversal of human cellular senescence: roles of the p53 and p16 pathways. EMBO J 22: 4212-4222, 2003.
5. Shay JW, Pereira-Smith OM and Wright WE: A role for both RB and p53 in the regulation of human cellular senescence. Exp Cell Res 196: 33-39, 1991.
6. Harley CB: Telomerase loss: mitotic clock or genetic time bomb? Mutat Res 256: 271-282, 1991.

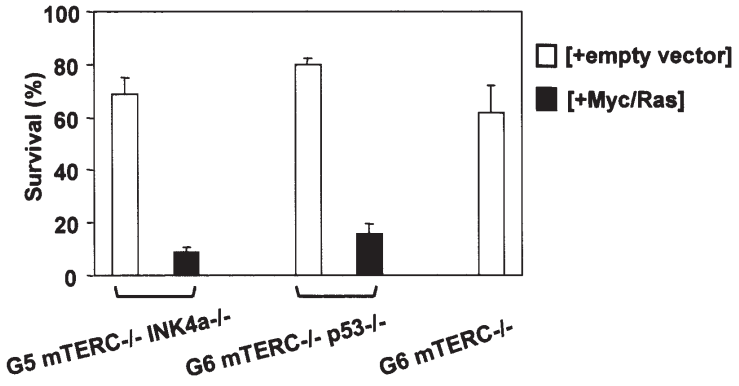
7. Zhang X, Mar V, Zhou W, Harrington L and Robinson MO: Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. Genes Dev 13: 2388-2399, 1999.
8. Chin L, Artandi SE, Shen Q, Tam A, Lee SL, Gottlieb GJ, Greider CW and De Pinho RA: p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. Cell 97: 527-538, 1999.
9. Karlseder J, Broccoli D, Dai Y, Hardy S and De Lange T: p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science 283: 1321-1325, 1999.
10. Elmore LW, Rehder CW, Di X, McChesney PA, Jackson-Cook CK, Gewirtz DA and Holt SE: Adriamycin-induced senescence in breast tumor cells involves functional p53 and telomere dysfunction. J Biol Chem 277: 35509-35515, 2002.
11. Preto A, Singhrao SK, Haughton MF, Kipling D, Wynford-Thomas D and Jones CJ: Telomere erosion triggers growth arrest but not cell death in human cancer cells retaining wild-type p53: implications for antitelomerase therapy. Oncogene 23: 4136-4145, 2004.
12. Shats I, Milyavsky M, Tang X, Stambolsky P, Erez N, Brosh R, Kogan I, Braunstein I, Tzukerman M, Ginsberg D and Rotter V: p53-dependent down-regulation of telomerase is mediated by p21/waf1. J Biol Chem 279: 50976-50985, 2004.
13. Gonzalez-Suarez E, Flores JM and Blasco MA: Cooperation between p53 mutation and high telomerase transgenic expression in spontaneous cancer development. Mol Cell Biol 22: 7291-7301, 2002.
14. Stampfer MR, Garbe J, Nijjar T, Wigington D, Swisshelm K and Yaswen P: Loss of p53 function accelerates acquisition of telomerase activity in indefinite lifespan human mammary epithelial cell lines. Oncogene 22: 5238-5251, 2003.
15. Wong KK, Chang S, Weiler SR, Ganesan S, Chaudhuri J, Zhu C, Artandi SE, Rudolph KL, Gottlieb GJ, Chin L, Alt FW and De Pinho RA: Telomere dysfunction impairs DNA repair and enhances sensitivity to ionizing radiation. Nat Genet 26: 85-88, 2000.
16. Offer H, Erez N, Zurer I, Tang X, Milyavsky M, Goldfinger N and Rotter V: The onset of p53-dependent DNA repair or apoptosis is determined by the level of accumulated damaged DNA. Carcinogenesis 23: 1025-1032, 2002.
17. Lowe SW, Ruley HE, Jacks T and Housman DE: p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74: 957-967, 1993.
18. Greenberg RA, Chin L, Femino A, Lee KH, Gottlieb GJ, Singer RH, Greider CW and De Pinho RA: Short dysfunctional telomeres impair tumorigenesis in the INK4a$^{\Delta 2/3}$ cancer-prone mouse. Cell 97: 515-525, 1999.
19. Lee KH, Rudolph KL, Ju YJ, Greenberg RA, Cannizzaro L, Chin L, Weiler SR and De Pinho RA: Telomere dysfunction alters the chemotherapeutic profile of transformed cells. Proc Natl Acad Sci USA 98: 3381-3386, 2001.
20. Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, De Pinho RA and Greider CW: Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 91: 25-34, 1997.
21. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D and Mulligan RC: Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc Natl Acad Sci USA 90: 3539-3543, 1993.
22. Silvia G, Nicola M, Maurizio Z, Jeremy BS, Ernesto C and Carlo AR: Pericentromeric organization at the fusion point of mouse robertsonian translocation chromosomes. Proc Natl Acad Sci USA 98: 171-175, 2001.
23. Petersen S, Saretzki G and Zglinicki TV: Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. Exp Cell Res 239: 152-160, 1998.
24. Miranda EI, Santana C, Rojas E, Hernandez S, Ostrosky-Wegman P and Garcia-Carranca A: Induced mitotic death of HeLa cells by abnormal expression of c-H-ras. Mutat Res 349: 173-182, 1996.
25. Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, Grosveld G and Sherr CJ: Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19^{ARF}. Cell 91: 649-659, 1997.
26. Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH and Peters G: The alternative product from the human CDKN2A locus, p14^{ARF}, participates in a regulatory feedback loop with p53 and MDM2. EMBO J 17: 5001-5014, 1998.
27. Blank M, Mandel M, Keisari Y, Meruelo D and Lavie G: Enhanced ubiquitinylation of heat shock protein 90 as a potential mechanism for mitotic cell death in cancer cells induced with hypericin. Cancer Res 63: 8241-8247, 2003.
28. Dhar S, Squire JA, Hande MP, Wellinger RJ and Pandita TK: Inactivation of 14-3-3σ influences telomere behavior and ionizing radiation-induced chromosomal instability. Mol Cell Biol 20: 7764-7772, 2000.
29. Hollander MC and Fornace AJ Jr: Genomic instability, centrosome amplification, cell cycle checkpoints and Gadd45a. Oncogene 21: 6228-6233, 2002.
30. Weinberg WC, Azzoli CG, Kadiwar N and Yuspa SH: p53 gene dosage modifies growth and malignant progression of keratinocytes expressing the v-rasHa oncogene. Cancer Res 54: 5584-5592, 1994.
31. Byrd PJ, Grand RJ and Gallimore PH: Differential transformation of primary human embryo retinal cells by adenovirus E1 regions and combinations of E1A + ras. Oncogene 2: 477-484, 1988.
32. White JA, Carter SG, Ozer HL and Boyd AL: Cooperativity of SV40 T antigen and ras in progressive stages of transformation of human fibroblasts. Exp Cell Res 203: 157-163, 1992.
33. Damm K, Hemmann U, Garin-Chesa P, Hauel N, Kauffmann I, Priepke H, Niestroj C, Daiber C, Enenkel B, Guilliard B, Lauritsch L, Muller E, Pascolo E, Sauter G, Pantic M, Martens UM, Wenz C, Lingner J, Kraut N, Rettig WJ and Schnapp A: A highly selective telomerase inhibitor limiting human cancer cell proliferation. EMBO J 20: 6958-6968, 2001.
34. El-Daly H, Kull M, Zimmermann S, Pantic M, Waller CF and Martens UM: Selective cytotoxicity and telomere damage in leukemia cells using the telomerase inhibitor BIBR1532. Blood 105: 1742-1749, 2005.
35. Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK and Allshire RC: Telomere reduction in human colorectal carcinoma and with ageing. Nature 346: 866-868, 1990.
36. De Lange T, Shiue L, Myers RM, Cox DR, Naylor SL, Killery AM and Varmus HE: Structure and variability of human chromosome ends. Mol Cell Biol 10: 518-527, 1990.
37. Leri AL, Franco S, Zacheo A, Barlucchi L, Chimenti S, Limana F, Nadal-Ginard B, Kajstura J, Anversa P and Blasco MA: Ablation of telomerase and telomere loss leads to cardiac dilatation and heart failure associated with p53 upregulation. EMBO J 22: 131-139, 2003.
38. Herbig U, Jobling WA, Chen DJ and Sedivy JM: Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). Mol Cell 14: 501-513, 2004.
39. Harve PA, Yuan J, Hedrick L, Cho KR and Glazer PM: p53 inactivation by HPV16 E6 results in increased mutagenesis in human cells. Cancer Res 55: 4420-4424, 1995.
40. Hackett JA, Feldser DM and Greider CW: Telomere dysfunction increases mutation rate and genomic instability. Cell 106: 275-286, 2001.



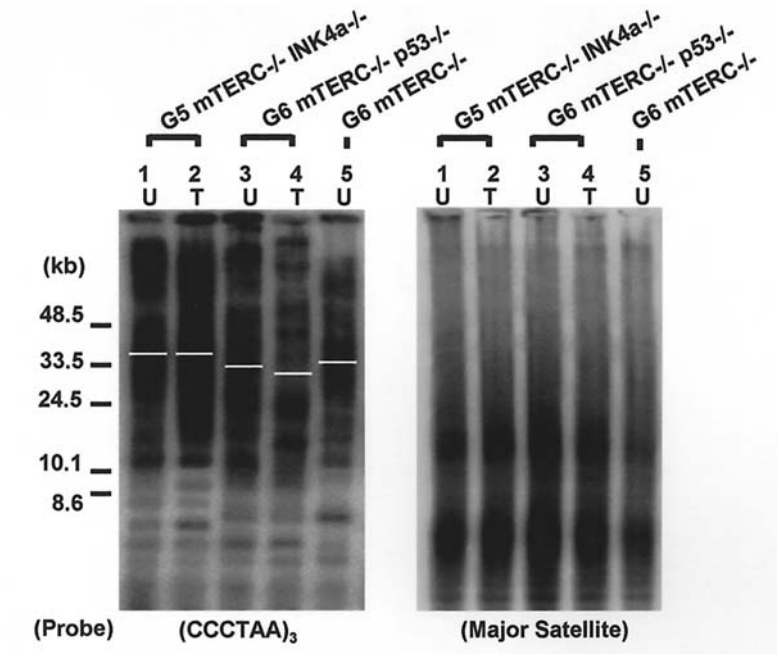
Supplementary Figure 1. Representative metaphase showing chromosomal end-to-end fusions, detected with TRF-FISH. Metaphase spreads of Myc/Ras-transformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ MEFs with or without mTERC reconstitution (+mTERC or +vector, respectively) were proved with Cy-3-labeled telomere specific $(CCCTAA)_3$ peptide nucleic acid probe and counter-stained with DAPI. Cy-3-labeled telomeres and DAPI-stained chromosome images were overlaid on triple band filter set (x1000), and then captured with a digital cooled CCD camera. The most observed type of fusion is chromosomal end-to-end fusion, but neither telomeric fusion nor telomeric association were observed. Arrows indicate the fusion point between p-arms of two chromosomes.



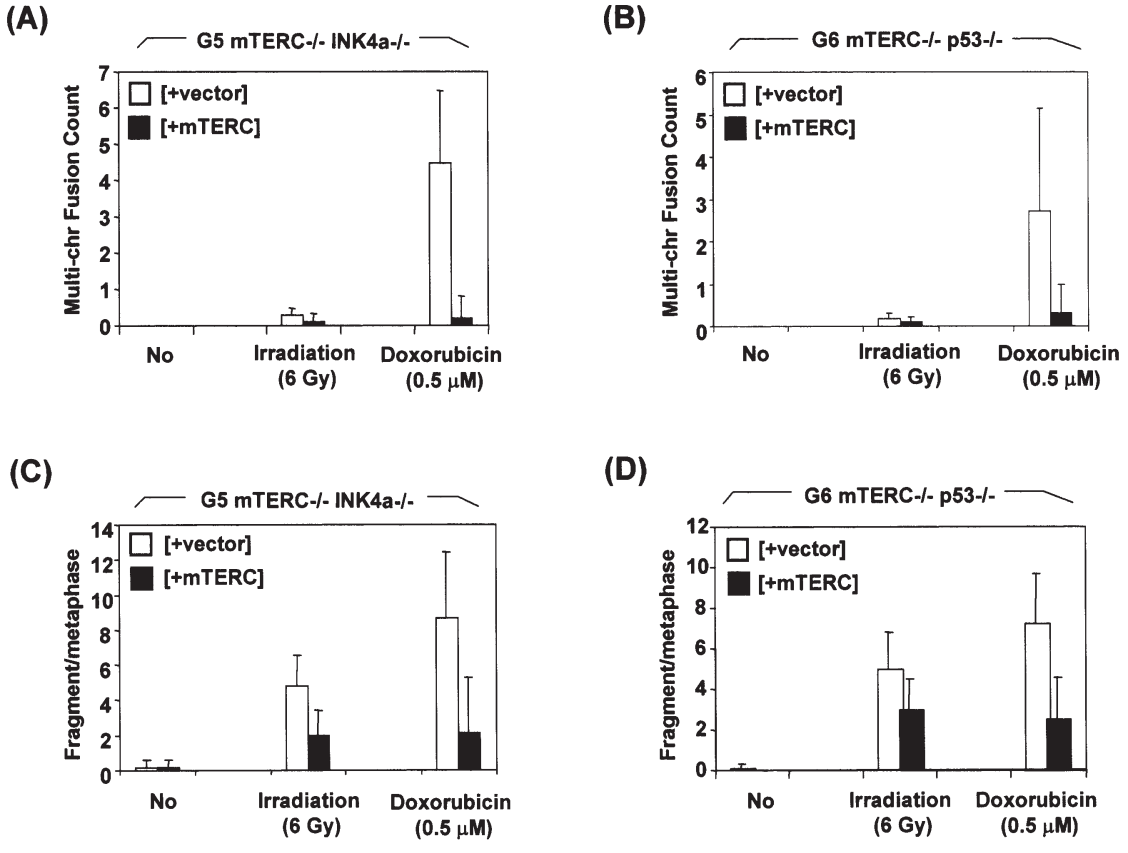
Supplementary Figure 2. Dose response curves of telomerase-deficient cells with and without telomerase reconstitution. The pooled populations passaged until PDL 220 as prepared in the legend of Fig. 2 were irradiated and survival rates were examined.



Supplementary Figure 3. Comparison of survival rates between transformed and untransformed late generation telomerase-deficient cells. Relative survival rates of Myc/Ras-transformed and their corresponding untransformed G5 mTERC$^{-/-}$INK4a$^{-/-}$ or G6 mTERC$^{-/-}$p53$^{-/-}$ cultures, and untransformed G6 mTERC$^{-/-}$ cultures were determined by trypan blue exclusion method 3 and 7 days after exposure to radiation. Representative survival data measured 7 days after radiation were presented.



Supplementary Figure 4. Comparison of telomere lengths of transformed and untransformed late generation telomerase-deficient MEFs determined by TRF-Southern blot hybridization. Telomere lengths of G5 mTERC$^{-/-}$INK4a$^{-/-}$ (lanes 1 and 2), G6 mTERC$^{-/-}$p53$^{-/-}$ (lanes 3 and 4), and G6 mTERC$^{-/-}$ MEFs (lanes 5) were measured by TRF-Southern blot hybridization. U and T, cells without and with Myc/Ras transformation, respectively. White bar on each lane indicates the mean telomere length of individual clones.



Supplementary Figure 5. Formation of multi-chromosomal fusions by doxorubicin, but not irradiation. The incidence of multi-chromosomal fusions (A and B) and chromosomal fragmentation (C and D) in response to 48 h of exposure to 0.5 μM doxorubicin or 6 Gy radiation in G5 mTERC$^{-/-}$INK4a$^{-/-}$ and G6 mTERC$^{-/-}$ p53$^{-/-}$ cells with or without mTERC reconstitution (closed bars or open bars, respectively).